Steroid Analysis

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Preface to the Second Edition

The second edition of this book has drawn heavily on the first edition but a huge amount of research on steroid analysis has been published over the last 15 years. As a result, the Editors decided to let the first edition of this book stand on its own and direct readers interested in pre-1995 steroid analysis to it, simply because the post-1995 research can on its own fill the second edition. We have tried to keep a balance but equally have allowed authors of each chapter a significant degree of freedom to approach their particular topics as they thought fit – they are after all the experts in their field. We hope that readers will agree that we have got the balance right.

In re-writing or updating these chapters, we have been greatly assisted by the developments in the availability of research publications electronically. Huge strides have been made in this area since 1995 and the ability to read a paper on one's computer rather than trekking to the British Library is a tremendous advantage. The editors wish to express their gratitude to their respective institutions (St. Bartholomew's and the Royal London School of Medicine, Queen Mary University of London and Kings College London) for providing us with electronic access to research journals from our home computers. Without such access, it would have been impossible to complete this book. Increasingly it is being recognised that while the purpose of research is to discover new scientific facts, discoveries must be disseminated to the scientific community. Unfettered and easy access to research publications is therefore vitally important. In the main, we have experienced very little difficulty in accessing research papers published in the last 20 years, though difficulties in accessing pre-1980 publications still persist, which is sad because the 'old methodology' may still offer solutions to today's problems. Regrettably, there still are journals which we have been unable to access – articles published in these journals are therefore not cited, unless, as has occurred on numerous occasions, authors of research papers in these journals have kindly provided us with copies of their publications. Where this has been done, we are very grateful. We recognise the difficulties which publishers, often learned societies which rely on income from their journals, have in allowing unrestricted access to their journals, but some solution must be found; otherwise those journals which do not allow access will not be cited and low citation rates will discourage authors from submitting their work to them.

At a late stage in the production of this book, we realised that enzyme nomenclature had moved on since the 'dehydrogenase' and 'hydroxylase' days and that cytochrome P450s have now been codified (Nelson et al. (2004); Nelson (2006)) and attempts are being made to re-name steroid dehydrogenase/reductase enzymes (Kavanagh et al. (2008); Persson et al. (2008)). Some chapter authors have been strict and have used the proper CYP names but others have not. We have not requested any author to change the steroid enzyme names which they have used, as in all cases, their terminology is clear and understandable to other colleagues in this area. We have, however, asked authors to ensure that there is no ambiguity in their text when referring to the enzyme or when referring to the gene which codes for the protein – mutations of course take place in genes and may be reflected in altered aminoacid sequence and enzyme activity of the expressed protein.

We are immensely grateful to our colleagues in this endeavour, who have produced their chapters and put up with all the problems inherent in any multi-authored book such as this. We thank all our colleagues throughout the world who have willingly given their permission for us to reproduce their work. We hope that our cumulative endeavours satisfy our readers but we encourage anyone who finds errors or disagrees with opinions expressed herein to write to the editors or authors to let us know their views. Good opinions are nice but to avoid complacency send us the bad opinions as well.

We would like to thank our publisher (Springer) for being so patient with us and for their gentle encouragement during the course of chapter delivery. It is 18 years since David Kirk died in the course of production of the first edition – we miss him now as much as we did then.

Finally and most importantly we thank our wives, Margaret and Dorothea, for putting up with us and not complaining too much when things needed to be done, finding that we were 'too busy with the book' to help. Their contribution has been considerable and without them it would have been a more onerous and lonely task.

Hugh L.J. Makin
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Chapter 1
Structure and Nomenclature of Steroids

Alexander Kasal

1.1 Structure of the Steroid Skeleton

1.1.1 Parent Hydrocarbons

A formula of a steroid compound is easily recognized by its four-membered hydrocarbon core (Fig. 1.1a). My little grand daughter called it "a little cottage", more advanced beginners may recall the term "cyclopentenoperhydrophenanthrene". All the thousands of natural and synthetic steroids are derivatives of that core. Ring A is the cyclohexane ring on the left; it is attached to another six-membered ring B. The C ring follows, the D ring is a cyclopentane system.

Numbering of the core is shown in the most prolific steroid – cholesterol (Fig. 1.1b): it starts at the A ring, continues at B, C, and then D rings. Methyl groups at angular positions 13 and 10 are ascribed numbers 18 and 19, the numbering then continues in the side chain. The numbers are used to describe positions at which substituents or multiple bonds are attached in individual compounds.
Most steroid compounds are derived from the following six basic hydrocarbons:

Gonanes (Fig. 1.2a, $R^1 = R^2 = H$), having hydrogen atoms at carbons 13 and 10
Estranes, with $R^1 = H$, R^2 = methyl; the word rings in trivial names like estradiol, estrone (Applezweig, 1964)
Androstane, with $R^1 = R^2$ = methyl, reminds us of androsterone and androgens
Pregnane (Fig. 1.2b), its echo – pregnancy – points to gestagens
Cholane (Fig. 1.2c), found mainly in cholic alcohols and acids
Cholestane (Fig. 1.2d), the foundation of sterols and their derivatives

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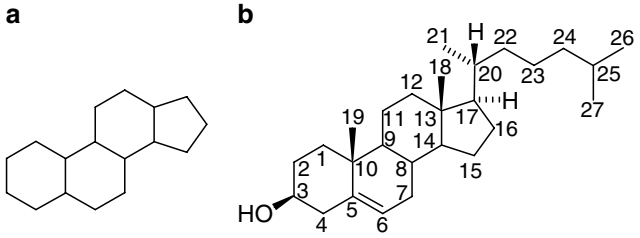

Fig. 1.1 (**a**) Perhydricyclopentenophenanthrene and (**b**) cholesterol and steroid locants

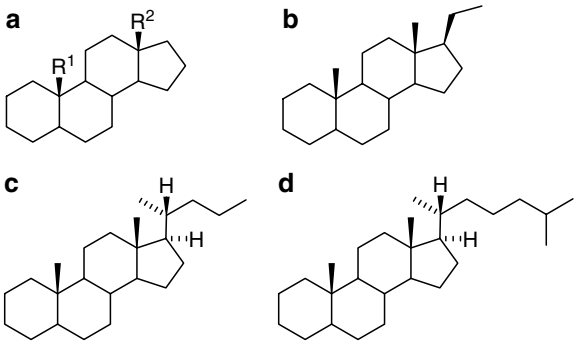

Fig. 1.2 Basic steroid hydrocarbons: (a) gonanes, estranes, androstanes; (b) pregnanes; (c) cholanes; (d) cholestanes

1.1.2 Configuration

(a) *Absolute Configuration* It is fortunate, for scientists and publishers alike, that the absolute configuration of the natural steroid molecule, established by X-ray analysis, corresponds to the one originally and arbitrarily chosen as the conventional representation when the structure of molecular framework in all other respects first became known.

While most steroids are directly or indirectly derived from a relatively small number of natural steroid raw materials, enantiomeric (i.e., mirror image molecules, not super imposable by any rotation of a molecule) steroids are available by total synthesis only. Before enantioselective reactions were contrived, products of total syntheses were 1:1 mixtures of standard steroids with natural absolute configuration and their enantiomers. These mixtures were called racemates. For example, estradiol thus formed was a mixture of estradiol and *ent*-estradiol (Fig. 1.3). It was designated by the prefix *rac*: as *rac*-estradiol, formerly, (±)-estradiol (the symbol indicates that racemates are not optically active).

For a long time, enantiomers were thought to be devoid of biological activity. Recent results, however, have suggested that enantiomers may be active though not via the usual steroid receptor route (Covey et al., 2001). Enantiomers of estradiol, androgens, and progesterone have all been shown to have neuroprotective actions

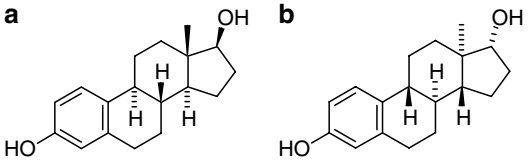

Fig. 1.3 (**a**) Estradiol and (**b**) *ent*-estradiol

as antioxidants or via GABA receptors (Klinger et al., 2002; Simpkins et al., 2004; Li et al., 2006; Van Landingham et al., 2006; Wang et al., 2006; Katona et al., 2007). Optimistic steroid chemists may find that Alice is not the only adventurer in the looking glass world …

(b) *Relative Configuration* Although steroids are in reality not planar molecules, they are represented on paper as planar projection, as illustrated in figures in this book. One has to keep in mind that hydrogen atoms or substituents bound to skeleton point either above the plane (in that case, the bond is drawn as a solid or preferably a wedged line) or below the plane (a broken or preferably a dashed line). The solid line bond is termed a "β-bond", the broken line an "α-bond". If the configuration at a certain point is not known, the bond is drawn as a wavy line and expressed by a Greek letter ξ ("xi").

For all single-bonded substituents at *secondary* carbon atoms in the ring structure, it is necessary to indicate the α- or β-configuration. It is often helpful, but is not obligatory, to include the stereochemical indicators for substituents at the tertiary sites (C-8, 9, 14, and 17), even when these correspond to the natural configuration, which is normally understood (e.g., 9α-fluoro- or 17α-hydroxy- in "17-hydroxyprogesterone").

These rules have been modified several times, however, not all users follow the most recent recommendations, and thus, one has to understand that formulae and names may be slightly different even in recent papers. For example, 3α-hydroxy-5α-pregnan-20-one (Fig. 1.4a), a steroid modulator of γ-aminobutyric acid in neurones, is only exceptionally not called "allopregnanolone". Equally, as another hormone – 3β-hydroxyandrost-5-en-17-one (Fig. 1.4b) – is most often named "dehydroepiandrosterone" (DHEA).

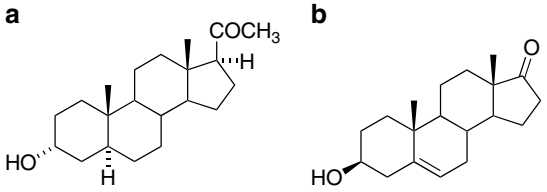

Fig. 1.4 (**a**) 3α-Hydroxy-5α-pregnan-20-one and (**b**) 3β-hydroxyandrost-5-en-17-one

Any steroid compound contains at least six chiral centers, which should theoretically lead to many configurational isomers. In reality, however, the situation is much simpler: configuration at several centers (8β, 9α, 10β, 13β) is constant in all natural

products and synthetic products based on them. The C14-configuration is α in most steroids; only a group of cardiac-active glycosides have the 14β configuration. Although a CIBA conference in 1950 defined the 5β configuration as "natural" (and their 5α – enantiomer as unnatural – allo, i.e. reversed), at the moment, the proportion of 5α and 5β steroids is almost equal. Thus a half of known steroids have the so-called all *trans* configuration: A and B rings are *trans* to each other (i.e., C10–C19 bond is *trans* to C5–H5); equally, B and C, and C and D rings are *trans*. The other half differ in the *cis* arrangment of A and B rings (see solid lines radiating from carbons 5 and 10). Therefore, C5 should be expressed always (with a solid or broken line connecting the hydrogen atom); some authors may forget this detail, which bears a great uncertainty on the whole conformation of the compound (see axial and equatorial bonds, page 8) because both isomers (see e.g. 5α-pregnane and 5β-pregnane in Fig. 1.5) greatly differ both in their chemistry and biological activity. The C5-configuration is not expressed if a compound contains a 4(5) or 5(6) double bond.

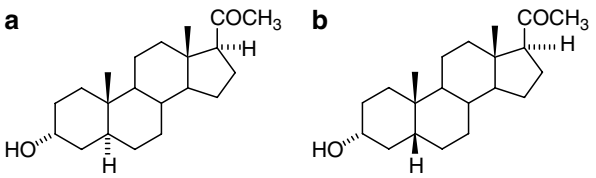

Fig. 1.5 (**a**) 3α-Hydroxy-5α-pregnan-20-one and (**b**) 3α-hydroxyandrost-5β-pregnan-20-one

When there is no ambiguity (i.e., if the C–H bonds are 8β, 9α, and 14α), hydrogen atoms at the bridgehead C8, C9, and C14 are omitted. Exceptionally, if in any of these cases, the configuration is different (unnatural), it must be shown in the name and formula.

Configuration of substituents at the side chain used to be based on a Fisher projection. Fieser and Fieser (1959) proposed an extension of the α/β system, based on the Fischer projection of the side chain, which has been widely used over many years, especially for the naming of such compounds as 'pregnanediol' (5β-pregnane-3α, 20α-diol, according to Fieser and Fieser). It is essential to this system that carbon with the highest number (i.e., the methyl group in Fig. 1.6) is placed on the top and groups projected to left and right are nearer the observer than groups projected in top and bottom positions. In such a conformation, 20α configuration has the substituent on the right side (Y = OH, X = H), 20β configuration is reversed (X = OH, Y = H).

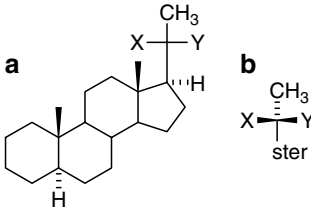

Fig. 1.6 (**a**) Fischer projection of 20 substituted 5α-pregnane derivatives; (**b**) a simplified scheme

The same applies to other positions of the cholestane side chain: always, the same trick is used: any molecule is reduced to a scheme like Fig. 1.7a in which a single carbon is considered and rest of the molecule – however complex – shrinks to four substituents: e.g., brassinolide – a plant growth hormone (Fig. 1.7b) has a complex structure of the steroid core, still, when C24 configuration is considered, its C24 configuration is expressed in the same simple manner: the single carbon 24 is arranged as in Fig. 1.7b: the highest number – C25 carbon – is put on top and below the plane of the paper; the 24-methyl group then appears at the right side and thus deserves the α configuration.

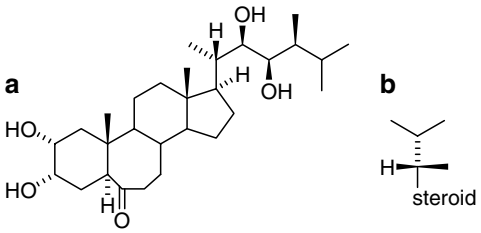

Fig. 1.7 (**a**) Brassinolide, a 24α-methyl steriol and (**b**) schematic view at carbon 24

More recently, International Union of Pure and Applied Chemistry (IUPAC) formulated a recommendation to conform to a wider use of the *R*/*S* system for designating the stereochemistry of the side chain. The procedure of assigning *R* or *S* configuration consists of two steps using the *sequence* and *conversion* rules.

According to the former one, "groups about an asymmetric atom shall be arranged in the order of decreasing atomic number of the atoms by which they are bound to it." This quotation of the IUPAC recommendation is simple e.g., for fluoro-bromo-chloro-methane (Br > Cl > F) or in isotopic derivatives (tritium > deuterium > protium) where priority is easily assigned. It is not so transparent if two or more atoms, attached directly at the chiral centre, are identical (e.g., carbon). Then continuation of the sequence rule should be applied. It reads, "if the relative priority of two groups cannot be thus decided, it shall be determined by a similar comparison of atom numbers of the next atoms in the groups, or, if this fails, of the next" (Cahn et al., 1966; Prelog V and Helmchen G, 1982).

In our former example of 20-hydroxy derivatives (Fig. 1.6) we easily assign the hydroxyl the highest priority (atomic number of oxygen and carbon is 16 and 12, respectively). The H-20 hydrogen atom (the atom number is 1) has the least priority. Two other substituents (carbons C-17 and C-21) compete and the race is decided at the second stage only: C-17 is bound to two other carbons, while C-21 to hydrogens only: C-17 has the second highest priority, C-21 is the third. If the priority were not decided by the second atom in each chain, the search would continue to the third atom in each chain until a deciding difference would be found.

Another rule should be mentioned here: Multiple bonds are treated as duplicated or triplicated single bonds: thus the carbon–carbon double bond counts as if each of the carbon atoms is attached to two other carbon atoms; similarly, the carbon–oxygen double bond is counted as if the carbon atom is bound to two oxygen

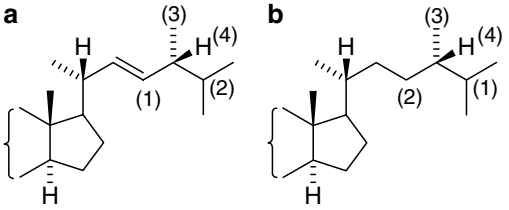

Fig. 1.8a The sequence rule in (**a**) ergosterol and (**b**) dihydroergosterol

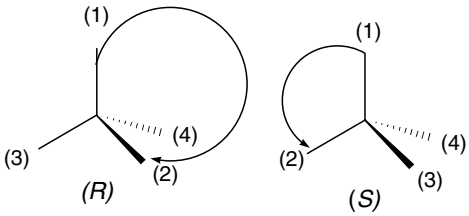

Fig. 1.8b The conversion rule

atoms. Thus, C-24 carbon is treated differently in ergosterol and dihydroergosterol (see Fig. 1.8a).

Having assigned priority to individual substituents at each centre, we turn to the *conversion* rule, which instructs us to view the substituents around the asymmetric atom in question (i.e., C-20 in this case) from an external point on the site remote from the substituent of least priority (usually a hydrogen atom): if then passing from the highest priority substituent to other two substituents according to decreasing priority has the clockwise sense, the configuration is defined to be *R* (from the Latin "*rectus*" meaning "right"). The reverse sense of order is defined as *S* (from the Latin "sinister", i.e., "left") (see Fig. 1.8b).

Motorists might prefer another model: the bond between the carbon considered and the least preferred substituent (usually hydrogen) can be visualized as a shaft holding a steering wheel which carries the remaining substituents; if one turns the wheel from the most preferred substituent to the less preferred one, and the sense is a clockwise one, the configuration is *R*.

The above operations must be carried out for each chiral centre in the molecule, except where the absolute configuration of the fundamental skeleton is implicit in the name, as it is for the skeletal structures of natural steroids. In this case it is necessary to designate *R* or *S* configurations only in side chains, or at any chiral centres not of fixed configuration defined by the class of compound. Thus if a polysubstituted side chain name is based on ergostane (e.g., epibrassinolide, Fig. 1.9), one need not express the C-24 configuration; if it is based on cholestane, the C-24 configuration has to be given.

While for the Fischer projection we had to abstract the particular segment of a molecule and put the highest carbon on top, there is no need to observe strict formalism in the *R*, *S* system: the sequence rule (the assignment of priority to substituents) can be done in any form of structure description, the conversion

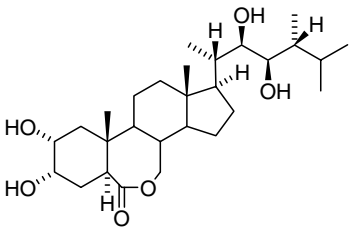

Fig. 1.9 (22*R*,23*R*,24*R*)-2α,3α,22,23-Tetrahydroxy-7-homo-24-methyl-5α-cholestabicarvi- 5,7-lcatone, or (22*R*,23*R*)-2α,3α,22,23-tetrahydroxy-7-homo-5α-ergostabicarbo-5,7-lactone (epibrassinolide)

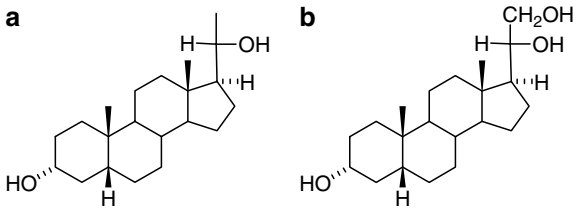

Fig. 1.10 (**a**) (20S)-5β-pregnane-3α,20-diol ('pregnanediol') and (**b**) (20*R*)-5β-pregnane-3α, 20,21-triol

rule (viewing of the space arrangement) can easily be applied anywhere without other requirements.

Although the *R*, *S* system is the most universal system capable of solving general problems with less simple skeletons, the Fischer system may seem favorable in some particular aspects. For instance, if configuration of a center remains constant throughout a reaction sequence, the Fischer-based configuration stays the same while the *R*, *S* description may change with changes of the vicinity of given carbon center. Thus, Fischer's 20β-alcohol (Fig. 1.10) keeps its unchanged configuration and configuration assignment through the hypothetical oxidation of compound (a) to compound (b), but its *R*/*S* assignment is changed.

Analogously, the brassinolide side chain of Fig. 1.11a is derived of campesterol, i.e., of 24α-methylcholest-5-en-3β-ol (Fig. 1.11b): the Fischer projection shows the same assignment in both cases, the *R*, *S* system does not (24*S* for brassinolide, 24*R* for campesterol) in spite of the fact that C-24 configuration in both cases is the same.

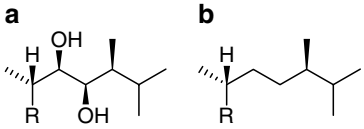

Fig. 1.11 (**a**) Brassinolide side chain and (**b**) campesterol side chain

1.1.3 Conformation: the Three-Dimensional Shapes of Steroids

The steroid ring system is represented in the formulae given in preceding figures as a planar projection but has, in reality, a 3D shape. Each saturated six-membered ring normally adopts a puckered shape like single-ring compound – cyclohexane.

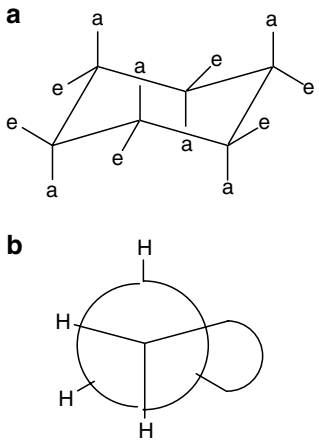

Fig. 1.12 (**a**) Chair conformation of cyclohexane; (**b**) Newman projection

The 'chair' form (Fig. 1.12a) is the most stable (minimum energy) conformation. It allows all the C–C–C bond angles to lie close to the tetrahedral value (~109.5°), and at the same time provides maximum separation of the bonds, which radiate from each linked pair of carbon atoms. This so–called 'staggered' or 'gauche' conformation, which minimizes torsional strains, is best seen from a 'Newman' projection of ethane (Fig. l.12b), in which the molecule is viewed along a C–C bond. The bonds at the front and rear carbon atoms in the diagram appear to make 60° angles with each other. Each ring is actually slightly flattened, so that torsion angles are close to, but not exactly, 60°.

Bonds to the cyclohexane ring fall into two groups, according to their geometric relationship to the ring structure. Those which lie close to the average plane of the ring are termed 'equatorial' bonds ('e' in Fig. 1.12a), and those, which are perpendicular to the average plane, are termed 'axial' bonds ('a' in Fig. 1.12a). The axial/equatorial distinction has considerable importance for chemical (i.e., reactivity) and physical properties (e.g., chromatographic behavior, spectroscopic characteristics) of steroids.

Whilst each six-membered ring approximates in shape to the chair conformation of cyclohexane, the five-membered ring D cannot form a complete chair. Like its monocyclic analogue – cyclopentane, it adopts a non-planar conformation which is often of 'half-chair' or 'envelope' type, adapting its shape in each individual steroid to minimize the strains associated with its mode of linkage to ring C and with any substituents present (Duax and Norton, 1975; Griffin et al., 1984).

The axial/equatorial classification of bonds is straightforward in 5α-steroids (Fig. 1.13a), where all the rings are similarly oriented. In the 5β-series, however, the A/B-*cis* junction causes a sharp bend in the ring structure, with ring A and the other three rings forming a roughly L-shaped whole (Fig. 1.13b). The difference between 5α- and 5β-steroids has a significant effect on their chromatographic mobilities and on their spectroscopic and chemical properties. One immediate consequence, apparent from an inspection of Fig. 1.13a and b, is that the respective axial or equatorial characters of the α- and β-bonds around ring A are reversed by the change of

configuration at C-5. This is easily seen, for example, by looking at the 3α-bond, which is axial in 5α-steroids but equatorial in 5β-steroids. A similar reversal of conformation with respect to ring A is seen at each of the other locants in ring A.

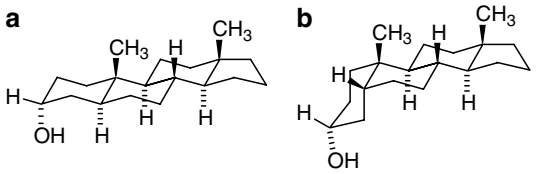

Fig. 1.13 (**a**) 5α-Androstan-3α-ol; (**b**) 5β-androstan-3α-ol

The bonds from five-membered ring D are not truly axial or equatorial: the terms quasi-axial and quasi-equatorial are commonly used to indicate their approximation to these conformations. Similar terminology applies in rings which contain unsaturation, and are partially flattened by the presence of a C=C double bond. Such rings are best described as 'half-chairs', with significant deviation from ideal chair geometry.

1.1.4 Functional Groups

Individual steroids of each skeletal class are characterized by the nature and locations of substituent groups, together with any unsaturation. These 'functional groups', as well as determining the overall shape of the molecule, determine its chemical and physical properties too, and contribute to its specific interactions with cell constituents, including hormone receptors and metabolizing enzyme systems.

Virtually all natural steroids have an oxygen function at C3, which may be in the form of a hydroxyl group in either 3α- or 3β-configuration or a phenolic (acidic) hydroxyl attached to an aromatic A ring in the estrogen series (Figs. 1.14 and 1.15). In certain natural products and in the main urinary metabolites of steroid hormones, the hydroxyl group may be masked by being in the form of a derivative (so called 'conjugates', see Section 1.1.3.1(e)); these derivatives are mostly of very polar nature; being more water-soluble, they are used as a means to rid the body of already spent steroids.

The other oxygen function commonly found at C-3 is a carbonyl (or 'oxo') group, where oxygen is double-bonded to carbon and the compound has the characteristics of a ketone (Fig. 1.15). All hormonally active steroids however, except estrogens, have the Δ^4-3-oxo group in the A-ring and catabolism of these active steroids involves inactivation by tetrahydro-reduction in the A-ring. 5α-Dihydrotestosterone formed in androgen-target cells is an exception to this, retaining potent activity even though the Δ^4 double bond is reduced. 5α-Dihydrotestosterone is even claimed to be the true androgen hormone (Degtyar et al., 2006).

Hydroxyl or carbonyl groups may, in principle, replace hydrogen at any of the skeletal sites where the requisite number of bonds is available. Examples of all possible

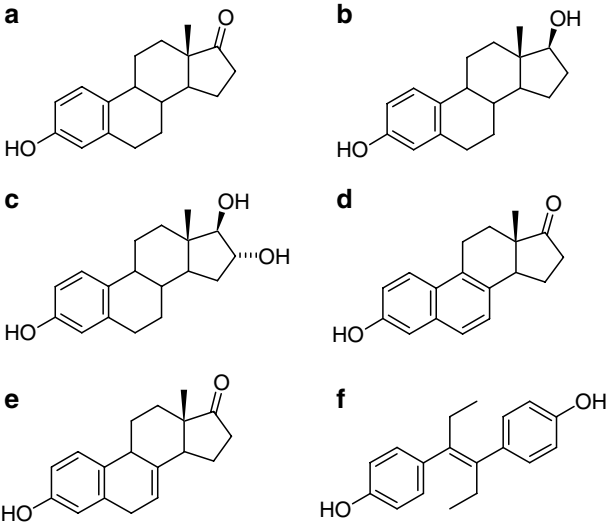

Fig. 1.14 (**a**) Estrone (3-hydroxyestra-1,3,5(10)-trien-17-one), (**b**) estradiol (estra-1,3,5(10)-trien-3,17β-diol), (**c**) estriol (estra-1,3,5(10)-trien-16α,17β-diol), (**d**) equilenin (3α-hydroxy-estra-1,3,5,7,9-pentaen-17-one), (**e**) equilin (3α-hydroxy-estra-1,3,5(10),7-tetraen-17-one), and (**f**) DES (diesthylstilbestrol)

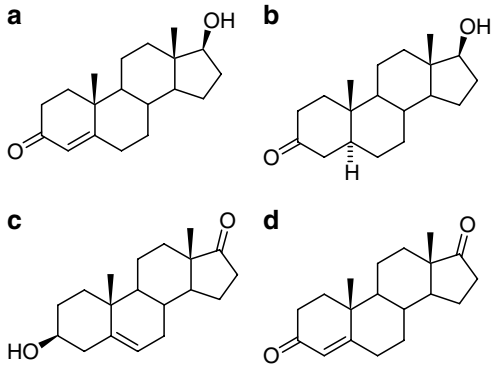

Fig. 1.15 (**a**) Testosterone (17β-hydroxyandrost-4-en-3-one), (**b**) dihydrostestosterone (17β-hydroxy-5α-androstan-3-one), (**c**) DHEA (dehydroepiandrosterone, 3β-hydroxyandrost-5-en-17-one), and (**d**) androstenedione (androst-4-en-3, 17-dione)

sites of hydroxylation, and of almost all possible sites for carbonyl groups, have been identified among the many steroids of natural origin. The most usual locants for substituents, other than C-3, include C-11, where the principal corticosteroids and their metabolites have either an 11-oxo or an 11β-hydroxy group, and C-17, where a 17-oxo group occurs in estrone and in various compounds of the androgen series, a 17β-hydroxy group in estradiol and testosterone, and a 17α-hydroxy group in some of the natural steroids of the pregnane type (e.g. the glucocorticoids, and 'pregnanetriol'). Whenever a single-bonded group like hydroxyl (OH) replaces one

of the hydrogen atoms at a methylene (CH_2) site of a steroid molecule, its configuration must be specified, as in the preceding examples.

The other most common sites for hydroxylation include C-21, in the corticosteroids, C-7 and C-12, in the principal bile acids, and C-1 and C-25, in the hormonally active metabolites of vitamin D (see Section 1.1.3.2).

Substitution of hydrogen atoms in the angular methyl groups (C-18 or C-19) is relatively frequent in metabolic processes. Even the conversion of testosterone into estradiol and estrone is launched by oxidation at the carbon C-19. The most important natural product of this type is aldosterone, where C-18 is oxidized to the level of an aldehyde (see Fig. 1.17f), although the aldehyde group is masked by hemiacetal formation (see Chapter 2, p. 62). Carboxyl groups are found mainly in the bile acids (Fig. 1.17), where the terminal C-24 position of cholane side chain is oxidized to a carboxyl group in the commonest series, the cholanic acids (cholic, deoxycholic, chenodeoxycholic, lithocholid and other). Carboxylic acids may also occur at other sites (e.g., C-18, C-19, C-21, C-26).

Unsaturation is the other most common feature found in steroids. Alkene-type double bonds (C=C) occur mainly at the 4,5- or 5,6-position. 7-Dehydrocholesterol and ergosterol, the provitamins D, are 5,7-dienes. Vitamins D_2 and D_3 themselves are conjugated trienes (see Section 1.1.3.2), whereas the estrogens (Fig. 1.14) have a fully unsaturated (aromatic) ring. In equilenin (Fig. 1.14d), both A and B rings are aromatic. Equilin (Fig. 1.14e), one of dihydro derivatives of equilenin, has become the most widely sought remedy against osteoporosis. The non-steroidal synthetic estrogen – diethylstilbestrol (Fig. 1.14f) also possesses aromatic rings, which appear to be essential feature for estrogenicity. Figures 1.4–1.7 illustrate some of the principal estrogen hormones and their metabolites. The system of nomenclature is discussed in Section 1.1.2.

The list of basic steroid structures should mention compounds which represent the source of material for large scale steroid production: nowadays, most steroids are produced by partial synthesis starting from diosgenin (Fig. 1.16a), which is degraded into dehydropregnenolone (3β-hydroxypregna-5,16-dien-20-one) and then converted into androstane and pregnane products. Sterols (e.g., sitosterol – Fig. 1.16c), present in industrial waste, can also be used as starting material for oxidation by *Mycobacterium* mutants into androst-1,4-diene-3,17-dione (ADD). Cholanic acids (e.g., Fig. 1.16e) were the starting material for the synthesis of 11-substituted pregnane compounds. Some other materials were also used but have been pushed out of business mainly due to low cost of diosgenin.

1.2 Steroid Nomenclature

In early days of steroid biochemistry, three major groups were involved in isolation and identification of naturally occurring steroids, allocating each new compound a letter of the alphabet in sequence as they were isolated. Inevitably steroids were not isolated in the same sequence and thus cortisol, the main glucocorticoid, was

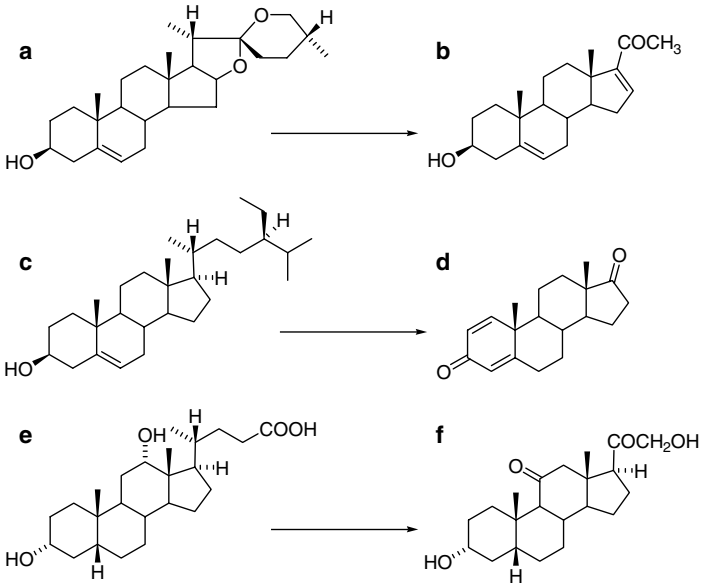

Fig. 1.16 (**a**) Diosgenin ((25*R*)-spirost-5-en-3β-ol), (**b**) dehydropregnenolone (3β-hydroxypregna-5,16,diene), (**c**) β-sitosterol (24*R*)-cholest-5-en-3β-ol, (**d**) ADD (androsta-1,4-diene-3,17-dione), (**e**) deoxycholic acid (3α-12α-dihydroxy-5β-cholanic acid), and (**f**) 3α-21-dihydroxy-5β-pregnane-11,20-dione

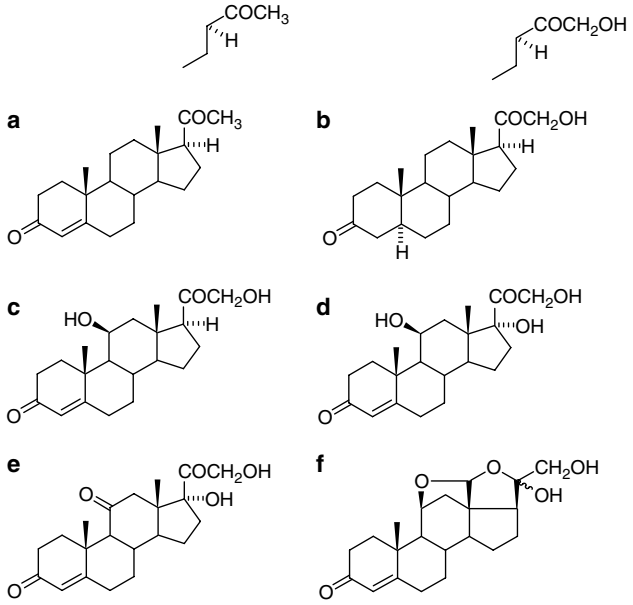

Fig. 1.17 (**a**) Progesterone (pregn-4-ene-3,20-dione), (**b**) deoxycorticosterone (21-hydroxy-pregn-4-ene-3,20-dione), (**c**) corticosterone, 11β,21-dihydroxypregn-4-ene-3,20-dione), (**d**) cortisol (11β,17,21-trihydroxypregn-4-ene-3,17-dione), (**e**) cortisone (17-hydroxypregn-4-ene-3,11,20-trione), and (**f**) aldosterone (11β,21-dihydroxypregn-4-ene-3,20-dione)

Kendall's compound F, Reichstein's compound M and Wintersteiner's compound F. This confusion is admirably resolved in a review by Reichstein and Shoppee (1943). Although these alphabetic designations have long since been formally replaced by IUPAC recommended names, elderly biochemists are still wont to use them as a convenient shorthand (e.g. THF is tetrahydrocortisol and THS is tetrahydro-11-deoxycortisol).

Many trivial names are still used more often than their orthodox equivalents; e.g., everybody speaks about progesterone but few name it as pregn-4-ene-3,20-dione (Fig. 1.17a). These names have faded out, though some other still linger on due to the increasing number of authors of medicinal and biological vocation who consult textbooks of their youth. Then 5α-androstane was called androstane, 5β-androstane was testane or etiocholane; 5α-pregnane was called allopregnane, 5β-pregnane was just pregnane; similarly, 5α-cholane was termed allocholane, and 5β-cholane cholane; cholestane meant 5α-cholestane, its 5β-isomer was called coprostane.

Table 1.1 gives trivial names that are still widely used for steroid hormones and for some of their principal precursors and metabolites. Some are contractions of the full UPAC/IUB-approved systematic names, while others derive from names of their source or biological activity. Many of the corticosteroids are also commonly

Table 1.1 Selected trivial names of some steroids

Aldosterone	11β,18-epoxy-18-hydroxypregn-4-ene-3, 20-dione (18,11-hemiacetal)
Androsterone	3α-Hydroxy-5α-androstan-17-one
Chenodeoxycholic acid	3α,7α-Dihydroxy-5β-cholan-24-oic acid
Cholesterol	Cholest-5-en-3β-ol
Cholic acid	3α,7α,12α-Trihydroxy-5β-cholan-24-oic acid
Corticosterone (Kendall's B[a], Reichstein's H)	11β,21-Dihydroxypregn-4-ene-3,20-dione
Cortisol[b] (hydrocortisone, Kendall's F[a])	11β,17,21-Trihydroxypregn-4-ene-3,20-dione
Cortisone (Kendall's E[a], Reichstein's F[a])	17,21-Dihydroxypregn-4-ene-3,11,20-trione
Dehydrocorticosterone (Kendall's A)	21-Hydroxypregn-4-ene-3,11,20-trione
Dehydroepiandrosterone (DHEA)	3β-Hydroxyandrost-5-en-17-one
Deoxycholic acid	3α,12α-Dihydroxy-5β-cholan-24-oic acid
Deoxycorticosterone (DOC, Reichstein's Q)	21-Hydroxypregn-4-ene-3,20-dione
Deoxycortisol (Reichstein's S)	17,21-Dihydroxypregn-4-ene-3,20-dione
Estradiol-17α[b]	Estra-l,3,5(10)-triene-3,17α-diol
Estradiol-17β[b]	Estra-l,3,5(10)-triene-3,17β-diol
Estriol[b]	Estra-1,3,5(10)-triene-3,16α,17β-triol
Lithocholic acid	3α-Hydroxy-5β-cholan-24-oic acid
Pregnenolone	3β-Hydroxypregn-5-en-20-one
Progesterone	Pregn-4-ene-3,20-dione
Testosterone	17β-Hydroxyandrost-4-en-3-one
Ursodeoxycholic acid	3α,7β-Dihydroxy-5β-cholan-24-oic acid

[a] Designation according to Kendall.

[b] Cortisol is identical with Reichstein's compound *M* and Wintersteiner's compound *F*.

designated by single letters of the alphabet, which originated from the order in which they were isolated from adrenal extracts by Kendall, Reichstein, or Wintersteiner (Fieser and Fieser, 1959). The trivial names of steroids, and the alphabetic designations of corticosteroids, are almost universally employed by those working in medical areas. International non-proprietary names (INNs) are available for many synthetic steroids in pharmaceutical use (Hill et al., 1991).

The nomenclature rules were put into detailed and definitive form in the early 1970s and were revised in 1989 (Moss, 1989). Steroids illustrated in this book are generally given both their full systematic names and their approved trivial names or INNs and common synonyms. The rules for steroid nomenclature follow quite closely those for other organic compounds, with special features to accommodate the stereochemical aspects and wide variety of structural and substitution types which come under the broad classification of steroids. We are concerned here only with those relatively straightforward rules that are sufficient for naming the steroids of biomedical importance that occur in the present text. The reader interested in more specialized aspects of nomenclature is referred to the full (40-page) statement of the rules (Moss, 1989; http://www.chem.qmul.ac.uk/iupac/steroids).

1.2.1 Procedure for Naming a Steroid

The system for naming a steroid is outlined in the following paragraphs. The reader should take note of punctuation, including hyphenation, of names that are given here as examples, and those which appear on later pages.

(a) *Hydrocarbon Class* Select the appropriate hydrocarbon skeleton (see Fig. 1.2). Note that the name of each saturated hydrocarbon follows the rule for simple hydrocarbons (methane, ethane, etc.) in having the ending '-ane'. If the steroid is saturated at C-5, prefix the skeletal name with C-5 configuration (e.g., 5β-pregnan-20-one). If there is an unnatural or uncommon configuration at any other ring junction position, indicate it (e.g., 5β, 14β, 17α-pregnan-20-one) (Fig. 1.18).

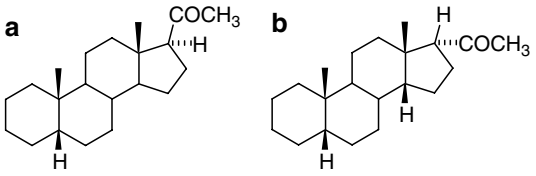

Fig. 1.18 (**a**) 5β-Pregnan-20-one and (**b**) 5β,14β,17α-pregnan-20-one

(b) *Unsaturation* Any unsaturation is indicated by replacing the terminal 'ane' by 'ene', 'diene', 'triene', 'yne', etc., according to the number of double (or triple) bonds present, preceded by locants. Unsaturation between consecutively-numbered carbon atoms (e.g. 5,6) is indicated by the lower locant (see cholest-5-ene

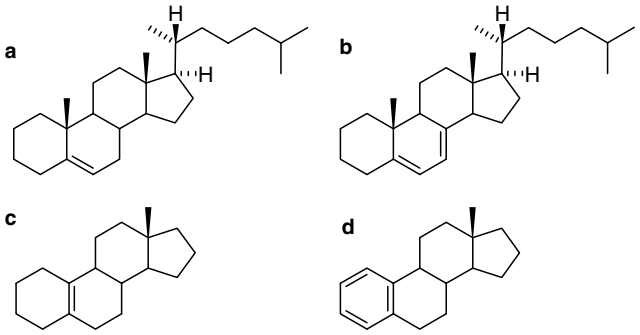

Fig. 1.19 (**a**) Cholest-5-ene, (**b**) cholesta-5, 7-diene; estr-5(10)-one, (**c**) estr-5(10)-ene, and (**d**) estra-1,3,5(10)-triene

and cholesta-5,7-diene; Fig. 1.19). Note, in the latter example, that 'a' (e.g., cholesta-5,7-diene) is added to the stem of the name when a consonant follows, to aid pronunciation. Non-consecutive locants, as, for example, a double bond between C-5 and C-10, both must be indicated, with the higher number in parentheses (e.g. estr-5(10)-ene). The aromatic ring A of estrogens is expressed as in 'estra-l,3,5(10)-triene' (Fig. 1.19). These locants for unsaturation are chosen in preference to those of the alternative Kekule form: 1(10),2,4.

However, when there is a possibility of using all single rather than compound locants, the former are preferred, as in estra-l,3,5,7,9-pentaenes (see equilenin, Fig. 1.14). The use of expressions like Δ^5 to denote unsaturation is no longer approved, except in generic terms (e.g., 'Δ^5 steroids').

The preceding two steps define full hydrocarbon skeleton of steroid. Further, it is necessary to designate all substituents, as described in the following paragraphs.

(c) *Substituent Atoms or Groups* Substituent groups are indicated either in suffix or in prefix form, as in general organic nomenclature. If more than one of the common types of substituent group is present it is necessary to select the one of highest priority, from those listed in Table 1.2, to comprise the suffix. Thus, for example, an oxo group has higher priority than a hydroxy group and so on: testosterone is named as 17β-hydroxyandrost-4-en-3-one and not 3-oxoandrost-4-en-17β-ol.

The selected suffix, in multiple form (di-, tri-, etc.) if necessary, and with locants, follows the skeletal hydrocarbon name, with omission of the final 'e' of the hydrocarbon if a vowel follows. Examples include 17β-hydroxyandrosta-4, 6-dien-3-one and androsta-1,4-diene-3,17-dione (Fig. 1.20), and pregn-4-ene-3, 20-dione (Fig. 1.17).

Any remaining substituents are indicated as prefixes, in alphabetical order, multipliers (di-, tri-, etc.) are ignored in this alphabetical ordering (thus, 2β,3α-diamino-9α-fluoro-5α-androstan-17-one is correct and 9α-fluoro-2β,3α-diamino-5α-androstan-17-one not). Locants precede the substituent names to which they

Table 1.2 Substituents in order of priority for selection of suffix

Group	Suffix form	Prefix form
Carboxylic acid	-oic acid[a]	—
Lactone	-lactone[b]	—
Ester or salt (of carboxylic acid)	-oate[c]	—
Aldehyde	-al	oxo-
Ketone	-one	oxo-
Hydroxyl	-ol	hydroxy-
Amino	-amine	amino-

[a]The alternative suffix '-carboxylic acid' is used if the acid group represents a carbon atom additional to those of the parent hydrocarbon skeleton.
[b]The lactone locants are preceded by 'o', replacing the terminal 'e' of the hydrocarbon, e.g. cholano-24,17-lactone; the alternative suffix '-carbolactone' is used if the lactone carbonyl group represents an added carbon atom.
[c]The alkyl group or cation precedes the main part of the name, e.g. methyl 5α-cholan-24-oate, or sodium 5α-cholan-24-oate.

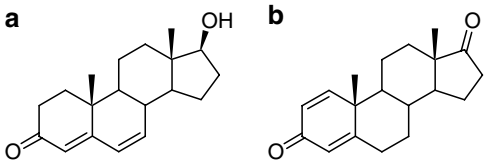

Fig. 1.20 (**a**) 17β-Hydroxyandrosta-4-,6-dien-3-one and (**b**) androsta-1,4-diene-3,17-dione

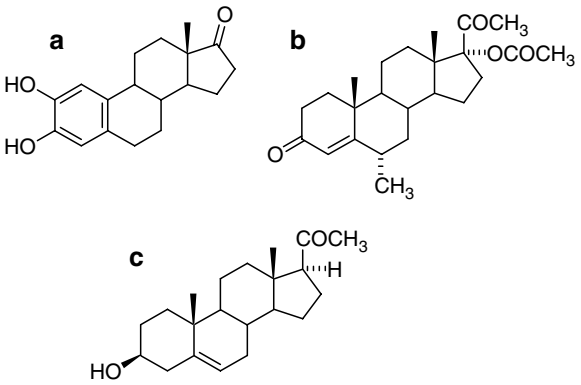

Fig. 1.21 (**a**) 2,3-Dihydroxyestra-1,3,5(10)-trien-17-one (2-hydroxyestrone), (**b**) 6α-methyl-3,20-dioxopregn-4-en-17β-yl acetate ('medroxyprogesterone acetate'), and (**c**) 3β-hydroxypregn-5-en-20--one ('pregnenolone')

refer. Composite suffixes (e.g. 'olone', as in 'pregnenolone'), are not permitted by IUPAC/IUB rules, although they are likely to remain in everyday use because of their convenience in trivial names like the one cited. Figures 1.4–1.7 contain several examples that illustrate use of prefixes. Others are given in Fig. 1.21.

(d) *Esters, Ethers, and Other Derivatives of Alcohols* In most recent recommendations, esters are named by replacing the terminal 'e' of the hydrocarbon name, or 'ol' of the alcohol name, by 'yl', to generate the radical name (compare 'ethane', which becomes 'ethyl'); 'yl' is preceded by its locant and any

multipliers, in the usual way. The acyloxy group is then indicated in anionic form, leading to names like cholest-5-en-3β-yl acetate ('cholesteryl acetate'; compare 'ethyl acetate').

Since esters take precedence over oxo (aldehyde or ketone) groups, systematic names of common steroidal esters do not necessarily derive directly from those of their parent alcohols. Thus medroxyprogesterone is 17β-hydroxy-6α-methylpregn-4-ene-3,20-dione, while its acetate is 6α-methyl-3,20-dioxopregn-4-en-17β-yl acetate (Fig. 1.21). Analogously, testosterone acetate is 3-oxoandrost-4-en-17β-yl acetate, the older form – 17β-acetoxyandrost-4-en-3-one – is no longer recommended.

For esters of diols and triols, names of the forms 'androst-5-ene-3α,17β-diyl diacetate' and '3α-hydroxyandrost-5-en-17β-yl acetate' are likely to be encountered. Where a steroid has an accepted trivial name, which implies the locants of any hydroxyl groups present, it is permissible and often convenient to use prefixes of the form '*O*-acyl' (e.g. '3-*O*-acetylcholic acid', Fig. 1.22).

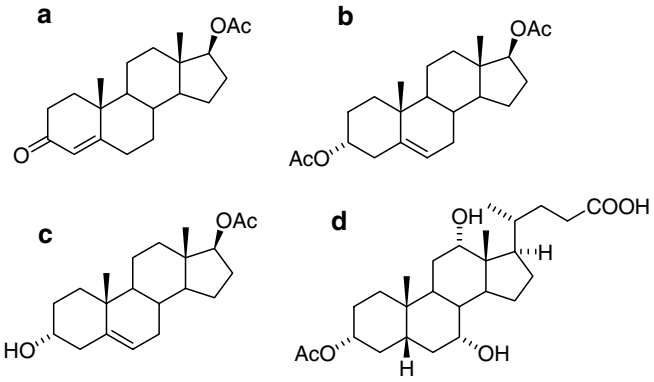

Fig. 1.22 (**a**) 3-Oxoandrost-4-en-17β-yl acetate (17β-acetoxyandrost-4-en-3-one; 'testosterone acetate'), (**b**) androst-5-ene-3α,17β-diyl acetate (3α,17β-diacetoxyandrost-5-ene), (**c**) 3α-hydroxyandrost-5-en-17β-yl acetate (androst-5-ene-3α,17β-diol 17-acetate), and (**d**) 3α-Acetoxy-7α,12α-dihydroxy-5β-cholan-24-oic acid (3-*O*-acetylcholic acid)

Steroid sulphates are named similarly. Thus sulphate of 3β-hydroxyandrost-5-en-17-one ('dehydroepiandrosterone'; DHEA) is 17-oxoandrost-5-en-3β-yl sulphate (more correctly, 'hydrogen sulphate' for the acidic form). Strictly, such names refer to the anion; the cation may be specified if appropriate (e.g. sodium 17-oxoandrost-5-en-3β-yl sulphate, sodium DHEA sulphate, DHEAS) (Fig. 1.23).

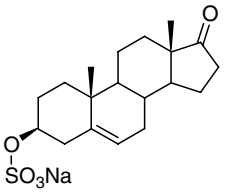

Fig. 1.23 Sodium 17-oxoandrost-5-en-3β-yl sulphate (sodium DHEA sulphate)

Ethers of hydroxy derivatives are indicated in prefix form (e.g., 3β-methoxy-, or 21-trimethylsilyloxy-). The nomenclature rules do not specifically cover the important urinary metabolites known as 'glucuronides', or similar sugar derivatives. The term 'glucuronide' is widely used and understood as an abbreviation for glucosiduronic acids, or even the fuller version glucopyranosiduronic acids, and their salts, the glucosiduronates (glucopyranosiduronates) (Fig. 1.24). Unless otherwise specified, these names refer to the β-D-isomers (Fig. 1.17), derived by linkage of a steroid alcohol to the hemiacetal carbon atom of D-glucuronic acid.

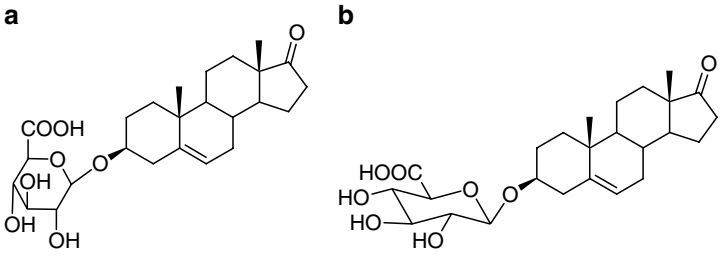

Fig. 1.24 A glucuronide, with the sugar moiety shown in (**a**) conventional and (**b**) conformational form

(e) *Derivatives of Carboxylic Acids* Esters and salts are generally named by use of suffixes (Table 1.2), as are amides (e.g., cholan-24-amide). The important conjugates of bile acids with glycine and taurine ($RCONHCH_2CO_2H$ and $RCONH(CH_2)_2SO_3H$, respectively, where R is the steroid residue) are not covered by the nomenclature rules: they are commonly indicated by the prefixes 'glyco' and 'tauro', respectively (e.g., glycocholic acid), or alternatively named as, for example, *N*-cholylglycine and *N*-cholyltaurine.

(f) *Geometric Isomerism* Unsaturation in the intact ring system needs no geometric indication, being fixed by ring geometry. In the side chain, the older terms *cis* and *trans* as descriptors of configurations about double bonds are now replaced by the more precise sequence rule terms (*Z*) and (*E*), respectively.

The four bonds that radiate from the two ends of a C=C double bond are coplanar. Since rotation about the double bond is prevented by π-bonding, the possibility of isomerism arises. In simple cases of 1,2-disubstituted alkene-type bonds, substituents may be *cis* or *trans* related. However, these terms are not applicable when three or four substituents are present on the unsaturated centers.

The following system is now preferred for use in all cases. The sequence rules, as summarized above (see Section 1.2b), are applied in turn to the pair of atoms or groups at each end of double bond. If atoms or groups of higher priority at each unsaturated atom lie on the same side of double bond, the configuration is *Z* (German: *zusammen*, together). If higher-priority atoms or groups are on opposite sides the configuration is *E* (German: *entgegen*, opposite). In 1,2-disubstituted

C=C bonds, *Z* corresponds to *cis*, and *E* corresponds to *trans* (Fig. 1.25) (Figures A.4 and A.5). Similar principles are used in defining the configurations about C=N bonds in oximes and related compounds.

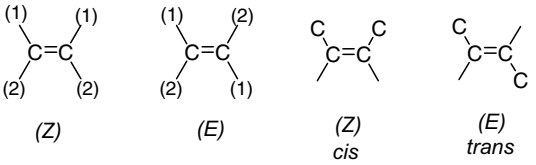

Fig. 1.25 *Z* and *E* configurations

The rules as summarized above should enable the reader to understand the assignments of configuration that occur in Table 1.3 and elsewhere in this chapter. Note that systematic nomenclature places *R*/*S* and *E*/*Z* descriptors, with locants if necessary, in parentheses at the beginning of the name of a compound.

Ergosterol (Fig. 1.26), for example, has the (22*E*)-configuration. The sequence rule is recommended also for describing the configuration around double bonds in vitamin D series (Section 1.1.3.2).

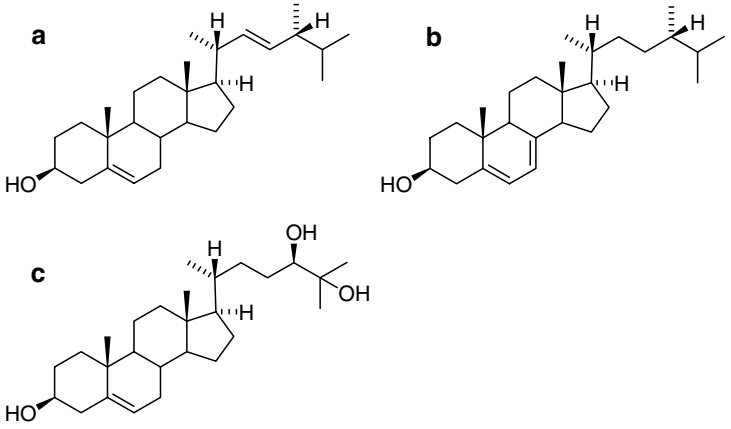

Fig. 1.26 (**a**) (22*E*)-Ergost-5,7,22-trien-3β-ol (ergosterol), (24*R*), (**b**) ergosta-5,7-dien-3β-ol (22,23-dihydroergosterol) (24*S*), and (**c**) (24*R*)-cholest-5-ene-3β,24,25-triol ((24*R*)-24,25-dihydroxycholesterol)

(g) *Skeletal Modifications* Absence of a carbon atom from one of the fundamental hydrocarbon structures is indicated by the prefix nor-, preceded by the locant of the missing carbon atom. The use is illustrated by 19-norpregnane (Fig. 1.27). Several of the synthetic ovulation inhibitors are strictly 19-nor-17α-pregnane derivatives: the simplest is 'norethisterone' (17β-hydroxy-19-nor-17α-pregn-4-en-20-yn-3-one; Fig. 1.27). 'Ethynylestradiol' (Fig. 1.27) is named systematically as 19-nor-17α-pregna-l, 3,5(10)-trien-17-yne-3,17-diol (17β- can be stated for clarity if desired). Note that 'estrane' is used in preference to '19-norandrostane',

although the trivial name '19-nor-testosterone' is commonly used for the anabolic steroid 17β-hydroxyestr-4-en-3-one.

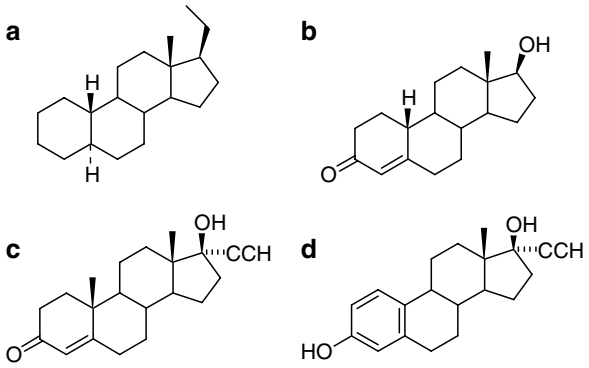

Fig. 1.27 (**a**) 19-Nor-5α-pregnane, (**b**) 17β-hydroxyestr-4-en-3-one (19-nortestosterone), (**c**) 17β-hydroxy-19-nor-17α-pregn-4-en-20-yn-3-one (17α-ethynyl-17β-hydroxyester-4-en-3-one, 19-norethisterone), and (**d**) 19-nor-17α-pregn-1,3,5(10)-trien-20-yne-3,17β-diol (17α-ethynylestra-1,3,5(10)-triene-3,17β-diol; ethynylestradiol)

Other uses for the nor- prefix occur if a ring is contracted (e.g. 4-nor-5α-androstane, or 7-norcholesterol, see Fig. 1.28). Formerly, these compounds were termed A-nor-5α-androstane and B-norcholesterol, respectively, which is not recommended any more. Missing carbons in the side chain are also termed in this way (e.g., in 24-nor-5β-cholan-23-oic acid or 23,24-dinor-5β-cholan-22-oic acid, Fig. 1.28).

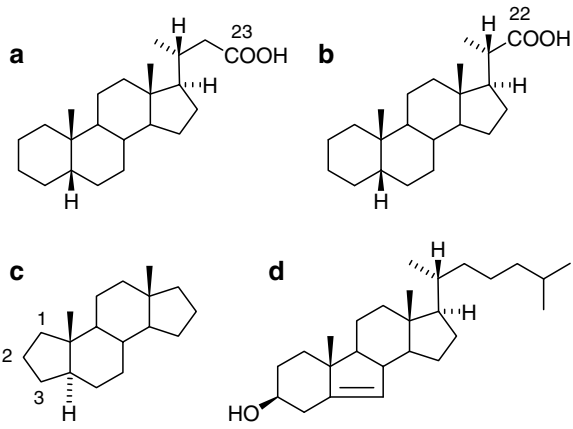

Fig. 1.28 (**a**) 24-Nor-5β-cholan-23-oic acid, (**b**) 23,24-dinor-5β-cholan-22-oic acid, (**c**) 4-Nor-5α-androstane (A-nor-5α-androstane), and (**d**) 7-norcholest-5-en-3β-ol

The prefix homo- similarly indicates added carbon atoms. Until recently an enlarged ring has been indicated as, for example, 'D-homoandrostane', but it is now recommended that the extra carbon be specified instead by its locant (Fischer et al. 2003).

This results in names of the form '17a-homo-5α-androstane' (Fig. 1.29). Scission of a ring is signified by the prefix seco-, with the locants of two carbon

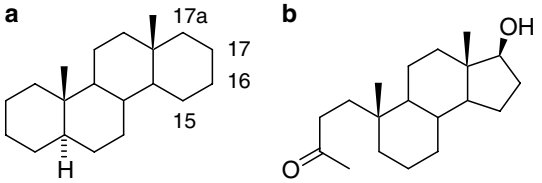

Fig. 1.29 (**a**) 17a-Homo-5α-androstane (D-homo-5α-androstane) and (**b**) 17β-hydroxyl-4(5) secoandrostan-3-one (4,5)-secodihydrotestosterone

atoms where the break occurs (Hu Y and Covey DF, 1993). The best known examples are the compounds of the vitamin D3 series (Fig. 1.30), which are derivatives of 9,10-secocholesta-5, 7,10(19)-triene, or the corresponding 9,10-secoergostanes.

1.2.2 Nomenclature of the Vitamin D Series

On UV irradiation of 5,7-unsaturated steroids, corresponding photoisomers are formed. History of D vitamins knows lumisterols, tachysterols, precalciferols and eventually calciferols (IUPAC-IUB, 1982). 7-Dehydrocholesterol eventually yields cholecalciferol or calciol, its systematic name being (5*Z*,7*E*)-(3*S*)-9,10-secocholesta-5,7,10(19)-trien-3-ol, Fig. 1.30b, $R^1 = R^2 = R^3 = H$. Since the B ring is split and the A ring is rotated, the substitutents which used to be above the plane are below and use of Greek letters is ambiguous. Therefore, the (*R*/*S*) system is also used for designating configurations of substituents in ring A. Other photoisomers are not given in this brief summary, they differ in the position and configuration of the double bonds between rings A and C (see Fig. 1.7).

Skeletal aspects of vitamin D nomenclature are discussed above. Table 1.3 gives trivial and systematic names for some of the more important compounds, by way of illustration. Additional hydroxyl groups may be indicated by prefixing the recommended trivial names, e.g. (1*S*)-l-hydroxycalciol.

Table 1.3 Nomenclature in the vitamin D series

Current trivial name[a]	Recommended trivial name[a]	Systematic name[a]
Cholecalciferol (vitamin D3)	Calciol or cholecalciferol	(5*Z*,7*E*)-(3*S*)-9,10-secocholesta-5,7,10(19)-trien-3-ol, Fig. 1.30b, $R^3 = H$
25-Hydroxycholecalciferol	Calcidiol	(5*Z*,7*E*)-(3*S*)-9,10-secocholesta-5,7,10(19)-triene-3,25-diol, Fig. 1.30b, $R^1 = OH$, $R^2 = R^3 = H$
lα,25-Dihydroxycholecalciferol	Calcitriol	(5*Z*,7*E*)-(l*S*,3*R*)-9,10-secocholesta-5,7,10(19)-triene-l,3,25-triol, Fig. 1.30b, $R^1 = R^2 = OH$, $R^3 = H$
(24*R*)-1α,24,25-Trihydroxycholecalciferol	Calcitetrol	(5*Z*,7*E*)-(l*S*,3*R*,24*R*)-9,10-secocholesta-5,7,10(19)-triene-l,3,24,25-tetrol, Fig. 1.30b, $R^1 = R^2 = R^3 = OH$

[a]Corresponding names in the ergosterol series are ergocalciferol (vitamin D2), ergocalciol, ergocalcidiol, etc., with systematic names based upon (22*E*)-9,10-secoergosta-5,7,10(19),22-tetraene.

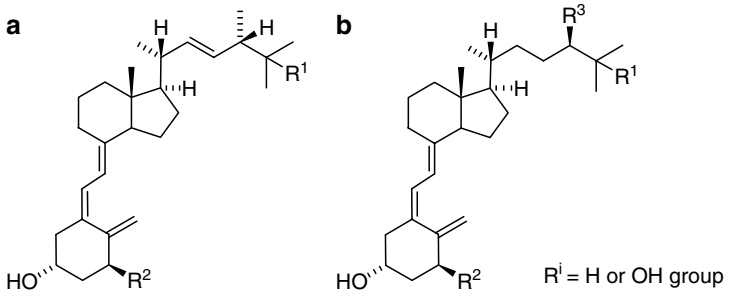

Fig. 1.30 (**a**) Ergocalciferol (D2 vitamin) and its hydroxyl and dihydroxy derivatives and (**b**) cholecalciferol (D3 vitamin) and its hydroxy, di- and trihydroxy derivatives

1.2.3 Nomenclature of Steroids Derived of a Heteroatom-Containing Skeleton

Although even other compounds can be classified as derivatives of basic steroid hydrocarbons, it would be cumbersome in some cases. Although diosgenin is actually a derivative of the cyclic form of 3β,16β,26-trihydroxycholest-5-en-22-one, the IUPAC rules recommend the use of (25ξ)-5ξ-spirostane as the ground for

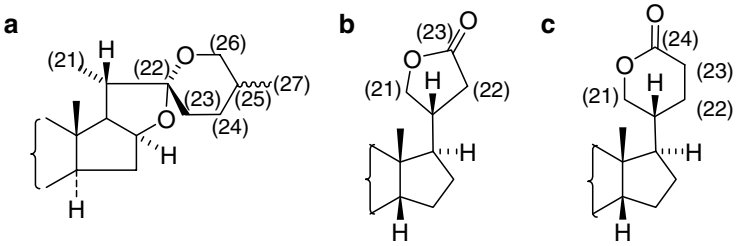

Fig. 1.31 (**a**) Spirostane, (**b**) cardanolide, and (**c**) bufanolide

naming these compounds (Fig. 1.31a).

Similarly, cardanolide is a lactone derived of 24-nor-14β-cholanic acid (Fig. 1.31b), as well as bufanolide is a lactone derived of 14β-cholanic acid (Fig. 1.31c).

Notice the 14β-configuration in cardanolide and bufanolide: this configuration is considered "normal" in cardioactive glycosides. Aglycons of some of them (strophantidin and scillarenin) are shown in Fig. 1.32.

In spirostans, both 25*R* and 25*S* derivatives are found in the nature, thus C-25 configuration has also to be specified. Figure 1.32a shows the structure of diosgenin, the most important raw material for industrial production of steroids. The chair conformation of the spirostane six-membered ring means that the 25-isomers differ in having the 25-methyl group in axial (25*S*) or equatorial (25*R*) position.

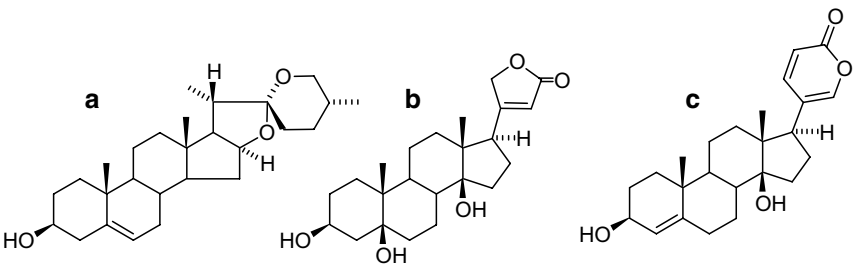

Fig. 1.32 (**a**) Diosgenin, (**b**) strophantidin, and (**c**) scillarenin

Another biogenic element can be found in steroids: nitrogen. Plants of the genus *Solanum* contain glycosidic alkaloids whose aglycons can also be named as cholestane derivatives. In spite of that new names were designed: 5ξ-solanidan and 5ξ-solasodan (Fig. 1.33). Solasodin and solanidin are the most frequently found aglycons of this kind.

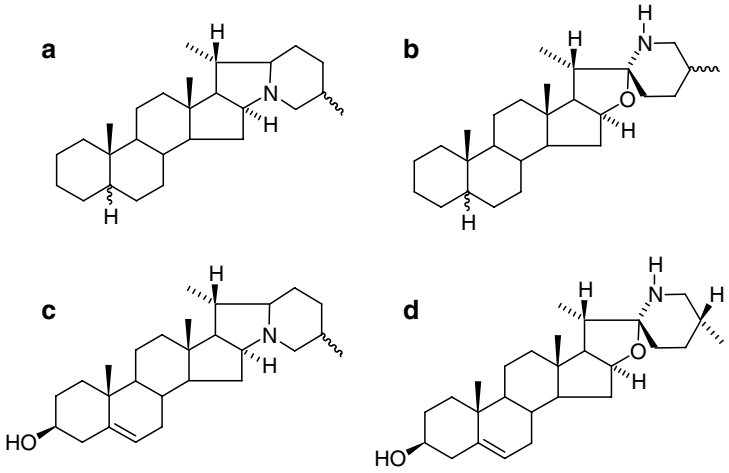

Fig. 1.33 (**a**) 5ξ-Solasodan, (**b**) 5ξ-solanidan, (**c**) solasodin, and (**d**) solanidin

Steroid skeleton can be found in yet another group of alkaloids: several alkaloids from *Holarrhena antidysenterica* and others are derivatives of (20*S*)-*N*-methyl-18,20-imino-5α-pregnane (Fig. 1.34), the steroid core, however, is called 5α-conane. The most abundant alkaloids of this family are conessine and holarrhimine.

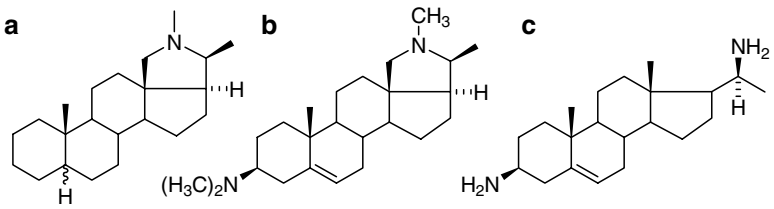

Fig. 1.34 (**a**) 5ξ-Conane, (**b**) conessine, and (**c**) holarrhimine

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Chapter 2
Spectroscopic Methods of Steroid Analysis

Alexander Kasal, Milos Budesinsky and William J. Griffiths

2.1 Introduction: Historical Perspective

Modern chemical laboratories contain equipment capable of measuring many of the physical properties of single chemical compounds and mixtures of compounds, particularly their spectral properties, which can, if interpreted correctly, provide valuable information about both structure (of single compounds) and composition (of mixtures). Over the past 50 years, the author have witnessed enormous progress in the technical capabilities of this equipment. Automation and speed of analysis have greatly improved the ease of use and the versatility of the technology.

As an example, in the early days, measurement of the ultraviolet spectrum (UV) of a single compound could take up to 20 min – the operator had first to select the wavelength manually, use another knob to compensate a gauge, wait until the pointer found its stable position, mark the value found into a graph and repeat the procedure again and again with a changed wavelength. A long complicated process was thus required to achieve information about conjugation of multiple bonds in a compound (e.g. as in estrogens, '4-en-3-ones' and vitamin D-type compounds). Later, tunable UV devices became part of analytical and preparative separation instruments (e.g. high performance liquid chromatography, HPLC), and today with the advent of computer data-handling, nobody is surprised by the speed at which they automatically monitor the UV output signal or even control the process of separation.

Infrared spectra (IR), though in the past very slowly produced on special paper that was susceptible to mechanical damage, could give a real insight into molecular

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structure, freeing an experimenter from the need to prepare derivatives proving the existence of this or that functional group. Laborious 'fingerprint' comparison with the spectra of authentic reference samples was used for identification of individual steroids. Only much later, when spectrometers were linked to a computer with its own database to compare the sample in question with known standards, did paper printouts of the spectra cease to be the only means of storing evidence.

Mass spectroscopy (MS) was in the early days limited to volatile and low molecular weight compounds and derivatisation was part of the routine in any MS laboratory. Standard nuclear magnetic resonance (NMR) apparatus, which gives information about single atoms and their surrounding atoms, did not exist in those days and the method was then considered only to be useful for the demonstration of heterogeneous flaws in industrial products.

One has to admire the classical chemists of the late nineteenth and early twentieth centuries, who had none of these instruments, and yet produced results appreciated even today. For instance, Reinitzer (1888) discovered the molecular formula of cholesterol merely by precise elemental analysis of a series of cholesteryl ester dibromides and his formula remains unchallenged today. The quality and purity of his samples, as determined by melting points, have not been surpassed.

An interested reader may not necessarily personally use the techniques mentioned above but should, however, understand the information which these techniques can provide and the application of these methodologies to the examination of steroid structure. The novice seeking information about general aspects of the electromagnetic spectra, their terminology, and the units employed, is advised to read the introductory section of any of the number of student texts, listed in the bibliography at the end of this chapter. This chapter will summarise the scope and limitations of each technique in the steroid field and is addressed particularly to the non-specialists who can derive benefit from an appreciation of the potential of these techniques in solving problems which arise during investigations involving steroids. Knowledge of steroid chemistry is a prerequisite to an understanding of steroid biochemistry – an increasingly important topic in biology and medicine. Potential readers of this book may therefore not be chemists and it is hoped that this chapter may prove useful. It is not intended to provide a comprehensive treatment of any of the topics. For this, the reader is referred to appropriate standard textbooks (Williams and Fleming, 1987; Kirk, 1989; Kemp, 1991), and specialist books cited therein. This chapter is to be regarded as an introduction to the physicochemical methods available to steroid chemists both in the past and today. Some of the electromagnetic techniques may not be as popular as they once were but still remain valuable and should not be ignored simply because they have been around for a long time. Other methodologies, particularly mass spectrometry (MS) and nuclear magnetic resonance are now more readily available to non-specialists and are increasingly finding application in steroid analysis – because of their increasing importance in this area, they are dealt with at greater length and in separate sections that follow.

2.2 Ultraviolet Absorption Spectroscopy and Related Methods

2.2.1 Introduction

When organic compounds absorb UV radiation, the UV light induces transitions of electrons between different energy levels. The transitions of interest in the present context are mainly from ground-state π-orbitals to unoccupied π-orbitals of higher energy. The latter are termed antibonding π-orbitals, and are designated by the symbol π*. After undergoing this transition by the absorption of a photon of appropriate energy, the molecule is said to be in the excited state.

Compounds which can readily undergo π→π* transitions include conjugated dienes, trienes, and conjugated α,β-unsaturated ketones, esters or lactones, with structural features of the types C=C–C=C, C=C–C=C–C=C, or C=C–C=O, respectively. They also include aromatic rings, such as the phenolic ring A of the estrogens. All such conjugated compounds absorb UV radiation strongly in the wavelength range between 220 and 350 nm, which is accessible to standard commercial UV–visible spectrophotometers. Figure 2.1 illustrates a typical UV absorption spectra. The wavelength (λ) is normally expressed in nanometers (nm, 10^{-9} m); older units such as angström (Å) and millimicron (mμ) are not used any more. Old data can be recalculated according to the following equation:

$$1 \text{ nm} = 10 \text{ Å} = 0.001 \text{ μ}$$

Any UV-absorbing part of a molecule is called a chromophore: this historic term derives from coloured substances, which contain functional groups (chromophore; chromos is colour in Greek) responsible for the absorption of radiation in the visible region of the electromagnetic spectrum (400–800 nm). Most of the steroids are white crystals, although a few coloured derivatives are also known (e.g. the 2,4-dinitrophenylhydrazones of steroid ketones are yellow or red).

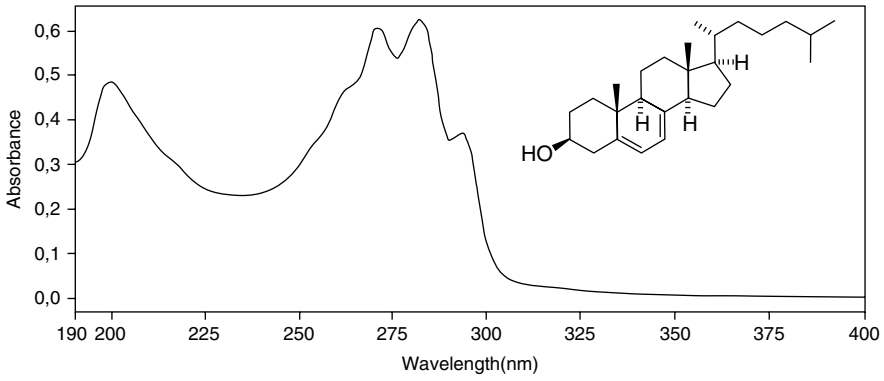


Fig. 2.1 UV spectrum of cholesta-5,7-dien-3β-ol

Electronic transitions also occur in other classes of organic compounds, which have no conjugated unsaturated systems, but the spectra in such cases are of relatively little practical value to organic chemists. For instance, absorption by isolated ethylenic double bonds in aliphatic compounds occurs at 170 nm and cannot therefore be recorded with standard commercial UV spectrophotometers. Special instruments are designed for absorption, which has a low probability and thus weak extinction (a small extinction coefficient ε). If, however, –C=C– bands are attached to one or two tertiary carbon atoms, their absorption occurs at a slightly longer wavelength ("red shift", see below) of about 200 nm and can then be detected quantitatively with many instruments (Fig. 2.2).

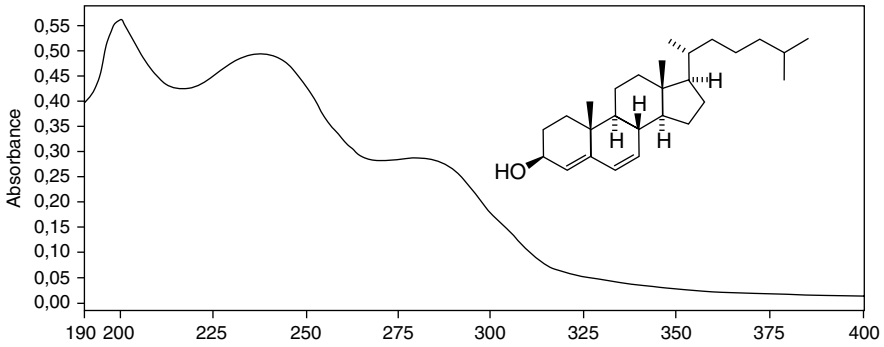


Fig. 2.2 UV spectrum of cholesta-4,6-dien-3β-ol

Equally, isolated C=O groups show an extremely weak (ε 20–100) absorption arising from the excitation of an electron from a non-bonding lone pair of the oxygen atom ($n \rightarrow \pi^*$ transition), in the wavelength range 280–320 nm. Such transitions, although "forbidden" by the so-called symmetry rules, do occur because of the loss of symmetry as the molecule vibrates. Since the extinction coefficient of these bands is from one to three orders smaller than usual, they have to be measured with correspondingly increased sample concentration or cell thickness. When the carbonyl group is conjugated with a double bond, the $n \rightarrow \pi^*$ transition of the C=O is overshadowed by a very strong $\pi \rightarrow \pi^*$ absorption of the C=C–C=O system.

Even a weak band of an isolated carbonyl group may have its diagnostic value in structural problems: for instance, in the presence of a neighbouring axial Br atom, the C=O band at 300 nm has its absorption shifted by about 30 nm (a red shift), while an equatorial Br atom has practically no effect on the C=O absorption. Other neighbouring groups' effects are also recognisable from this band.

In accordance with Lambert's Law, the light absorbed is a fixed fraction of the incident light, irrespective of the source intensity. Beer's Law states that the absorption is proportional to the number of absorbing molecules. Together these laws lead to Eq. 2.1:

$$\log_{10} (I_0 / I) = \varepsilon l \cdot c \tag{2.1}$$

where I_0 and I are the intensities of the incident and transmitted radiation, l is the path length of the absorbing solution in cm, c is the concentration of the solution in mol/L, and ε is the *molar absorptivity*, more commonly referred to by its older name as the *molar extinction coefficient*. Equation 2.1 leads directly to ε being in units of $dm^3\ mol^{-1}\ cm^{-1}$, the reciprocal of the units of the product $l{\cdot}c$. The units of ε are not generally expressed, when referring to the absorption maximum (λ_{max}) of an organic chromophore. The value of ε is a characteristic of a particular compound at each wavelength. When the molecular weight is unknown, precluding use of ε, the alternative expression $E^{1\%}_{1\,cm}$ is used. This is the value of $\log_{10}(I_0/I)$, the so-called *absorbance* (A) (formerly known as *optical density*) for a 1% solution in a cell of path length 1 cm.

2.2.2 Instrumentation

Conventional UV–visible spectrophotometers used to be based upon a scanning mono-chromator system. This produced a radiation beam of a wavelength, which changed through the required spectral range over a period of the order of minutes. The beam was split into two equal parts; one passed through the sample cell, where absorption of energy occurred according to the nature of the sample, the other passed through the reference cell with the solvent used. The light intensities of emerging beams were measured by photomultiplier tubes or photodiodes and the difference in light intensities of the two beams was plotted as a function of wavelength to give the absorption spectrum of the sample.

Modern instruments operate on a different principle, the whole spectral range being scanned almost instantaneously by a diode array. This comprises many hundreds of photodiodes, each of which collects the light received at a particular wavelength. Scan time to 'read' the entire array is typically in the range 5–100 ms, providing very rapid acquisition of the spectrum. Computer processing of the data permits the use of graphic displays, and storage and mathematical manipulation of the spectra.

2.2.3 Measurement of Spectra

Spectra are normally obtained for accurately weighed samples in very dilute solutions. A concentration in the approximate range 1–2 mg in 10 mL of solvent is typical when measuring the $\pi \rightarrow \pi^*$ transition of a conjugated system in a steroid. A silica cell, usually of 1 cm thickness ($l = 1$, in Eq. 2.1), is filled with the solution and put in the sample compartment of the spectrophotometer, with an identical cell filled with the pure solvent in the reference beam of the instrument. The intensities of light transmitted by the two cells are compared automatically as the spectrometer reads over the chosen wavelength span. This may cover the whole range from 220 nm through the visible spectrum, or be limited to that part of the spectrum where an absorption band is

expected. The spectrum is normally plotted on a chart as the value of $\log_{10}(I_0/I)$ against the wavelength. From the measured value of $\log_{10}(I_0/I)$ at the absorption maximum (λ_{max}), together with the path length of the cell (l), and the molar concentration of the solution (c), ε can be calculated by application of Eq. 2.1.

The spectral chart normally shows one or more broad humps, each of them centered upon the absorption maximum for the chromophore concerned. Simple chromophores like conjugated dienes or enones give a single absorption maximum, although absorption occurs over a wide band of wavelengths, and gives a very broad peak, which spreads on either side of the maximum to a total width of some 35–40 nm. The absorption band may contain a number of inflections or 'shoulders'. These are indications of vibrational fine structure; the wavelengths of shoulders are often quoted, as well as λ_{max}, to aid comparison of spectra. They can be of considerable value in the identification of particular compounds when compared with reference spectra.

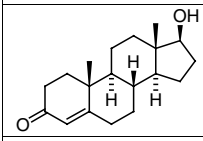
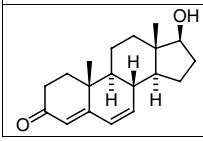
2.2.4 Solvents

The choice of solvent is limited to those which are transparent to UV radiation over the wavelength range of interest, dissolve the sample at a concentration sufficient for measurement and do not react chemically with it. Ethanol and methanol mostly fulfil these conditions. They are transparent down to about 205 nm, dissolve steroid samples well and scarcely interact with them (they may occasionally lead to esterification of steroid acids, which would not affect the value found; methanol, however, can add to a 3-oxo steroid forming a hemiacetal, which would reduce its already weak absorption). Water can be used to below 210 nm, but is rarely a suitable solvent for steroids. Diethyl ether and acetonitrile are suitable for many polar steroids, and are transparent to ~210 nm. Hydrocarbon solvents such as hexane or cyclohexane can be used down to about 190 nm for steroids of low polarity. All solvents should be of 'spectroscopic grade', available from commercial suppliers, to avoid errors due to traces of UV-absorbing impurities such as aromatic hydrocarbons.

Allowance must be made in many cases for shifts in UV absorption maxima depending upon the dipole moment of the solvent. The $\pi \rightarrow \pi^*$ transition, particularly of an α,β-unsaturated ketone, generates an excited state which is more polar than the ground state. Polar solvents, therefore, interact more strongly with the excited state, lowering its energy and thus shifting the absorption band to a somewhat longer wavelength ("*a red shift*" or "*bathochromic effect*"): e.g. when changing from hexane to ethanol, the shift is about 10–15 nm. The changes are not uniform, they depend on the structure of a chromophore; a shift in the absorption maximum of the 3-keto-Δ^4-system is smaller than that of the 3-keto-$\Delta^{4,6}$-system. Interestingly, the extinction coefficient is also affected by solvents (see Table 2.1); furthermore, a finger print structure of a band is usually lost in more polar solvents.

In contrast, the weak $n \rightarrow \pi^*$ transitions of ketones, including α,β-unsaturated ketones, have their maxima in the region of 280 nm in hydrocarbons but are 'blue shifted' (*hypsochromic effect*) on changing to hydroxylic solvents in the range 265–270 nm.

Table 2.1 The effects of solvents on UV absorption (modified from Neudert and Röpke, 1965)

Compound	Solvent		
	Isooctane	230	17,900
	Ether	232	18,100
	Chloroform	240	18,200
	Methanol	240	17,000
	Acetic acid	243	15,000
	Isooctane	268	29,300
	Ether	272	28,000
	Chloroform	281	30,000
	Methanol	281	26,100
	Acetic acid	284	26,500

2.2.5 *Effect of pH*

The UV spectra of UV active systems, whose mesomerism, tautomerism or dissociation can be influenced by H^+ concentration, alter in appearance, when the pH value is changed. Besides the shifts or changes in intensity of the band maxima, new bands may also appear. This is particularly true for compounds whose chromophoric or auxochromic groups themselves dissociate or undergo enolisation such as –COOH, $–NH_2$ or phenolic –OH groups. In such cases significant changes occur in a spectrum, when the pH is altered. For instance, estradiol in methanol (pH 7.0 and less) has a normal maximum at 280 nm and a shifted maximum in an alkaline solution (287 nm at pH 11.9, 295 nm at pH 13.0) (Fig. 2.3).

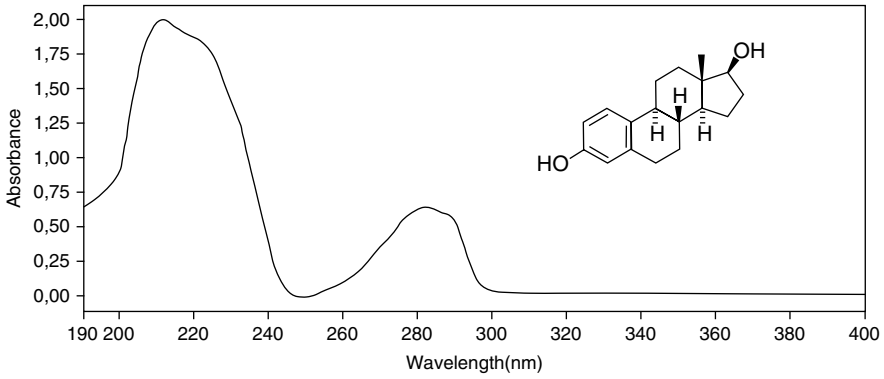


Fig. 2.3 UV spectrum of estra-1,3,5(10)-triene-3,17β-diol (i.e. estradiol) in ethanol

2.2.6 UV Absorption of Common Chromophores

Many UV absorption spectra covering all the types commonly found in steroids are given in an atlas (e.g. Neudert and Ropke, 1965). The most important chromophores found in steroids are given here.

(i) *Conjugated Dienes and Trienes* For the simplest conjugated diene, buta-1,3-diene, λ_{max} is about 217 nm ($\varepsilon \sim 21{,}000$). Inclusion of the conjugate diene system in a ring structure, or the presence of substituents on the unsaturated carbon atoms, increases the wavelength of maximum absorption. In 1941, R.B. Woodward proposed an empirical rule to correlate the known absorption data with the structure of dienes. The rule has been modified by A.I. Scott, and then by Fieser and Fieser. It now allows the prediction of λ_{max} with reasonable precision, often to within 2–3 nm of the observed value, for a great variety of conjugated diene and polyene chromophores. The relevant rules are summarised in Table 2.2. A diene, or the diene component of a longer conjugated system, is assigned the parent value of 214 nm. Increments corresponding to the effects of other structural features present are added to the parent value to obtain the final calculated value of λ_{max} for the particular chromophore.

In applying increments taken from Table 2.2, only those substituents bonded directly to the unsaturated carbon atoms of the conjugated system are taken into account. A ring residue is any carbon–carbon bond which is not part of the conjugated system but originates at one of the unsaturated carbon atoms and comprises part of a ring structure. An exocyclic double bond is a double bond in which one of the two unsaturated carbon atoms is part of a particular ring, the other of the pair being outside that ring. The steroidal 4,5 double bond is perhaps the most common example. It lies within ring A, but is exocyclic with respect to ring B. Structures of different types of dienes are shown in Fig. 2.4.

Table 2.2 Calculation of λ_{max} (nm) for conjugated dienes and polyenes (see Fig. 2.4)

Parent diene system	214
Increment if the diene is homoannular	+39
Increment for each additional double bond in a conjugated triene or polyene	+30
Increment for each alkyl substituent or ring residue	+5
Increment for exocyclic location of a double bond	+5
Increments for heteroatom substituents	
–OAlkyl	+6
–SAlkyl	+30
–Cl or –Br	+60
–NR2 (R = alkyl)	+5

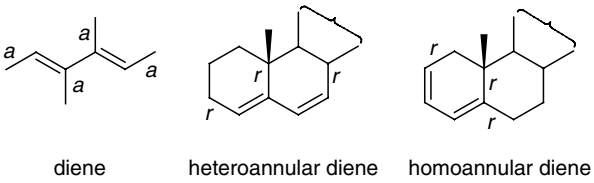
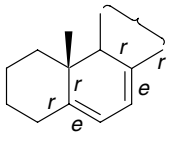


Fig. 2.4 Structures of different types of dienes; a = alkyl substituent, r = ring residue

Examples of calculations for dienes and trienes

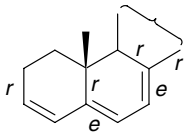
Parent diene system	214
3 ring-residue C–C bonds (r)	15
Exocyclic character (e) of the 5,6 double bond	5
Total	**234**
Found (in ether)	234

(a) Steroidal 5,7-diene



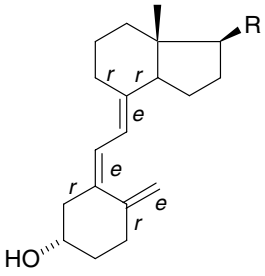
	nm
Parent diene system	214
Increment for homoannular diene	39
4 ring-residue C–C bonds (r)	20
Exocyclic character (e) of both double bonds	10
Total	**283**
Found (in ether)	280

(b) Steroidal 3,5,7-triene



	nm
Parent diene system	214
Increment for extra conjugated C=C	30
Increment for homoannular diene	39
4 ring-residue C–C bonds (*r*)	20
Exocyclic character (*e*) of two double bonds	10
Total	**313**
Found	316

(c) Steroidal 5,7,10(19)-triene (vitamin D series)



	nm
Parent diene system	214
Increment for extra conjugated C=C	30
4 ring-residue C–C bonds (*r*)	20
Exocyclic character (*e*) of each double bond	15
Total	**279**
Found	265

The large discrepancy in the last example illustrates a weakness of the rules for calculating λ_{max}, in that they assume that the conjugated system of C=C bonds lies approximately in a plane. This condition is met in cases (a), (b), and (c). The triene system of vitamin D (the last case), however, deviates significantly from planarity by virtue of the chair conformation of ring A (Kolodziejski et al., 2005). This forces the 5(6) and 10(19) double bonds to lie in different planes (Fig. 2.5). Their π–π* interaction and the effectiveness of conjugation are thereby reduced. This cautionary example points to the need for consideration of molecular geometry, conveniently with the aid of models, when applying the rules to conjugated systems more complicated or more flexible than those in the first three examples given above.

(ii) *Conjugated Unsaturated Ketones* The UV absorption of most interest here is that associated with the $\pi \rightarrow \pi^*$ transition of α,β-unsaturated ketones. The Woodward–Fieser–Scott rules for such systems are broadly similar to those for dienes, but differ in some details (Table 2.3).

The main difference when compared with the data in Table 2.2 lies in the distinct increments for substituents at the α, β, γ, and more remote positions along the unsaturated chain in conjugated dienones and polyenones. In contrast to a diene or

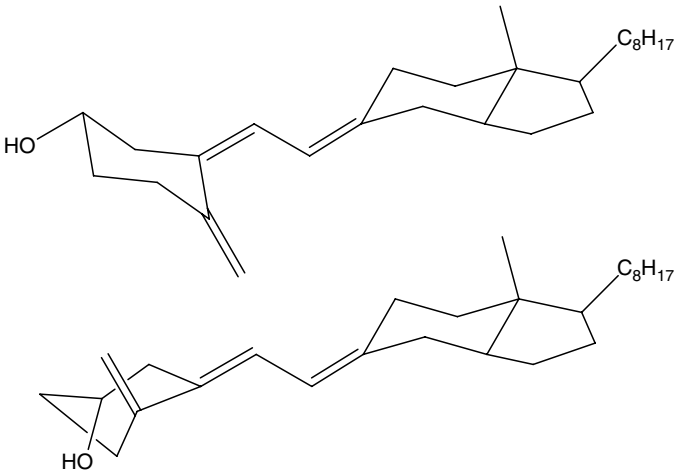


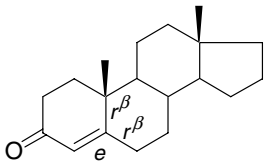
Fig. 2.5 Two major conformers of vitamin D in solution

Table 2.3 Calculation of λ_{max} for α,β-unsaturated ketones

$C^{\delta}=C^{\gamma}–C^{\beta}=C^{\alpha}–C=O$	nm
Parent value for α,β-unsaturated ketone (acyclic, or in a six-membered ring ketone)	215
Parent value (five-membered ring ketone)	202
Increment for each extra conjugated double bond	+30
Increment for a homoannular diene component	+39
Increment for exocyclic location of a double bond	+5
Increment for alkyl group or ring residue:	
At α position	+10
At β position	+12
At γ position or beyond	+18
Increments for heteroatom substituents:	
–OH at α position	+35
At β position	+30
At γ position	+50
–OAc at α, β, or γ position	+6
–Cl at α position	+15
At β position	+12

polyene, where the electron distribution is hardly polarised at all, the carbonyl group confers strong polarity, which extends through the conjugated system, and alters the magnitude of interactions with substituent groups at the various sites along the chain.

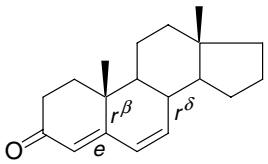
Examples of calculations for conjugated enones
(a) A steroidal 4-en-3-one



	Nm
Parent enone system	215
2 ring residues at β carbon (r^{β} in the formula)	24
Exocyclic character (*e*) of C=C	5
Total	**244**

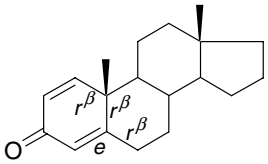
Steroidal 4-en-3-ones typically have λ_{max} at 240 ± 2 nm.

(b) A steroidal 4,6-dien-3-one



	nm
Parent enone system	215
Extra conjugated C=C	30
Ring residues at β carbon (r^{β} in the formula)	12
Ring residues at δ carbon (r^{δ} in the formula)	18
Exocyclic character (*e*) of the 4,5 C=C	5
Total	**280**
Found	**280**

(c) A steroidal 1,4-dien-3-one



This is a special case, known as a *cross-conjugated* diene. The two C=C bonds are not conjugated with each other, but each is conjugated to the carbonyl group. It therefore has to be treated as comprising *two* conjugated enones, rather than as a dienone with extended conjugation. The calculation for the 4-en-3-one component is made as in example (a), above. The 1-en-3 one component comprises the parent

enone (215 nm) with a single ring residue at the β position (r^{β}; +12 nm), giving a total of 227 nm. The spectrum (λ_{max} 244 nm) is essentially that of the 4-en-3-one component but often shows a broadening on the low-wavelength side indicative of an underlying and somewhat weaker band, attributable to the 1-en-3-one moiety.

Conjugated enones, which incorporate a homoannular diene (e.g. **8**, Fig. 2.6), are rare but do exist: e.g. a 2,4-dien-1-one has a maximum at 324 nm (Weissenberg and Glotter, 1977); calculated value: 325 nm (i.e. 215 nm for an enone +30 nm for an additional double bond +39 nm for the homoannular system +5 nm for the exocyclic Δ^4-double bond +36 nm for two substituents at the δ-carbon atom).

Another set of rules was devised for the calculation of the UV absorption of unsaturated acids and their esters (Williams and Fleming (1987)). Examples of calculation for bufadienolides and cardenolides are not given here because the UV spectra of the two basic types do not vary. These compounds are exemplified by cardioactive steroid glycosides scillaridin and digitoxin. Their aglycons scillarhenin (**9**) and digitoxigenin (**10**) are lactones of α,β-unsaturated acids: the latter type is a γ-lactone **10** (see Fig. 2.7) with a maximum of the UV absorption at 217 nm; the former is an unsaturated δ-enol-lactone with a system of three conjugated double bonds, its extended conjugation shifts the maximum to 300 nm.

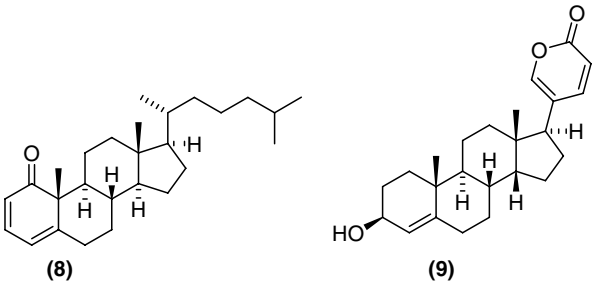


Fig. 2.6 Other conjugated dienes

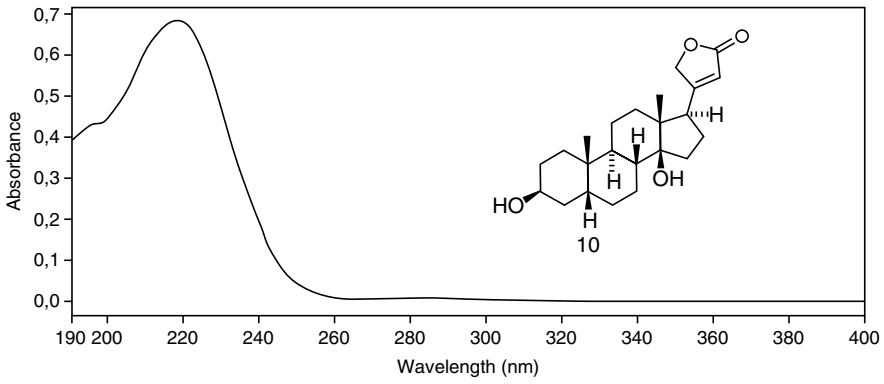


Fig. 2.7 UV spectrum of digitoxigenin or 3β,14-dihydroxy-5β-card-20(22)-enolide (**10**)

(iii) *Aromatic Chromophores* Benzene has a very strong absorption band at 184 nm (ε 60,000), and moderately strong absorption at 203 nm (ε 7,400), although these normally appear only as 'end absorption' down to the usual limit of measurement (~190 nm). In addition, there is a weak forbidden band at 254 nm ($\varepsilon \sim 200$).

Common substituents on the benzene ring, shift the last two of these maxima to longer wavelengths and in most cases increase the value of ε, especially for the forbidden band. A substituent of common interest in steroids is the phenolic hydroxyl group at C-3 in the estrogens. The UV absorption spectrum of estradiol shows a maximum at 280 nm (in ether, $\varepsilon \sim 2{,}000$), with strong end absorption at shorter wavelengths, which represents the beginning of the main maximum, lying below 200 nm. Another common substitution of the phenolic A ring is hydroxylation or halogenation in positions 2 and 4.

Estrogens in which both rings A and B are aromatic (equilenin (**11**) and its 17-hydroxy analogues, Fig. 2.8) show characteristically more complicated naphthol-type absorption, with principal maxima at 280 ($\varepsilon \sim 5{,}000$) and 342 nm ($\varepsilon \sim 2{,}500$), as well as very strong end absorption down to 200 nm. The UV spectrum of equilin (**12**), concomitant in nature with equilenin, is closer to estrone.

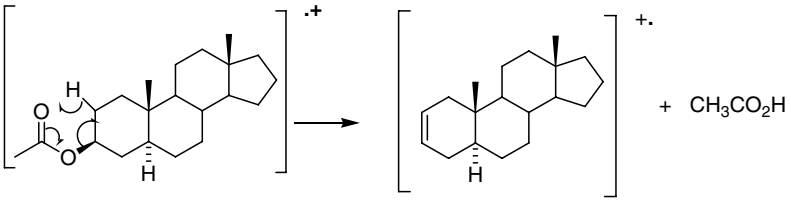


Fig. 2.8 Equilenin (**11**) and equilin (**12**)

2.2.7 *Related Phenomena*

Two other aspects of electronic excitation must be mentioned, although space does not permit any detailed discussion of either.

(i) *Fluorescence* Fluorescence is the emission of light by electronically excited, generally singlet-state molecules that have lost a part of the excitation energy as heat by collision with surrounding molecules (Lakowicz, 1999). The excited singlet state typically has a lifetime in the range 10^{-9}–10^{-6} s, and emits a photon of lower energy than that originally absorbed, as the molecule returns to the ground state. The UV–visible radiation is normally used for excitation. The measured beam of fluorescent light is perpendicular to the direction of the excitation light. The fluorescence spectrum is normally of a similar profile to the absorption spectrum but is displaced to less energetic, i.e., longer wavelengths.

Neither the σ–σ^* transitions of saturated steroids nor the n–π^* transitions of compounds with heteroatoms (e.g. ketones) give rise to florescence. The π–π^*

transitions of conjugated C=C bonds do, particularly, in those with aromatic rings. Thus in *intact steroids*, fluorescence spectroscopy is almost exclusively viable in analysis of aromatic compounds – estrogens. The background fluorescence used to be quenched by alkalisation and thus the concentration of the estrogen was calculated from the difference between the fluorescence values measured in neutral and alkaline media. For some studies a suitable fluorescent analogue could be utilised. For instance, $\Delta^{9(11)}$-dehydroergosterol (**13**, Rando et al., 1982; Schroeder et al., 1985, see Fig. 2.9) was exploited for probes of the role of cholesterol in micelles and cell membranes. Equally, 5,6,11,12-tetrahydrochrysene-2,8-diol (**14**), with a mild bond to the estrogen receptor, was used for the diagnosis of breast cancer (Bowen and Katzenellenbogen, 1997).

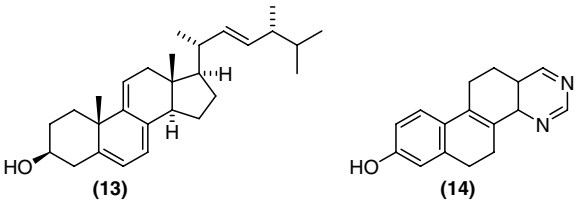


Fig. 2.9 Fluorescent analogues of cholesterol (**13**) and estradiol (**14**)

Fluorescence provided the basis for early analytical procedures for some of the steroid hormones in biological fluids. Various techniques were developed which involved brutal modification of the sample with strong acids and some other reagents (Görög and Szasz, 1978). Fluorescence was then generated not only in estrogens: e.g. methods for the determination of corticosteroids, after treatment with strong acids, have also been described. This treatment involved a complex sequence of reactions, which included protonation of hydroxy groups, elimination and carbocation rearrangements. Similar treatment was also used in thin layer chromatography where "chromophores", present in the analytes, were used to visualise the separation of components and identify some of them.

The fluorimetric assays, however, are prone to interference by fluorescing contaminants. Often they were complicated by 'fluorescence quenching', when much or all the absorbed energy was transferred to other molecules in solution, instead of being emitted as radiation. Usually, they had poor specificity, although for some combinations of analytes they were selective enough (e.g. the determination of mestranol beside gestagens present in contraceptive pills (Görög and Szasz, 1978).

Some steroids, even when devoid of a strong "fluorophore" (e.g. aromatic rings, conjugated double bonds), can still be analysed by methods based on fluorescence, when they are derivatised with reagents tagging the steroid by covalent linkage to a fluorescent moiety (e.g. naphthalen-based groups "dansyl" or "EDTN"). For instance, steroid alcohols and ketones can form dansyl derivatives with 5-dimethylaminonaphthalenesulphonyl chloride or 5-dimethylaminonaphthalene-1-sulphonohydrazide.

In a way, conjugates of steroids and bovine serum albumin, used in some very sensitive radio-immunoassay procedures (RIA), are also such derivatised analytes. Fluoro-immuno assay (FIA) has been developed as a safe alternative to RIA (Dandliker et al., 1977; Kobayashi et al., 1979; Barrows et al., 1980; Evrain et al., 1980; Chard, 1982; Bertoft et al., 1985; Lovgren, 1987; Kirk, 1989; Kimura et al., 2000) and has replaced it by permitting the measurement of antibody binding using fluorescence spectroscopy.

(ii) *Chiroptical Properties* These comprise optical rotation, optical rotatory dispersion, and circular dichroism (CD). Steroids, by virtue of their chirality, are optically active compounds. Measurement of specific optical rotation, generally at the wavelength of the yellow sodium D-line (589 nm), used to be an obligatory part of the characterisation of any new steroidal compound. The need to publish specific rotation values led to precise purification of the new products in the past. The practice has become less common in the last decade since the time some authors have supported their claim of having produced a pure new compound by using only a few signals of its NMR spectrum.

The specific optical rotation $[\alpha]$ is given by the following equation:

$$[\alpha] = \alpha / cl,$$

where α is the angular rotation of the plane of polarisation of a beam of plane polarised light, measured in a *polarimeter*, c is the concentration of the solution in g mL^{-1}, and l is the path length (cell length), expressed in decimetres (dm). The reader should consult standard chemistry textbooks for details of the polarimeter and its use. The value of $[\alpha]$ at the sodium D-line is given as $[\alpha]_D$; ideally, the temperature (°C) and solvent should also be specified (e.g. $[\alpha]_D^{25}$ ($CHCl_3$), i.e. measured in chloroform at 25°C).

Optical rotational data are still available for some thousands of steroids (Fieser and Fieser, 1959, p. 177; Jacques et al., 1965). This collection can be a useful tool for structure verification through prediction of optical activity of newly prepared compounds: Barton (1945) developed Freudenberg's "Rule of Shift" (Freudenberg, 1933) and formulated his method of molecular rotation differences (molecular rotation M = [α].molecular weight/100). He claimed that identical structural changes are accompanied by identical changes of molecular rotation (i.e. $\Delta M = M_2 - M_1 = M_4 - M_3$). The structural changes may involve even hypothetical reactions such as the "oxidation" of a cholestane side chain into a pregnane side chain. In both series (i.e. the "conversion" of compound **15** into **16** and **17** into **18**), the change of molecular rotation values should be the same. Thus from three known data the unknown fourth can be calculated (Fig. 2.10).

Optical rotation is a wavelength-dependent property of chiral compounds. Optical rotatory dispersion (ORD; Fig. 2.11) is the variation of [α] with wavelength (Djerassi, 1960; Crabbé, 1965). Over wavelengths far from any absorption band, the value of [α] varies only gradually, but if the optical rotation is plotted through an absorption band, it gives a typical S-shaped curve, with two extremes of opposite signs, known as a Cotton effect. The Cotton effect curve is conventionally designated

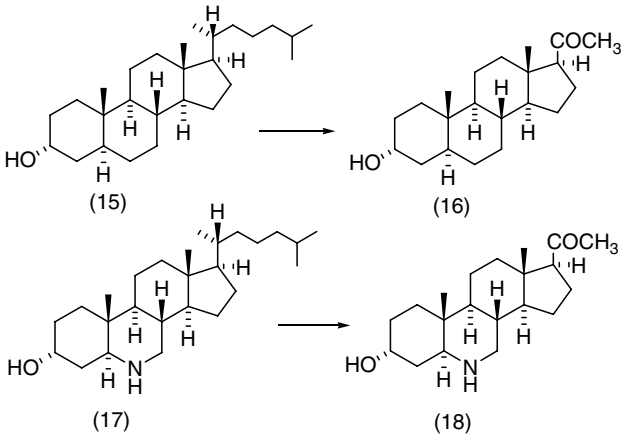


Fig. 2.10 Calculation of $[M]_D$ from known molecular rotations of compounds (**15**), (**16**) and (**17**)

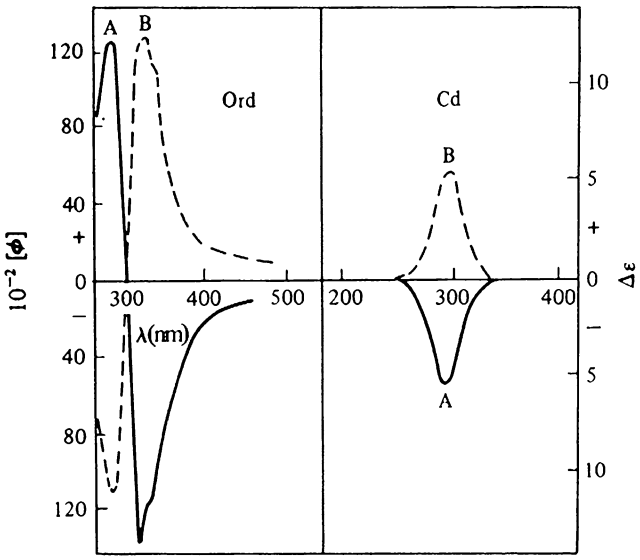


Fig. 2.11 ORD and CD curves for a pair of quasi-enantiomeric ketones. (A) 5α-androstan-16-one and (B) A-nor-5α-androstan-2-one (from Kirk, 1986, with permission)

as having either a positive or negative sign, according to the sign of the maximum at longer wavelength. The amplitude of the curve (*a*) is the total change in value of [α] from peak to trough, appropriately signed.

Another manifestation of the same phenomenon is circular dichroism (Crabbé, 1965; Fukushima and Matsui, 1969). It measures the difference between the extinction

coefficients (ε) for left- and right-circularly polarised beams, as a function of wavelength. The sign of ε is the same of that of the ORD amplitude, a. Earlier data on ORD can be used and converted into CD terms: $\varepsilon = a/40$.

The profile of a CD curve resembles that of the corresponding UV absorption spectrum. CD has largely surpassed ORD: in Chemical Abstract, CD is now mentioned 20 times more often than ORD. The two methods have the same applications and yield the same structural information.

ORD and CD are most valuable for the study of absolute configuration of steroidal ketones (Kirk, 1986) where the sign and magnitude of the Cotton effect in the region of the $n \rightarrow \pi^*$ absorption band (~280 nm) are related to the three-dimensional chiral structure of the molecule in the vicinity of the oxo group. This relationship is expressed in the so-called 'octant rule' (Djerassi, 1960). This set of empirical rules allows the prediction of the sign of the Cotton effect.

As shown in Fig. 2.12, using the carbonyl chromophore as the reference point, we can divide a cyclohexanone ring into eight octants by means of three planes (an observer should look through the C=O bond). Plane A is vertical, passing through C-1 and C-4. The horizontal plane B encompasses C-1 and its two adjacent carbon atoms: equatorially oriented substituents attached to these two "α-carbons" are practically in plane B. A third plane, C, is perpendicular to plane A and dissects the carbonyl group; it separates the four front and four rear octants. In most cases the rear octants behind the C plane suffice to make a prediction of the Cotton effect.

The octant rule states that substituents lying in these dividing planes A and B make substantially no contribution and can be almost ignored. Atoms situated in the far-lower-right (e.g. axial substituents in the α-position to the carbonyl carbon) and

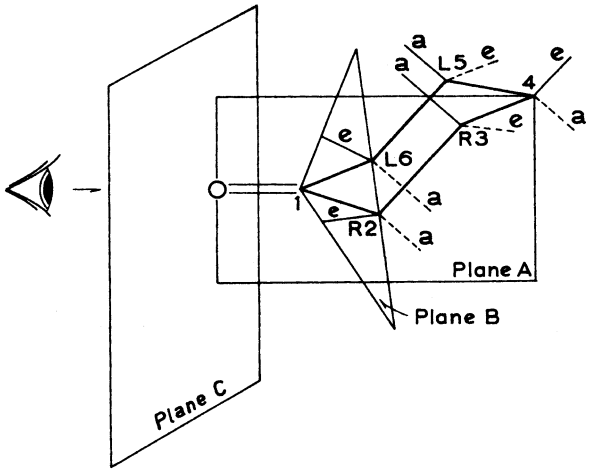
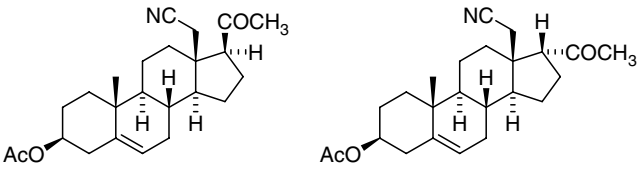


Fig. 2.12 Geometry of a cyclohexanone ring: planes A, B and C create eight octants, axial (a) and equatorial (e) bonds

far-upper-left (i.e. at the β-carbon) make a positive contribution, while those located in the far-lower-left and far-upper right octants produce a negative effect. The situation in a three-dimensional system is visualised best using molecular models. For instance, a model of 5α-cholestan-3-one shows that most carbon atoms are located in the far-upper-left octant and should exert positive Cotton effect. On the other hand, 5β-cholestan-3-one has most of the mass situated in the negative far-upper right octant.

Steroids with an oxo group in any of the rings are easily analysed by chiroptical methods (actually, steroid models were the original basis from which many general rules were formulated). Less straightforward is the understanding of the Cotton effect of open chain ketones, e.g. 20-oxopregnane derivatives. The side chain freely rotates around the C_{17}–C_{20} bond; certain rotamers, however, statistically prevail. Thus, e.g. 20-oxopregnanes **19** and **20** (Fig. 2.13) yield CD curves, which are almost mirror images of each other (Kasal and Černý, 1967).



19, a = +17000 **20**, a = –13000

Fig. 2.13 Optical rotary dispersion of 17ξ-pregnan-20-ones

CD curves get more complicated when a double bond is introduced into a molecule (Fig. 2.14). Thus both C=O groups in 19-nor-10α-androst-4-ene-3,17-dione (**21**) contribute to the positive hump of the $n \rightarrow \pi^*$ transitions around 290 nm, while the negative hump around 340 nm corresponds to $n \rightarrow \pi^*$ transitions of the α,β-unsaturated 3-ketone.

An additional double bond (as, in $\Delta^{1,4}$-dien-3-ones) shifts the position of the latter even further (to 365 nm), the shape of the curves then becomes more complex with a fine structure. Figure 2.15 shows the effect of additional conjugation on the region around 360 nm: a 4,6-dien-3-one **23** and a cross-conjugated diene **22** have a similar pattern but an opposite sign; conjugation with a γ,δ-oxido ring is less expressed than with an additional double bond: compound **24** shows a smaller effect on the position and magnitude of the Cotton effect

Other functional groups that have yielded useful structural information from chiroptical studies include olefins, α,β-unsaturated ketones, β,γ-unsaturated ketones, α,β-epoxy- and α,β-cyclopropano-ketones, lactones and lactams (for a review of previous work, see Kirk, 1986).

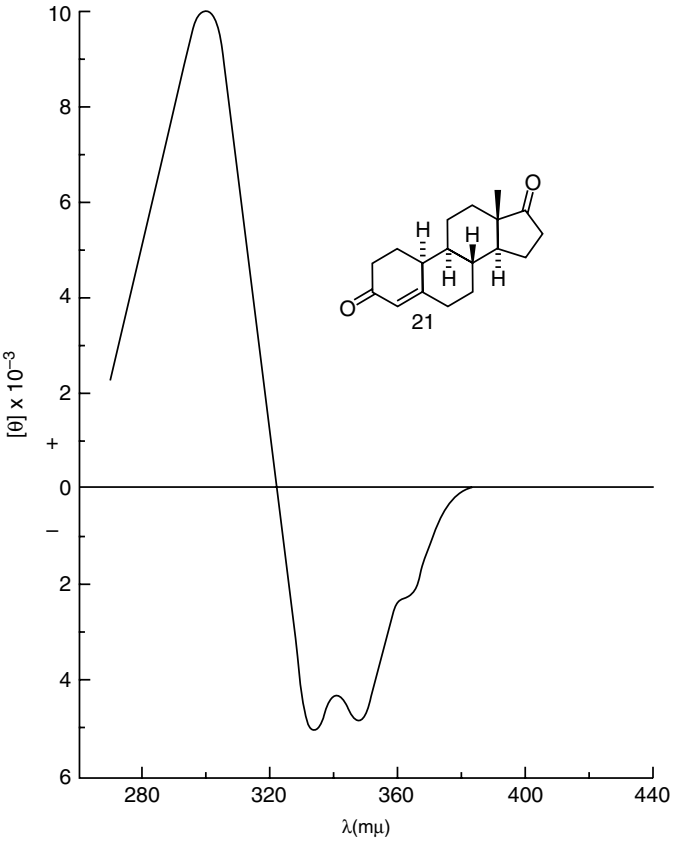


Fig. 2.14 CD of 19-nor-10α-androst-4-ene-3,17-dione (**21**)

The ring chirality rule for lactones (Jennings, 1965) often gives the correct sign of the Cotton effect near 215 nm. The octant rule was modified by adding an additional plane of symmetry which bisects the carboxyl group (O–C=O) assuming that both C–O bonds are equivalent; the molecule has to be viewed from above, projected on to the plane of the lactone ring. Thus brassinolide type lactones give a positive Cotton effect at 220 nm (most of the steroid mass is in the positive upper right back *E* sector), while its isomer (a 6-oxa-7-oxo derivative) gives a negative value (Garbuz et al., 1992). Individual contributions are more understandable when molecular models are used instead of structural formulae. The signs given are those of the back upper sectors (see Fig. 2.16).

In summary, the main applications have been for the determination of absolute configurations of chiral molecules from the signs and magnitudes of ketonic Cotton effects, and for the determination of configurations of local substituents in ketones of known absolute configuration.

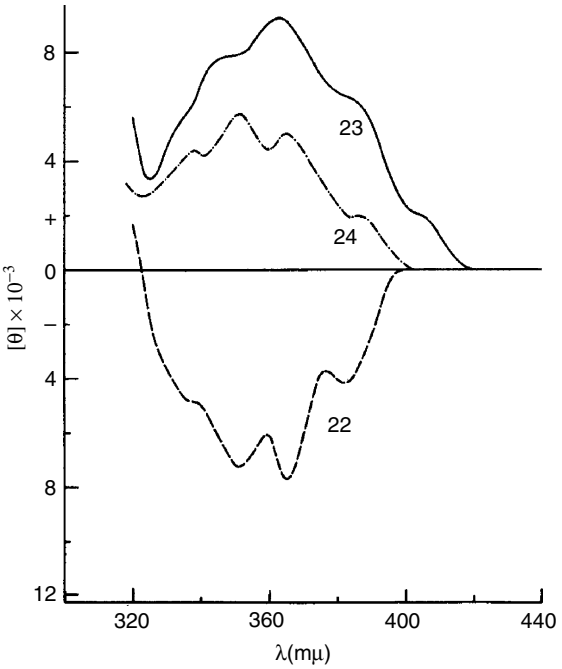


Fig. 2.15 Circular dichroism of (**a**) 6β,19-oxidoandrosta-1,4-dien-17-one (**22**); (**b**) 3,20-dioxo-pregna-4,6-dien-19-yl acetate (**23**); (**c**) 6α,7α-oxidopregn-4-ene-3,20-dione (**24**)

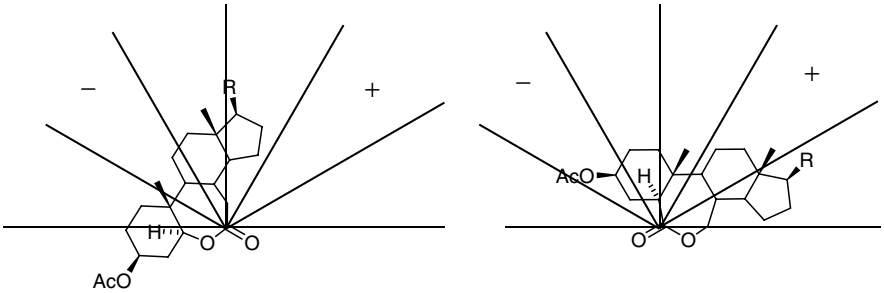


Fig. 2.16 The sector rule for a brassinolide analogue (*right*, positive contributions in the upper right sector) and isomeric 6-oxa-7-one (*left*, negative contributions in the upper left sector) (modified from Garbuz et al., 1992)

2.3 Infrared Absorption Spectroscopy

2.3.1 Introduction

Absorption of radiation in the IR region of the electromagnetic spectrum results in vibrational excitation of molecules because the vibrational frequencies of bonds lie within the IR range. Even in the lowest energy state (*ground state*), every bond in

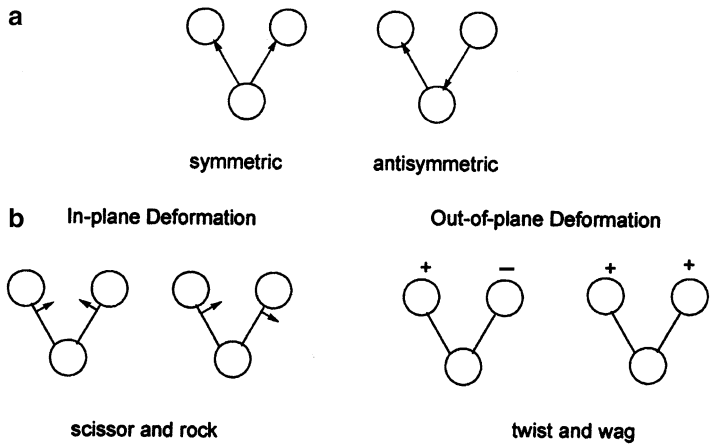


Fig. 2.17 Vibration modes in methylene groups. (**a**) Stretching modes; (**b**) bending or deformation modes. Similar AX_2 groups ($–NH_2$, $–NO_2$, etc.) and methyl groups behave similarly (from Kemp, 1991, with permission)

an organic molecule undergoes constant stretching (abbreviated as 'str.') and bending vibrations (Fig. 2.17), each with its own characteristic frequency. The amplitude of vibration is increased in distinct steps by the absorption of quanta of radiation having the same frequency as the bond vibrations.

When a beam of IR radiation passes through a sample of an organic compound, only those frequencies which correspond to molecular vibrations are liable to be absorbed. By plotting the absorbance against the frequency of the radiation, we obtain the IR spectrum of the compound.

A steroid possesses a large number of vibrational modes, each with its own characteristic frequency, so that the IR spectrum contains many absorption bands. However, the vibrations associated with the presence of particular functional groups, and a few other distinct structural features, usually give the strongest absorption bands in the spectrum. They can generally be picked out from the very complicated spectra produced by typical steroids.

In verbal description of IR spectra, the term *wave number* is used and the corresponding symbol is ν. It means the number of waves within 1 cm, thus the units are cm^{-1}. The wavelength (λ) can therefore be calculated from the following equation:

$$\nu = 1/\lambda$$

Unfortunately, most chemists confuse the term *wave number* with *frequency*. The latter term is, however, reserved for numbers relating to 1 s, the unit being s^{-1}.

The higher-energy end of the useful spectrum begins at 4,000 cm^{-1}, i.e. at wavelength of 2.5 μm (i.e. 1/4,000). The spectrum is usually recorded down to 250 cm^{-1}. Some early spectra were plotted on a linear wavelength scale in micrometre. Within the given range, we find absorption maxima (ν_{max}) corresponding to the common functional groups (hydroxyl, carbonyl, C=C double bonds, etc.), and many absorption bands

which are due to unassigned skeletal vibrations. IR spectra below 600 cm^{-1} correspond mostly to stretching vibration of the C–X bonds in halogen derivatives, however, these are only exceptionally found among steroids. Bending C–C vibrations of aliphatic chains are also reflected in this region, though with little diagnostic significance.

The value of IR spectra for organic chemists and biochemists is greatly enhanced by the fact that the strength of an absorption band depends upon the magnitude of the dipole associated with the particular vibrating group; Only those structural features whose vibrations involve significant change in the bond dipole are able to interact with the IR radiation. The strongest absorption bands therefore result from H–O, C=O, and other highly polarised bonds. The many C–H bonds in a steroid combine to give a fairly strong absorption band of little information for the experimenter. Unsymmetrical alkenes (e.g. Δ^4 or Δ^5 double bonds) and monosubstituted alkynes may give bands of medium intensity, especially when their polarity is enhanced by conjugation with a carbonyl group. Any double bond with a high degree of local symmetry (disubstituted or tetrasubstituted C=C) may fail to appear in the IR spectrum, unless it is part of a conjugated enone system and thereby assumes a dipolar character.

The many essentially non-polar C–C single bonds contribute no recognisable peaks to the spectra.

2.3.2 Preparation of Samples

Steroids, which are normally solids, are examined either in solution or in the solid state. Those, which fail to crystallise, may be studied as liquid films. Samples of the order of a milligram are normally used.

Very few organic solvents are transparent to IR radiation over the regions of interest. The choice is normally limited to carbon disulphide (CS_2), carbon tetrachloride (CCl_4) and chloroform ($CHCl_3$). Even these solvents absorb some IR radiation (Table 2.4 and Fig. 2.18), fortunately, in regions of the spectrum which are of relatively low importance for examining the structure of compounds. One has, anyway, to keep these regions in mind and disregard the corresponding part of the spectrum or measure the spectrum again in another solvent, which is completely transparent in that particular part. Highly polar steroids, unfortunately, often lack the necessary solubility in any of these solvents, leaving the solid-state technique as the only option.

Solid-state spectra are now the most commonly used for steroids. The sample may be ground to a powder with potassium bromide (KBr) which is then compressed into a translucent disc by means of a specially designed hydraulic press.

Table 2.4 Absorbing regions of solvents used for IR spectroscopy

Solvent	Absorbing regions (cm^{-1})
Carbon tetrachloride	1,560–1,550, 820–720
Chloroform	3,030–2,990, 1,260–1,180, 940–920, 860–550

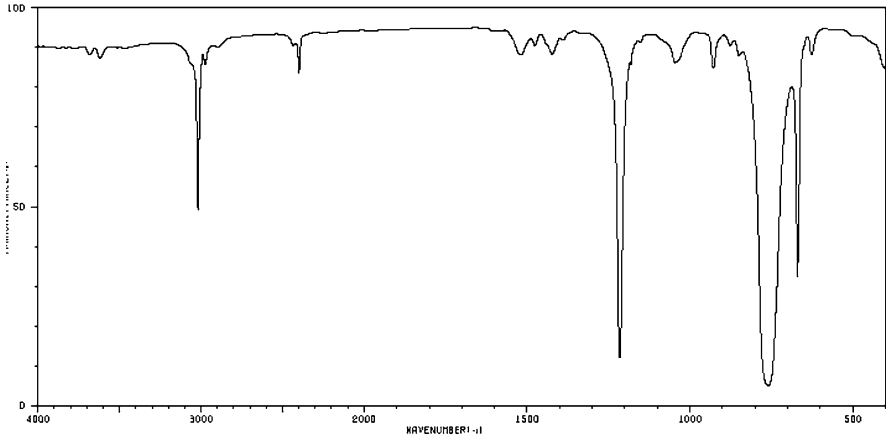


Fig. 2.18 Absorption spectrum of chloroform

Alternatively, the sample may be powdered and dispersed in a drop of highly purified hydrocarbon oil (Nujol, or a similar commercial product), by use of an agate mortar and pestle. Neither method is without disadvantages. KBr tends to give the sharper spectra, but often retains traces of moisture which result in a broad absorption band in the O–H str. region of the spectrum (~3,500–3,400 cm^{-1}). Nujol avoids this problem, but being a mixture of hydrocarbons, it supplies the spectrum with its own C–H str. and bending absorption bands. These may not be a problem in the spectrum of a steroid, which possesses so many C–H bonds that, except in certain special cases, little information can be derived from these parts of the spectrum. A common difficulty which may arise with solid-state spectra of steroids in either medium is the shifting of the very important C=O str. bands if a carbonyl group is intermolecularly hydrogen bonded to hydroxyl groups in the crystal structure of the compound (see hydroxyl/carbonyl interaction).

The KBr disc containing a steroid sample is placed directly into the IR beam of the spectrometer, in a suitable holder. All other types of sample have to be contained in a cell, with windows made of a material, which is transparent to IR radiation. For samples in solution, specially designed cells with optical windows composed of sodium chloride (NaCl) are suitable. The windows are both fragile and water-soluble, so have to be treated with very great care. Even the lower alcohols (methanol, ethanol) will gradually dissolve enough NaCl to cause damage, so cleaning must be done only by the use of those solvents which can be used to dissolve the samples (see above). Similarly, Nujol mulls or oily liquid samples are sandwiched between two plates of highly polished NaCl, which are then carefully clamped into a special holder. NaCl plates, like cell windows, have to be handled with the greatest care because of their fragility and the ease with which their polished optical faces can be damaged even by contact with moist surfaces, including fingers.

2.3.3 Instrumentation

The older type of IR spectrometer used to be designed to scan slowly through the wavelength range of interest, automatically comparing the intensity of a reference beam with that of the beam transmitted by the sample. The intensity difference is plotted as a function of wavelength, or more commonly wave-number. The wave-number scale on spectral charts is linear, but is often divided into two regions: the wave-numbers from 4,000 to 2,000 cm^{-1} are compressed into a shorter chart length than the rest of the spectrum, from 2,000 to 400 cm^{-1}, to allow for more detail in the latter region.

Almost all instruments are designed to plot absorption peaks downwards, from the 'baseline' at the top of the chart. The ν scale was formerly calibrated against the accurately known values for the main IR absorption bands produced by a film of polystyrene: IR spectrometers, particularly the mechanical configuration of a prism or a grating monochromator, were sensitive to changes of temperature and calibration was essential (the previous edition of this book still gave the IR spectrum of polystyrene for calibration). Modern Fourier-transformed spectrometers do not require any calibration; the wave-length is given by the built-in laser.

The wave-number at maximum absorption of a peak is reported as ν_{max}. Most scientific papers list only the most significant IR peaks when listing physical data for steroids. Atlases (Láng, 1978) of complete spectra are published for more common compounds, and some recent spectra can be found on the internet (http://riodb01.ibase.aist.go.jp/sdbs/cgi-bin/cre_index.cgi?lang=eng).

Intensities of IR absorbance are rarely measured for steroids, thus very accurate weighing of samples is generally unnecessary. The spectrometer sensitivity is conveniently set so that the strongest peaks lie in the range 50–90% of the full absorbance scale, and peak intensities are described relatively and qualitatively as strong (s), medium (m), or weak (w). In isolated cases, when intensity is important (the oxo group position, Fig. 2.19), relative intensities, found by the software treatment of the spectrum, are usually quite sufficient.

During the last decade, Fourier transform IR (FTIR) spectroscopy has been increasingly used (Kemp, 1991). FTIR instruments use a beam of broad-band IR radiation covering the whole range of the spectrum. Put simply, the beam is split into two; one of the beams passes through the sample, or both pass through the sample but with different path lengths, then the two are recombined to produce interference patterns, as an interferogram. A computer carries out a Fourier transformation of the interferogram to convert it into the form of a normal spectrum, for plotting on the chart. FTIR is especially useful for its speed, and because the computer can add together the results of several fast scans, providing greater sensitivity and better spectra, especially for small samples. Moreover, the data are stored by the computer in digital form, and so can be manipulated, for example to expand regions of special interest, or by subtraction of one spectrum from another, to reveal the spectrum of a minor component of a mixture.

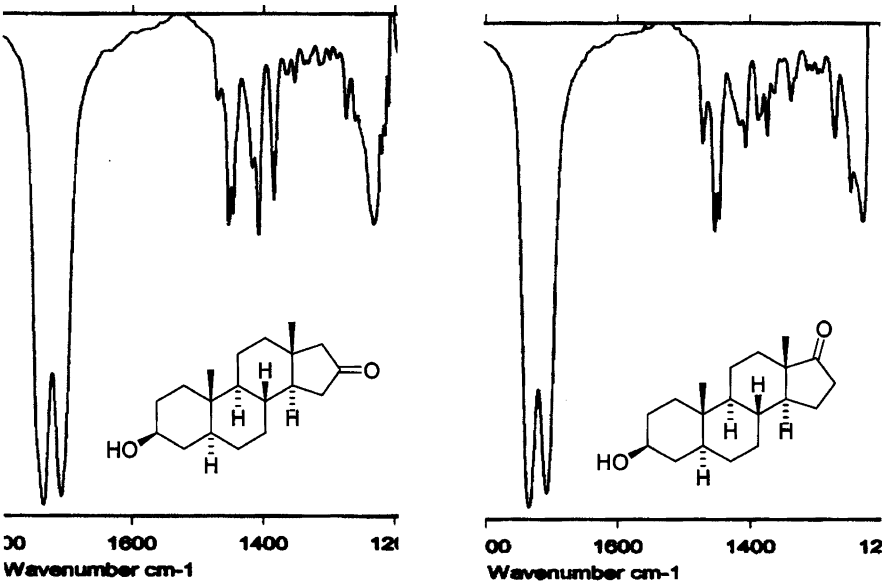


Fig. 2.19 CH_2–CO scissoring vibrations of cyclopentanone derivatives at ν 1,408 cm^{-1}. Two $COCH_2$ groups exist in 3β-hydroxy-5α-androstan-16-one, one in 3β-hydroxy-5α-androstan-17-one

2.3.4 Interpretation of IR Spectra

IR spectral peaks provide evidence of functional groups in the sample under investigation. The information that IR spectra can give about skeletal structure is very limited. The analysis of IR spectra of steroids depends upon recognition of those peaks which are characteristic of structural features. Most of the useful absorption bands lie above 1,500 cm^{-1}. The so-called 'fingerprint' region of the spectrum, below 1,500 cm^{-1}, often contains peaks that cannot be assigned to specific vibrations. Their pattern is characteristic of the particular steroid, and may be useful for identification of a compound by comparison of the spectrum with that of an authentic sample. Certain groupings of fingerprint peaks have been listed (Dobriner et al., 1953; Roberts et al., 1958) as characteristic of specific structural features, especially hydroxyl and carbonyl groups at the more important sites in steroids (C-3, -17 and -20). It is often a matter for skilled judgement whether a peak in the fingerprint region can be treated as having any structural significance; many are better ignored, except as fingerprint detail for purposes of comparison.

Figure 2.20 is provided as a first guide to the recognition of the most useful features of the IR spectrum of a steroid. More detail is contained in Tables 2.5–2.9, and is discussed below in relation to the structural information that it can afford. The data have been largely taken from tables compiled by Jones and co-workers (Roberts et al., 1958), augmented by reference to more recent listings by the Oxford

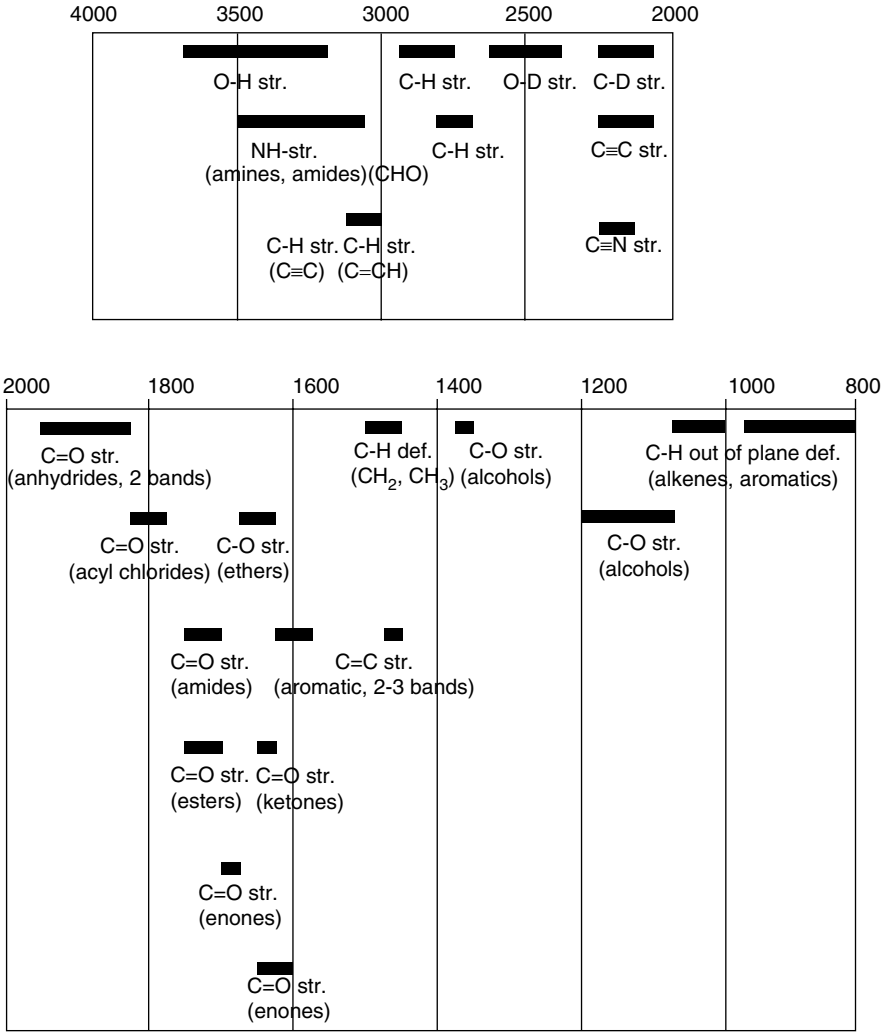


Fig. 2.20 Main regions of interest in IR spectra (cm^{-1})

group (Boul et al., 1971). Some typical IR spectra of steroids are given as Figs. 2.21–2.23.

(a) *The Hydrocarbon Skeleton* (*Table 2.5*) The steroid skeleton itself, being a saturated hydrocarbon, is not a source of many useful IR features. Any vibrational bands due to C–C bonds are very weak and are lost among others in the fingerprint region. Stretching vibrations of C–H bonds merge into one broad band, of complex structure, between 2,970 and 2,850 cm^{-1}, where Nujol also produces strong absorption. Rather weak but sharper bands between 3,100 and 3,000 cm^{-1} are characteristic of the stretching of C–H bonds at olefinic or aromatic positions,

Table 2.5 Characteristic group wave-numbers: C–H stretching bands

Group	Wave-number (cm^{-1})
–C=C–H	3,340–3,300
Aromatic C–H	3,100–3,000
C=CH_2 in 17-vinyl steroids	~3,085
C≡C–H in 17-ethynyl steroids	~3,300
C=C–H in steroidal 14-enes	3,055–3,050
C=C–H in six-membered rings	3,040–3,010
CH_3 and CH_2 groups	2,970–2,850
CH_3O group	2,830–2,815
O=C– H in aldehydes	2,750–2,700
C–D adjacent to carbonyl	2,275–2,100
C–D in other positions	2,180–2,100

whereas the C≡C–H group, present in 17α-ethynyl steroids, usually gives a sharp C–H str. band near 3,300 cm^{-1}. The stretching band of the relatively weak C–H bonds of a methoxy group occurs typically at about 2,830–2,815 cm^{-1}.

The other main spectral region affected by the skeletal structure lies between 1,500 and 1,300 cm^{-1}, where hydrocarbons give two broad and complex peaks arising from scissoring or bending vibrations of methylene and methyl groups (~1,475–1,445 and ~1,390–1,370 cm^{-1}, respectively). Just separated from these bands, often as shoulders, it may be possible to observe CH_2 scissoring bands for CH_2 attached to ketonic or olefinic carbon atoms (1,440–1,410 cm^{-1}). Intensity measurements have some potential as a method for identifying ring D ketones from their CH_2–CO scissoring vibrations at 1,408 cm^{-1} (Boul et al., 1971). Pregnan-20-ones (i.e. methyl ketones) usually show a methyl bending vibration at 1,360–1,356 cm^{-1}.

(b) *Unsaturation* (*Table 2.6*) The C=C str. band is rather weak for non-conjugated unsaturated steroids (e.g. Δ^5), and may be obscured by stronger carbonyl bands. It lies between 1,670 and 1,625 cm^{-1} for different C=C locations.

Conjugation and polar substitution increase the intensity of the C=C stretching band by breaking the inherent symmetry of the C=C bond. α,β-Unsaturated ketones, in particular, show C=C stretching bands of medium intensity (~1,635–1,605 cm^{-1}). Together with the carbonyl absorption (see below), this is one of the best ways of recognising a steroid of the important 4-en-3-one class of hormones, which include testosterone, progesterone, and corticosteroids. Steroidal 1,4-dien-3-ones give two C=C bands: a medium one at ~1,635 cm^{-1} and a weaker one at ~1,605 cm^{-1}. The 4,6-dien-3-one system similarly gives characteristic C=C bands at ~1,618 and ~1,587 cm^{-1}.

Simple conjugated dienes (e.g. $\Delta^{3,5}$) also give two bands (~1,650 and ~1,600 cm^{-1}), whereas the esters and ethers of 3,5-dien-3-ols, the enolic form of 4-en-3-ones, give relatively strong bands in the range 1,690–1,660 cm^{-1}.

The C≡C stretching band in ethynyl steroids (around 2,200 cm^{-1}) is very weak. Aromatic rings generally show two or three stretching vibrations in the region around 1,600 cm^{-1}, according to structural type.

Olefinic and aromatic compounds with at least one hydrogen atom bound to unsaturated carbon are additionally characterised by C–H out-of-plane bending

Table 2.6 Characteristic group wave-numbers for unsaturated steroids

	Wave-number (cm^{-1})	
Group	C=C or C≡C stretching	C–H bending[a]
C≡C		
17-Ethynyl	2,150–2,100[b]	
C=C		
1-ene	1,644[c]	754–752
2-ene	1,657–1,654[c]	774–772 and 663
3-ene	1,647[c]	773 and 761
4-ene	1,657[c]	
5-ene	1,672–1,667[c]	840 and 800
6-ene	1,639–1,633[c]	772, 739, 729 and 710–704
7-ene	1,672–1,664[c]	830 and 800
9(ll)-ene	1,645–1,635[c]	828–821
14-ene	1,648–1,646[c]	825–822, 810–808 and 801–800
16-ene	1,630–1,621[c]	715–710
22-ene (trans)	1,666–1,664[c]	974–970
3,5-diene	1,618 and 1,578	
5,7-diene	1,655–1,650 and 1,600	840–835 and 808
l-en-3-one	1,609–1,604	
4-en-3-one	1,620–1,612	880–860
5-en-7-one	1,633	
9(ll)-en-12-one	1,607	
16-en-20-one	1,592–1,587	
l,4-dien-3-one	1,636–1,632 and 1,608–1,603	888–887
4,6-dien-3-one	1,619–1,616 and 1,587	875–874
3,5-dien-7-one	1,627 and 1,598	
Enol acetate (six-membered ring or side-chain)	1,697–1,660	
Enol acetate (ring D)	1,620–1,615	
3,5-dien-3-ol acetate	1,670 and 1,639	
Bufa-20,22-dienolide	1,638–1,636 and 1,602	
Card-20(22)-enolide	1,624–1,619	
Aromatic rings		
Aromatic A; OH in 3	1,613–1,610, 1,590–1,589 and 1,503–1,490	
Aromatic A: OH in 2,3 and 3,4	1,625–1,623 and 1,503–1,490	
Aromatic rings A and B	1,625, 1,605 and 1,573	
Benzoates	1,604–1,603 and 1,586–1,584	708–703

[a] Tentative assignment; medium-intensity bands.
[b] Very weak sharp band.
[c] Weak, in absence of conjugation.

vibrations, which give medium-intensity bands in the fingerprint region, between 970 and 700 cm^{-1}. These bands are often the most prominent in that part of the spectrum, but must be interpreted with caution. A band at ~860 cm^{-1} is a feature of 4-en-3-ones unsubstituted at C-4. Aromatic compounds may show several bands in this region, indicative of the pattern of substitution on the aromatic ring (Williams and Fleming, 1987).

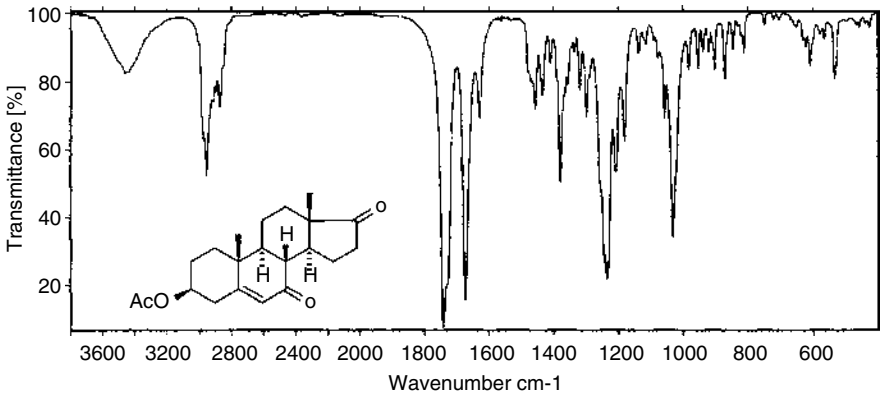


Fig. 2.21 Infrared spectrum of 7-oxo-DHEA (KBr)

When several groups of the same type exist in a molecule, all can often be identified. For instance, the spectrum of 3β-acetoxyandrost-5-ene-7,17-dione (Fig. 2.21) shows the presence of three carbonyl functions, although the acetoxy group ($\nu_{C=O}$ 1,731 cm^{-1}) appears as a shoulder at the stronger band of the cyclopentanone signal ($\nu_{C=O}$ 1,742 cm^{-1}). The third carbonyl in position 7 is well separated: see $\nu_{C=O}$ at 1,673 cm^{-1} and $\nu_{C=C}$ at 1,629 cm^{-1}. Also the signal at 1,408 cm^{-1} is of diagnostic value: it belongs to deformation vibrations of a CH_2 group next to a carbonyl group of a cyclopentanone system.

(c) *Hydroxyl Groups* (*Table 2.7*) The O–H str. band, in the region 3,625–3,200 cm^{-1}, is often among the most prominent in a steroid spectrum. Its appearance and position depend upon the state of association (hydrogen bonding) of the material. An unassociated hydroxyl group gives a single very sharp band (3,625–3,600 cm^{-1}), but hydrogen bonding, whether inter- or intramolecular, shifts and broadens the absorption band. Intramolecular hydrogen bonding between adjacent hydroxyl groups, or between a hydroxyl and a carbonyl group, gives a medium-sharp band (3,600–3,450 cm^{-1}), whereas intermolecular hydrogen bonding, either in the crystal structure or in solution, gives a broader band (3,550–3,200 cm^{-1}). For carboxylic acids, the O–H str. band becomes extremely broad and diffuse, extending down as far as 2,500 cm^{-1}.

Table 2.7 Characteristic group wave-numbers: O–H stretching bands

Group	Wave-number (cm^{-1})
O–H (hydroxyl)	
Non-associated O–H	3,625–3,600 (sharp band)
Intramolecularly associated O–H	3,600–3,450 (medium-sharp)
Intermolecularly associated O–H	3,550–3,200 (broad band)
Hydrogen bonded O–H in carboxylic acid	3,330–2,500 (very broad band)
O–D (deuterohydroxyl)	
Non-associated O–D	2,630–2,620 (sharp band)
Associated O–D	2,600–2,400 (broad band)

Many hydroxy steroids show a combination of associated and unassociated types of absorption, giving both a broad and a sharp band. It is often possible to distinguish between inter- and intramolecular association, with implications for molecular structure, by comparing spectra in solutions at different concentrations. If the interaction is intermolecular, low concentrations accentuate the 3,600 cm^{-1} band, whereas higher concentrations favour association, and so lead to an increase in the intensity of the broader lower-frequency band at the expense of the sharper one. Such spectral changes were used to investigate the state of aggregation of 5α-cholestanols in solution (Kunst et al., 1979). On the other hand, a spectrum which is independent of concentration, implies that hydrogen bonding is of an intramolecular type (i.e. between neighbouring functional groups). IR spectra therefore used to be a source of information on the conformation and configuration of the compound (Suga et al., 1972). Asymmetry in the shape of the O–H band may reflect contributions from different rotamers around the C–O bond (Boul et al., 1971). Deuterated hydroxyl groups show O–D str. bands at very much lower wave-numbers than O–H groups, the difference being about 1,000 cm^{-1}.

The C–O single bond str. vibration in alcohols is of only medium intensity and lies in the fingerprint region (1,060–1,000 cm^{-1}). It has relatively low diagnostic value, although use has been made of the observation that axial C–O bonds vibrate at the lower end of this frequency range while equatorial C–O bonds give peaks towards the higher end. Ethers of hydroxy steroids show C–O str. absorption in the range 1,200–1,080 cm^{-1}. The tertiary hydroxyl in the hydrogen-bonded 5α-hydroxy 6-ketone (see Fig. 2.22) has the C–O str. absorption at 967 cm^{-1} while its equatorial 5β-isomer (see Fig. 2.23) shows the same signal at a higher wave-number (1,014 cm^{-1}).

Experts in NMR spectroscopy are proud of being able to solve most structural problems. However, IR spectroscopy can easily uncover structures which would call for more delicate measurement, should a NMR spectrometer be used. Let us consider the above 5α-alcohol and its 5β counterpart (Fig. 2.23): the two display quite different IR behaviour (the carbon-tetrachloride spectra have the solvent

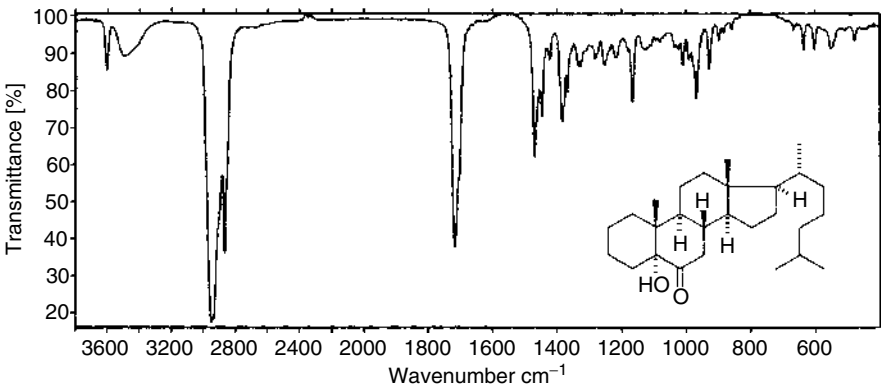


Fig. 2.22 IR spectrum of 5-hydroxy-5α-cholestan-6-one

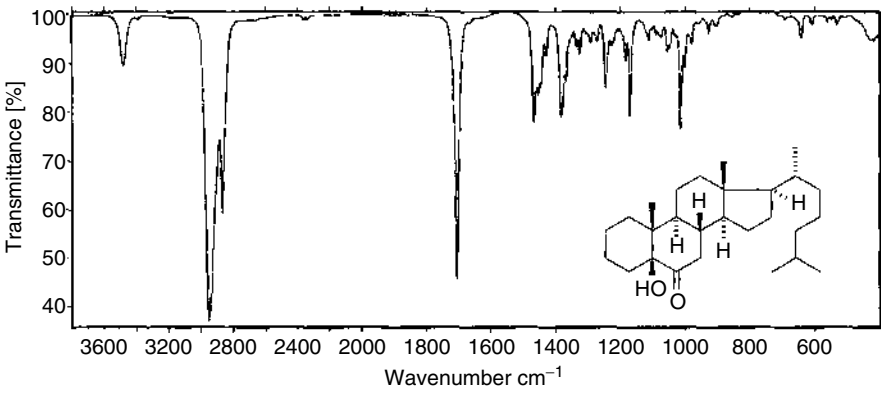


Fig. 2.23 IR spectrum of 5-hydroxy-5β-cholestan-6-one in carbon tetrachloride

region around 800 cm^{-1} deleted): In the 5α-alcohol, the hydroxy group is partly free (3,603 cm^{-1}) and partly intermolecularly hydrogen-bonded (3,494 cm^{-1}). Equally, the carbonyl region shows both the absorption of a free ketone (1,716 cm^{-1}) and of an intermolecularly associated ketone (a shoulder at 1,702 cm^{-1}).

On the other hand, the corresponding 5β-alcohol (Fig. 2.23, the spectrum measured under identical conditions) shows complete hydrogen bonding at both chromophores: the hydroxyl has only the bonded OH vibration (ν_{OH} at 3,481 cm^{-1}) and the oxo group is completely bonded ($\nu_{C=O}$ at 1,704 cm^{-1}). Apparently, the broad signal at 423 cm^{-1} belongs to the bonded hydroxyl.

(d) *Carbonyl Groups* (*Tables 2.3.5 and 2.3.6*)

Ketones All carbonyl compounds give a very strong C=O stretch peak in the region 1,800–1,650 cm^{-1}. For ketones, the wave-number is characteristic of the structural type, and especially of the ring size (Table 2.8). The most important distinction is between ketones in the five-membered ring D, which give C=O bands in the region 1,750–1,740 cm^{-1}, and those in any of the six-membered rings or in the side chain, which appear in the range 1,720–1,700 cm^{-1}. Jones' early compilation of a very large amount of data (Roberts et al., 1958) was later confirmed and augmented at Oxford University by an analysis covering mono-, di-, and tri-oxo steroids of many types (Boul et al., 1971): interactions between two or more carbonyl groups in the same molecule may increase the C=O str. frequencies by some 1–3 cm^{-1}.

α-Acetoxyketones and similar ketoesters show shifts of the ketonic carbonyl band to higher wave-number (Table 2.9), as a result of interaction between the two adjacent functional groups: $\nu_{(C=O)}$ of a 17-oxo steroid in chloroform is found around 1,737 cm^{-1} and that of an acetoxy derivative around 1,722 cm^{-1}, however, the 16α-acetoxy-17-ketone has two bands near 1,755 and 1,742 cm^{-1}.

Conjugation of the C=O group with a C=C bond shifts the absorption maximum to a lower frequency by some 30–40 cm^{-1} for α, β-unsaturated ketones. The best-known example is steroidal 4-en-3-ones, with the C=O band near 1,680 cm^{-1}. Table 2.9 includes other examples. Conjugation with additional double bonds shifts the C=O

Table 2.8 Characteristic group wave-numbers (cm^{-1}): C=O stretching bands

	CS_2 or $CC1_4$	$CHCl_3$
Aldehydes		
19-Aldehyde	1,730–1,722	1,723–1,717
Non-conjugated ketones		
(**1**, **4**, **6**, **11**, **12**, **20**, **22**)	1,714–1,704	1,707–1,698
(**3**, **7**, **24**)	1,719–1,711	1,709–1,700
(**16**, unsubstituted at C-17)	1,749	1,737
(**16**, with OH at C-17)	1,752–1,749	
(**17**-Ketone)	1,745–1,740	1,737–1,733
(A-nor-**2**-ketone)	1,745–1,740	1,737–1,733
(**17**, 14-ene)	1,754–1,752	
(**20**, with OH at C-17)	1,710–1,707; 1,697–1,690	1,710–1,700; 1,688–1,685
(**20**, with 16,17-methylene)	1,685	
(**11** and **17**)	1,752–1,748; 1,719–1,713	1,742–1,738; 1,711–1,707
Conjugated ketones		
(**1**, 2-ene)	1,682–1,680	
(**3**-Ketone, 1-ene)	1,684–1,680	1,672–1,670
(**3**-Ketone, 4-ene)	1,681–1,677	1,668–1,660
(**3** and **6**, 4-ene)	1,692	
(**7**-Ketone, 5-ene)	1,682–1,673	1,669–1,666
(7-Ketone, 8-ene)	1,667	
(**ll**, 8-ene)	1,660	
(**12**, 9(ll)-ene)	1,684–1,680	1,676–1,671
(**17**, 15-ene)	1,716	
(**20**, 16-ene)	1,670–1,666	1,662–1,652
Conjugated dienones		
(**3**, 1,4-diene)	1,671–1,663	1,666–1,660
(**3**, 4,6-diene)	1,669–1,666	
(**7**, 3,5-diene) 1663		
Carboxylic acids and esters		
Monomeric carboxyl group	1,758–1,748	
Dimeric carboxyl group	1,710–1,700	
Methyl cholanates	1,742–1,739	1,732–1,728
Methyl androstane-17β-carboxylate	1,739–1,735	
Lactone		
Cardanolides	1,793–1,786	1,778–1,775
Card-20(22)-enolides	1,786–1,780 and 1,757–1,755	1,790 and 1,750–1,747
3-Oxo-4-oxa-steroids	1,744–1,737	
17-oxo-17a-oxa-D-homosteroids	1,744–1,737	

absorption still further: e.g. a trienone – (i.e. gestrinone, see Fig. 2.24) has its $\nu_{C=O}$ at 1,649 cm^{-1}. Tetrahydrogestrinone (THG), the so-called anabolic 'designer drug' found recently in an athlete, has the same IR pattern, which proves that the four hydrogens did not occur at the conjugated enone system but the ethynyl group (Catlin et al., 2004). The C=O band is often rather broad, and at high resolution may split into two, separated by 2–3 cm^{-1}. The possible origins of this splitting have been discussed by James and Noyce (1971).

Table 2.9 Characteristic group wave-numbers for esters and ketoesters

Group	Wave-number (cm^{-1})		
	C=O stretching		
Solvent	CS_2 or CCl_4	$CHC1_3$	C–O stretching
Acetates			
Naphtholic 3-acetates[a]	1,770	1,762	1,209–1,204; 1,017–1,010
Phenolic 3-acetates[b]	1,767–1,764	1,758	1,209–1,204; 1,017–1,010
16-En-17-yl acetate	1,769–1,765		1,208–1,204; 1,099–1,096
Other enol acetates	1,758–1,749		1,225–1,206; 1,160–1,100
20,21-Diacetates	1,749	1,739–1,736	
Acetates (axial)	1,742–1,733	1,728–1,719	1,260–1,220; 1,080–1,020
Acetates (equatorial)	1,742–1,733	1,728–1,719	1,250–1,230; 1,025–1,016
Other esters			
Benzoates[c]	1,724–1,717	1,713–1,710	1,270[c]
Formates	1,729–1,725		1,180–1,175
Propionates	1,742–1,733	1,728–1,719	1,190–1,188,
Toluene-p-sulphonates[d]	–	–	1,190–1,189; 1,180–1,178[d]
Methanesulphonates	–	–	1,179–1,178
Ketoesters			
2-Acetoxy-4-en-3-one	1,748–1,737; 1,700–1,683		
6-Acetoxy-4-en-3-one	1,749–1,743; 1,688–1,685		1,234–1,232
16-Acetoxy-17-ketone	1,762; 1,750–1,748	1,757–1,753; 1,743–1,740	
17-Acetoxy- 16-ketone	1,765; 1,747	1,759; 1,737	
17-Acetoxy-20-ketone	1,742–1,736; 1,719–1,716		
21-Acetoxy-20-ketone	1,758–1,754; 1,736–1,724	1,750–1,745; 1,727–1,720	1,231; 1,078–1,053
Other acetoxyketones	1,755–1,748; 1,730–1,710		

[a]Naphtolic compounds have a and b rings aromatic.
[b] Phenolic steroids have a ring only aromatic.
[c]3-benzoyloxy ring a aromatics give three or four strong bands in the range 1,260–1,205 cm^{-1}.
[d]Tosylates have still another band at 1,099–1,097 cm^{-1}.

Solvent Effects The frequencies quoted above refer to spectra in CCl_4 or CS_2. Table 2.9 includes data for $CHCl_3$ as solvent, showing significant shifts to lower frequency in some cases. Solvent shifts of the C=O band of selected 3-oxo and other steroids have been measured for various solvents, relative to saturated hydrocarbons (James and Ramgoolam, 1978). Polar solvents solvate the carbonyl group and hence reduce the energy difference between the ground and excited states which is manifest in a reduced frequency. Some spectroscopic features found in the C–H str. region for carbonyl compounds have been noted above (see section (a) The hydrocarbon skeleton, above).

Aldehydes The aldehydic C=O str. frequency lies in the region 1,730–1,720 cm^{-1} and, as in ketones, shows some solvent sensitivity. A few compounds that are formally

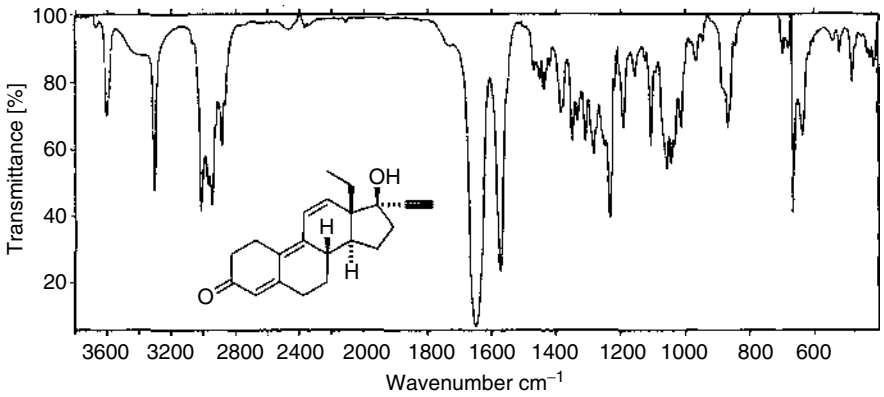


Fig. 2.24 Absorption spectrum of 17α-ethynyl-17β-hydroxy-18-homoandrosta-4,9,11-trien-3-one ("gestrinone")

aldehydic (e.g. aldosterone, Fig. 2.25) have the aldehyde carbonyl group masked by hemiacetal formation, so show no aldehyde C=O str. band (see Hydroxyl/carbonyl interactions, below). Aldehydes are also characterised by a distinctive C–H str. of the CHO group at ~2,750 cm^{-1}; it is usually weak and may appear only as an inflection on the lower-frequency side of the main C–H str. absorption band.

Hydroxyl/Carbonyl Interactions Intermolecular hydrogen bonding between a hydroxyl function and a carbonyl group of another molecule is a common feature of steroid crystal structures. Its effect is frequently seen in solid-state (KBr or nujol) spectra as a shift in the C=O str. to lower wave-number, often accompanied by band broadening. The O–H str. band is then of the associated type. The position of the carbonyl band can be deceptive in such cases, leading to possible misinterpretation: if this is suspected, it is best to rerun the spectrum in solution to obtain the normal C=O str. frequency.

It has been shown (Suga et al., 1972) that neighbouring hydroxyl and carbonyl groups of the same molecule may be hydrogen bonded to each other if the conformation is suitable. However, the expectation that the C-20 carbonyl group would be intramolecularly hydrogen bonded to 17α- and 21-hydroxy groups in corticosteroids has been shown to be erroneous, by IR studies involving competitive hydrogen bonding to added p-bromophenol (Eger et al., 1971).

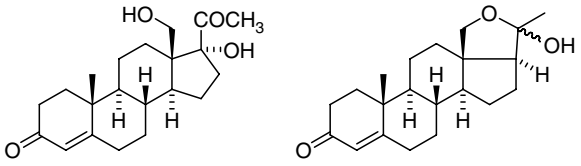


Fig. 2.25 18-Hydroxyprogesterone and its hemiacetal form

Another possibility in certain cases is the formation of internal hemiacetals, which may effectively mask the presence of a carbonyl group. 18-Hydroxyprogesterone is such a compound: the 18-hydroxy group combines with the neighbouring 20-carbonyl to form the 18,20-epoxy-20-hydroxy structure (Fig. 2.25), which is much more stable, so that the 20-oxo group fails to appear in the spectrum.

Carboxylic Acids and Their Derivatives The carboxyl O–H str. band has been described above. The C=O str. vibration is found at 1,710–1,700 cm^{-1} for free carboxylic acids in their normal hydrogen-bonded dimeric state; monomeric carboxylic acids are reported to absorb at ~1,750 cm^{-1}, but carboxylic acids rarely exist in this condition. Conversion to methyl or ethyl esters shifts the C=O str. band to the region of 1,750–1,735 cm^{-1}.

In lactones, cyclic esters between carboxyl and hydroxyl groups of the same molecule, the C=O str. band depends upon the ring size and any conjugation present (see Table 2.8).

Carboxylic esters of hydroxyl steroids show similar ester carbonyl frequencies (Table 2.9), but with variations according to the acyl component of the carboxylic acid. The acetate C=O str. frequency is normally about 1,740 cm^{-1}, but in phenolic and enolic acetates, and in α-acetoxyketones, it is shifted to 1,765–1,755 cm^{-1}. Esters of other saturated aliphatic acids (propanoates, butanoates, etc.) are similar to acetates, but formates (~1,730 cm^{-1}) and benzoates (~1,720 cm^{-1}) show significant differences.

All esters of hydroxy steroids are characterised additionally by two C–O str. vibrations, giving a distinctive total of *three* strong bands, which generally dominate the spectrum. The OC–O str. band (Fig. 2.26) is found near 1,240 cm^{-1} for acetates, or elsewhere in the range 1,270–1,160 cm^{-1} for other esters, according to the structural type (see Table 2.9). The OCO–C str. band occurs in the range 1,100–1,000 cm^{-1}, close to the C–O str. of alcohols, but with enhanced intensity.

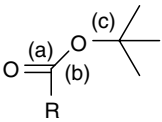


Fig. 2.26 Carbon–oxygen stretching bands in esters, (**a**) ~1,770–1,710 cm^{-1}; (**b**) ~1,270–1,160 cm^{-1}; (**c**) ~1,100–1,000 cm^{-1}

2.4 Nuclear Magnetic Resonance Spectroscopy

NMR spectroscopy is by far the most informative spectroscopic technique for the elucidation of molecular structure. Whereas IR spectroscopy concentrates on functional groups, and UV spectroscopy is limited to conjugated systems, NMR spectroscopy observes atoms of the molecular framework itself, and at the same time can reveal the presence and exact location of most of the common functional groups.

It is not possible in the space available here to give more than an outline description of the physical basis of NMR spectroscopy and of the instrumentation. The

reader can find detailed information on these topics elsewhere (Ernst et al., 1987; Sanders and Hunter, 1987; Williams and Fleming, 1987; Kemp, 1991; Grant and Harris, 1996; Macomber, 1998; Claridge, 2000; Levitt, 2001; Friebolin, 2005). We are concerned here with the ways in which the organic chemist can use NMR spectroscopy to solve structural problems.

2.4.1 Basic Principles of NMR Spectroscopy

Certain atomic nuclei, including protons ^{1}H, ^{13}C, and ^{19}F, possess *nuclear spin* (I) which gives them magnetic properties. Like bar magnets and compass needles, these nuclei tend to align their *magnetic moments* with that of an external magnetic field. The axis of spin precesses around the field direction, much as the axis of a spinning top precesses in the earth's gravitational field (Fig. 2.27a). Quantum restrictions allow magnetic nuclei to be orientated in $(2I + 1)$ ways; for ^{1}H, ^{13}C, and ^{19}F, each with spin $I = 1/2$, there are two possible orientations, one aligned with the applied field and the other opposing it (Fig. 2.27b). These are low-energy and high-energy states, respectively. The difference between their energy levels is directly proportional to the strength of the applied magnetic field. The energy difference is actually very small, giving a *Boltzmann distribution* such that there is only a minute excess of nuclei in the lower energy state at equilibrium (of the order of 1 in 10^5 or 10^6) resulting in a pure macroscopic magnetisation M_0 (Fig. 2.27c).

When radiofrequency (RF) radiation at the precession frequency is applied to atomic nuclei oriented in a magnetic field, energy is absorbed and the populations of nuclei in the two energy states become more nearly equal; this is the so-called *resonance* condition. *Saturation* occurs if the numbers of nuclei in the two states become exactly equal; no more energy can then be absorbed. The absorbed energy

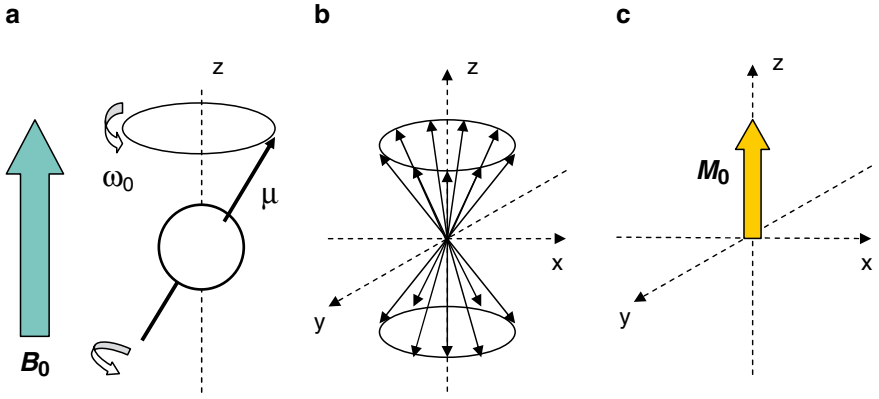


Fig. 2.27 Behaviour of nuclei with spin 1/2 in external magnetic field B_0

Table 2.10 NMR properties of selected nuclei

Nuclide	Natural abundance (%)	Spin I	Electric quadrupole moment eQ (10^{-28} m^2)	Magnetogyric ratio (10^7 rad T^{-1} s^{-1})	Relative sensitivity	Receptivity	Resonance frequency (B_0 11.47 T)
^{1}H	99.985	1/2	–	26.7519	1	1	500.00
^{2}H	0.015	1	2.87×10^{-3}	4.1066	9.65×10^{-3}	1.45×10^{-6}	76.75
^{3}H	0	1/2	–	28.5350	1.21	–	533.32
^{12}C	98.89	0	–	–	–	–	–
^{13}C	0.11	1/2	–	6.7283	1.59×10^{-2}	1.76×10^{-4}	125.72
^{14}N	99.63	1	1.67×10^{-2}	1.9338	1.01×10^{-3}	1.01×10^{-3}	36.12
^{15}N	0.37	1/2	–	−2.7126	1.04×10^{-3}	3.85×10^{-6}	50.66
^{16}O	99.76	0	–	–	–	–	–
^{17}O	0.04	5/2	-2.6×10^{-2}	−3.6280	2.91×10^{-2}	1.08 x $\times 10^{-5}$	67.78
^{18}O	0.20	0	–	–		–	–
^{19}F	100	1/2	–	25.1815	0.83	0.83	470.38
^{28}Si	95.30	0	–	–	–	–	–
^{29}Si	4.70	1/2	–	−5.3190	7.84×10^{-3}	3.69×10^{-4}	99.32
^{31}P	100	1/2	–	10.8394	6.63×10^{-2}	6.63×10^{-2}	202.40
^{32}S	95.06	0	–	–	–	–	–
^{33}S	0.74	3/2	5×10^{-2}	2.0534	9.73×10^{-2}	1.72×10^{-5}	38.35
^{34}S	4.18	0	–	–	–	–	–

is subsequently lost (*relaxation*), mainly by being dissipated, through various mechanisms, among nearby nuclei.

The resonance frequency is directly proportional to the strength of the applied field. For protons in a magnetic field of 11.74 T the resonance frequency is 500 MHz (MHz = megahertz, 10^6 cycles per second). Other nuclei resonate at different frequencies in the same magnetic field. The important NMR properties of selected nuclides are shown in Table 2.10.

2.4.2 NMR Spectrometer and Obtaining an NMR Spectrum

A schematic illustration of a modern *NMR spectrometer* is shown in Fig. 2.28. A stable – usually *superconducting magnet* – produces a homogeneous magnetic field. The field strength of the magnet is maintained by electronic locking, and homogeneity is maintained by shimming. An NMR tube containing the sample is placed into the probe. The probe contains a transmitter coil that produces oscillating magnetic field B_1 and serves also as the receiver coil in which the NMR signal is generated. All radiofrequency transmitters and receivers are located in a *console* that also contains an ADC converter, and units for control and regulation of homogeneity, pulse magnetic field gradients, sample temperature etc. A host computer at the *operator's workstation* controls all the parameters of the NMR experiments defined by the operator and stores the results. They can be saved in digital form in a computer memory and printed as hard copy NMR spectra.

Modern spectrometers operate by subjecting the sample to a single powerful pulse of broad-band radiation which covers the whole frequency range of interest

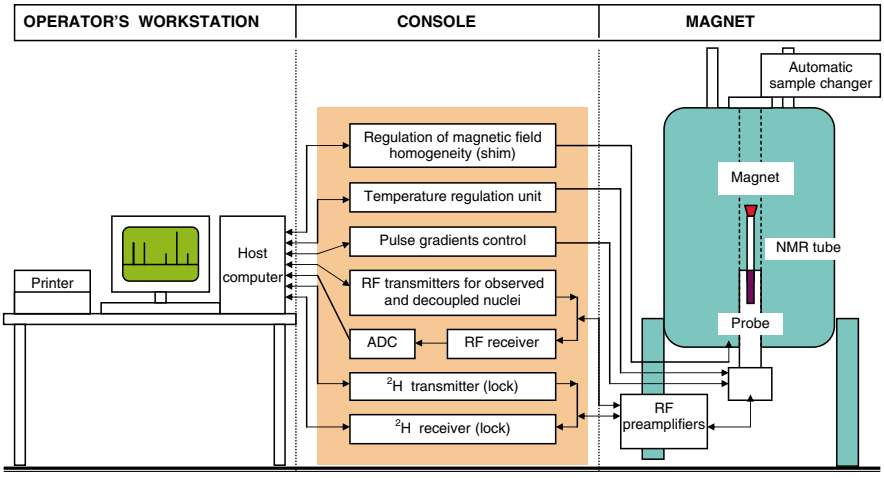


Fig. 2.28 Schematic picture of modern NMR spectrometer

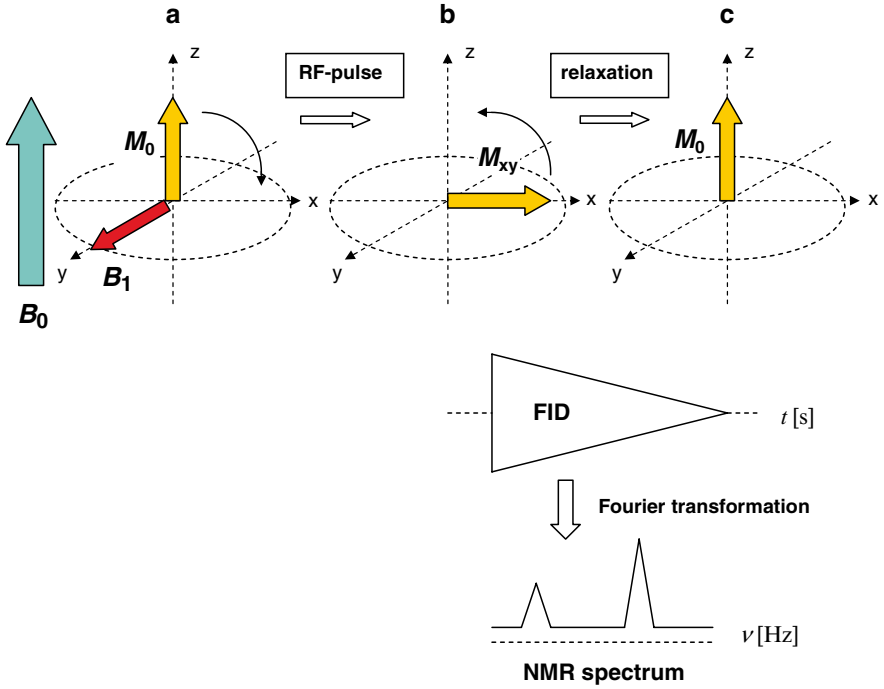


Fig. 2.29 Macroscopic magnetisation and the effect of RF pulse and following relaxation

and lasts only a few microseconds. The instrument is designed so that this pulse generates an oscillating magnetic field (B_1) perpendicular to the applied field B_0 (Fig. 2.29a). The pulse simultaneously disturbs the magnetisation of the nuclei which resonate across the whole range of frequencies, tipping the net magnetisation of the sample through an angle (θ) given by

$$\theta = \gamma B_1 t_p$$

where γ is the *magnetogyric ratio* (proportionality constant representing the strength of the nuclear magnet) and t_p is the *pulse length* (in μs). Figure 2.29b shows the situation after the flip angle 90° when the magnetisation is turned into the *x*,*y*-plane. After the pulse, the sample loses the absorbed energy as the nuclei relax back to their equilibrium distribution, over a time scale of the order of seconds (Fig. 2.29c). During this period (*acquisition time*) an RF receiver picks up the oscillation which is emitted, in the form of a complicated wave pattern known as the *free induction decay* (FID). The resulting data, in digital form, are subjected to the mathematical process of a *Fourier transformation* to obtain the spectrum. This is

done by a computer which both controls the spectrometer and collects and processes the data. The NMR spectra are usually obtained by summing the FIDs from a number of repeated pulses. The signal-to-noise ratio S/N is improved by a factor of $\sqrt{n}$, where n is the number of pulses employed.

2.4.3 NMR Parameters

There are five NMR parameters which can be extracted from NMR spectra. They will be briefly discussed in the following paragraphs.

2.4.3.1 Chemical Shift

The great value of NMR spectra to chemists results from the fact that identical nuclei in different environments within a molecule resonate at slightly different frequencies. In a magnetic field B_0, the electrons surrounding each nucleus circulate in such a way as to generate an induced field B_{ind} of opposite direction. The effective magnetic field B_{eff} is proportional to B_0 according to the relation:

$$B_{eff} = B_0 - B_{ind} = B_0 - B_0\sigma$$

where σ is so called *shielding constant* that reflects the chemical surrounding of nucleus. The nuclei with a different chemical surrounding are exposed to different B_{eff} (they have different shielding constants σ) and they resonate at different frequencies:

$$\nu = \gamma B_{eff}/2\pi$$

Since it is not practical to measure resonance frequencies in the absolute values (MHz), the frequency differences (in Hz) are measured from the resonance of a chosen standard substance as *chemical shifts*. However, the chemical shifts in frequency units depend on the spectrometer frequency ν_0 (or field B_0). For easy comparison of NMR data from different spectrometers the so called δ-*scale* with chemical shifts expressed in dimensionless units (parts per million, ppm), defined by the relation:

$$\delta\,[\text{ppm}] = [(\nu(\text{sample}) - \nu(\text{standard}))/\,\nu_0(\text{spectrometer})]\cdot 10^6$$

was introduced. The nuclei with higher δ-values are often said to be *deshielded* (*lowfield* and/or *paramagnetic shift*) while the nuclei with lower δ-values are shielded (*upfield* and/or *diamagnetic shift*). The chemical shift δ-scale and the direction of shielding and deshielding effects is shown schematically in Fig. 2.30.

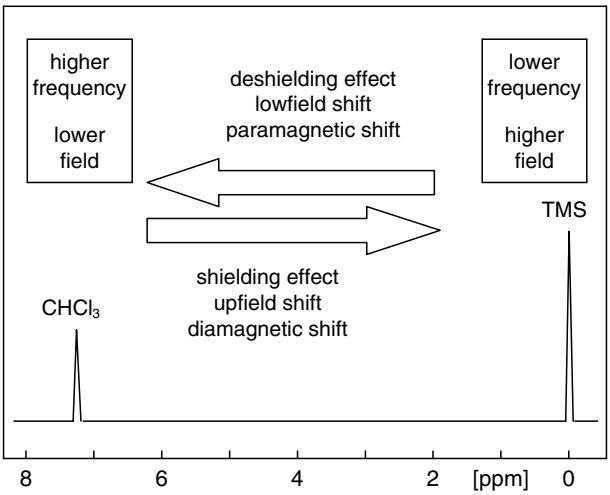


Fig. 2.30 Schematic ^{1}H NMR spectrum of $CHCl_3$ with TMS as standard. Definition of δ-scale, shielding and deshielding effects

2.4.3.2 Spin–Spin Coupling

The *direct* or *dipolar interactions* between nuclear spins through space are only observable in solid state NMR spectra while in solution they are averaged to zero by molecular motion. In solution there are other types of magnetic interaction – *indirect* or *scalar interactions* through covalent bonds – that cause the splitting of NMR signals into multiplets. The energy of scalar interaction is given by the *spin–spin coupling constant J* (in Hz), that can be measured as a frequency difference between two lines in a multiplet. Unlike chemical shift, the *J*-value does not depend on the field B_0. The size of *J* decreases in general with the number of bonds between coupled nuclei. According to the number of intervening bonds the coupling constants are described as ^{1}J (one-bond or direct couplings), ^{2}J (geminal couplings), ^{3}J (vicinal couplings), ^{4}J and ^{5}J (long-range couplings).

2.4.3.3 Signal Multiplicity

Signal multiplicity is the extent of splitting of the NMR signal due to spin–spin couplings. Signals which show no splitting are described as *singlets* (s). The multiplets with two, three and/or four equidistant lines (equal *J*-values) are called *doublets* (d), *triplets* (t) and/or *quartets* (q). When two or more different *J*-values produce a multiplet, this is referred to as two- or threefold multiplet, e.g. a doublet of doublets (dd), a doublet of triplets (dt), or a doublet of doublets of doublets (ddd).

2.4.3.4 Intensities of Signals

The absolute values of the intensities of NMR signals are complex function of parameters of sample, spectrometer and experimental conditions. On the other hand relative intensities of signals are (under proper conditions of the NMR experiment) directly proportional to the concentration of nuclei in solution. In the case of pure compounds they reflect the relative numbers of resonating nuclei while in the case of mixtures they correspond to the relative numbers of molecules of individual compounds present in the mixture. The relative intensities are therefore used in the structural analysis to determine numbers of protons in individual signals as well as in quantitative analysis for determination of the sample purity and/or molar ratio of components in the mixture. The addition of a known amount of standard allows the estimation of the amounts of compounds in the sample.

2.4.3.5 Relaxation and Relaxation Times

Relaxation refers to all processes which regenerate the equilibrium distribution of nuclear spins at their energy levels.

Spin–lattice (*longitudinal*) *relaxation* involves energy exchange between a given nuclear spin and fluctuating magnetic or electric fields in the lattice (the collection of neighbouring magnetic nuclei of the sample). It restores an excess of the nuclei at the lower energy level (Boltzmann distribution).

The *spin–lattice relaxation time* T_1 is the time constant of the return of longitudinal magnetisation M_Z to its equilibrium value M_0 (see Fig. 2.31a).

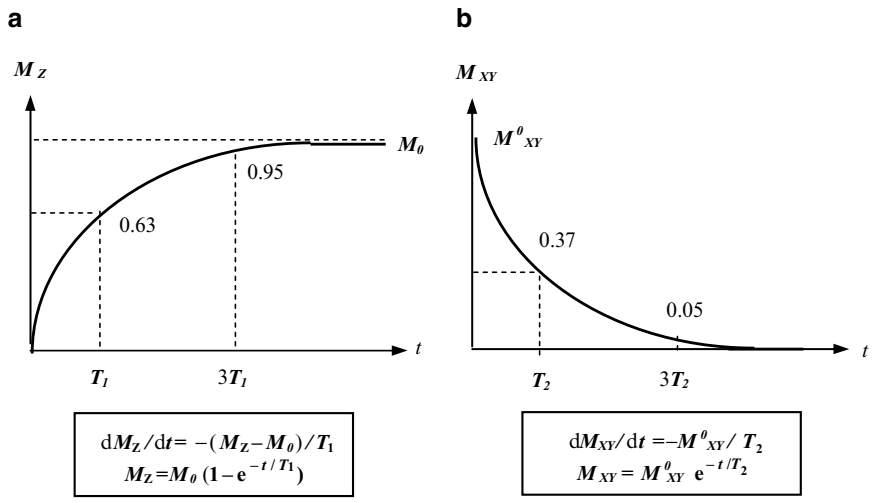


Fig. 2.31 (**a**) Return of longitudinal magnetisation M_Z to its equilibrium value M_0; (**b**) decay of transverse magnetisation M_{XY} produced by the NMR excitation to its zero equilibrium value

Spin–spin (*transversal*) *relaxation* can be accomplished either by mutual energy exchange between two nuclei or by inhomogeneities in the magnetic field B_0. In either case, this relaxation is an entropy driven process which does not change the energy of the target spins.

Spin–spin relaxation time T_2 is the time constant of the steady decay of transverse magnetisation M_{XY} produced by the NMR excitation to its zero equilibrium value (see Fig. 2.31b). The value of T_2 of a given nucleus determines the half-width ($\nu_{1/2}$) of its NMR signal that is given by the relationship:

$$\nu_{1/2} = 1/\pi T_2 + \nu^*$$

where $1/\pi T_2$ is the natural half-width and ν^* is the line broadening caused by inhomogeneities of field B_0.

The efficiencies of both types of relaxation depend critically on the similarity of the oscillation frequency (or correlation time) of the interacting nuclei in the lattice and resonance frequency of the target nuclei. The relaxation times therefore reflect molecular dynamics.

2.4.4 Factors Influencing the NMR Spectrum

The observed NMR spectrum can be influenced by internal factors (symmetry of the molecule, order of spectrum, dynamic processes) and external factors (spin decoupling, magnetic field B_0, solvent, concentration, temperature). Their effects on the observed NMR spectrum are shortly discussed in the following paragraphs.

2.4.4.1 Symmetry of Molecule and Equivalence of Nuclei

The NMR spectrum reflects the symmetry of the molecule. Nuclei of the same isotope in the molecule can display the following type of equivalence.

Chemical shift equivalence shows the nuclei with same chemical shift (nuclei are isochronous) as the result of either symmetry of the molecule and/or accidental combination of shielding effects.

Nuclei related by one or more symmetry elements (centre, axis, plane) as a result of the symmetry of the molecule and/or the motional averaging (e.g. interconverting conformers). display *symmetry equivalence*. Symmetry equivalent nuclei have the same chemical shift.

Magnetically equivalent nuclei display not only the same chemical shifts but also the same coupling constants with each of the other nuclei in the molecule. The mutual spin–spin couplings of magnetically equivalent nuclei are not observed in NMR spectra.

2.4.4.2 Order of the NMR Spectrum and Spin Systems

First order spectra (*multiplets*) are observed when the coupling constant is small compared with the frequency difference of chemical shifts between the coupled nuclei. The following rules are valid for the first order spectra of nuclei with spin $I = 1/2$:

Maximum number of lines (m) in the multiplet of a given nucleus is:

$$m = (n_a + 1)(n_b + 1)...$$

where n_i are numbers of magnetically equivalent nuclei coupled to given nucleus. If all J-values are identical then $m = n + 1$.

The *relative intensities* of the individual lines are given by coefficients of the Pascal triangle:

Doublet	1:1
Triplet	1:2:1
Quartet	1:3:3:1
Pentet	1:4:6:4:1

Chemical shift corresponds to the centre of multiplet.

Width of multiplet (W) corresponds to the sum of coupling constants of a given nucleus.

Notation of Spin Systems The isolated group of mutually coupled nuclei is called a spin system. The notation of spin systems reflects number of nuclei, type of equivalence and strength of couplings. It is convenient to label individual protons in coupled systems using letters of the alphabet. The next letters of the alphabet (A, B, C, D, …) are used for non-equivalent nuclei with signal separation comparable with the splitting (strongly coupled nuclei). Terms like A, M, X are used for widely separated nuclei (weakly coupled). Magnetically equivalent nuclei have the same letter with the index reflecting the number of those nuclei (e.g. A_3, B_2, …). Symmetrically equivalent nuclei are denoted as A, A′, B, B′,… Some examples of common spin systems are shown in Fig. 2.32.

Second order spectra (where chemical shift differences are comparable with J-values) display complex spectral patterns. The accurate values of NMR parameters (δ and J) cannot be simply read from the spectrum and require simulation–iteration analysis. Programmes for such calculations are available either as a standard part of the NMR software package or as special programmes (e.g. *gNMR*, *PERCH*).

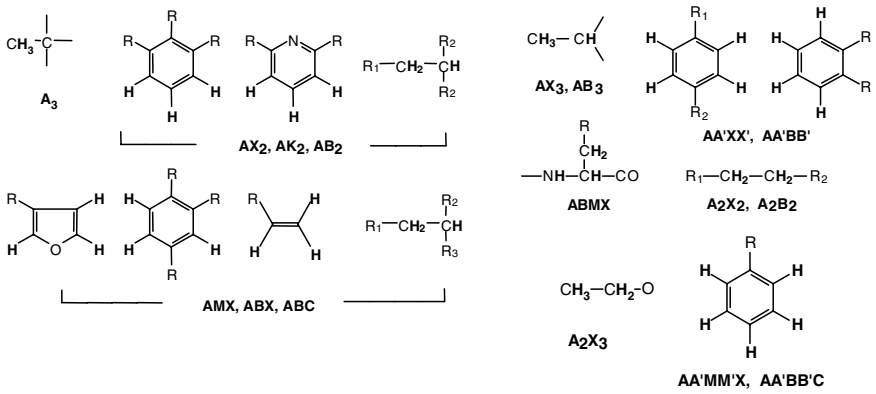


Fig. 2.32 Some examples of common three-, four- and five-spin systems

2.4.4.3 Dynamic Processes

NMR methods can be used to study both the basic types of chemical processes: reversible reactions (leading to an equilibrium mixture) and irreversible reactions (proceeding in one direction to the final reaction products).

The NMR study of *irreversible reactions* is based on the quantitative determination of starting compounds and reaction products in NMR spectra repeatedly measured in the proper time intervals.

Reversible processes (interconversion of conformers, valence tautomerism, proton transfers, etc.) are commonly called *chemical exchange* (one or more nuclei exchange their site in the molecular environment) in NMR. The appearance of the spectrum for a compound undergoing exchange depends on the relative magnitude of the exchange rate constant (k) compared to the difference in chemical shift between the sites ($\Delta\nu$). When $k << \Delta\nu$ (slow exchange), the spectrum consists of sharp signals for each site in the interconverting structures. When $k >> \Delta\nu$ (fast exchange) each set of exchanging nuclei gives rise to one sharp signal whose chemical shift is the average of all interconverting sites. When $k \approx \Delta\nu$, each set of exchanging nuclei gives rise to one very broad signal (coalescence). The estimation of thermodynamic parameters of chemical exchange requires a computer line-shape analysis of spectra over a wide range of the k values (temperature interval).

Many rapid chemical exchanges involve formation of *guest–host complexes* between two or more molecules. The observed chemical shift is then related to the equilibrium constant K for complex formation. The use of *lanthanide shift reagents* in NMR spectroscopy belongs to this category.

Enantiomers dissolved in common (achiral) solvents exhibit identical NMR spectra. However, if such molecules are placed into an asymmetric medium (chiral solvent or chiral shift reagent) the formation of dynamic diastereoisomeric complexes may lead to the splitting of signals and resolution of enantiomers present in the original racemic mixture.

2.4.4.4 Spin Decoupling and Nuclear Overhauser Effect

Spin decoupling (double resonance) is an NMR technique in which a second oscillating magnetic field B_2 is applied. When signals in a spectrum are split by mutual spin coupling, it is possible to remove the coupling and so simplify the spectrum. For the simplest AX system, this is done by strongly irradiating nucleus A at its resonance frequency, causing it to flip rapidly between spin states, while applying the observing RF pulse and acquiring the spectrum. The X nucleus then sees only an averaged spin state of nucleus A, and is decoupled from it giving a singlet instead of doublet. If the A and X nuclei are the same isotope (e.g. protons), this experiment is referred to as *selective homonuclear decoupling*. If A and X nuclei are different (e.g. ^{1}H and ^{13}C), then it is referred to as *selective heteronuclear decoupling*.

Selective homonuclear proton decoupling allows the determination of mutually coupled protons (typically separated by two or three bonds), and thus helps in structure determination and signal assignment. Nowadays homonuclear decoupling experiments have been efficiently replaced by two-dimensional correlation spectroscopy (2D-H,H-COSY), that permits simultaneous detection of all coupled protons in a sample.

In ^{13}C NMR spectroscopy, different kinds of heteronuclear spin decouplings can be used. In *proton broadband decoupled* ^{13}C NMR spectra a strong irradiation field B_2 is placed in the middle of the ^{1}H spectrum covering the whole frequency range of proton shifts. The spectrum then displays singlets for the carbon atoms of the molecule.

In a *gated proton decoupling* the decoupler is switched on during the so called relaxation delay and switched off during data acquisition. The obtained proton-coupled ^{13}C NMR spectra contain carbon multiplets with intensity enhancement by the nuclear Overhauser effect (NOE, see below). This method is used when J(C,H) are required for structure analysis.

Inverse gated decoupling, with the proton decoupler switched on only during data acquisition, provides proton decoupled ^{13}C NMR spectra with suppressed NOE. The relative intensities of signals are comparable and this technique can be used in a quantitative analysis of mixtures.

The *nuclear Overhauser effect* causes a change in intensity (usually increase) during decoupling experiments. The maximum intensity enhancement (NOE) depends on the magnetogyric ratios of the coupled nuclei. In the homonuclear case (proton–proton decoupling) the NOE is always less than 50%, while in heteronuclear case (proton decoupling in ^{13}C NMR spectra) NOE enhancement is commonly close to a maximum value of 200%.

2.4.4.5 Effect of Magnetic Field, Solvent, Concentration and Temperature

Higher magnetic field B_0 increases the energy difference ΔE between spin states of nuclei ($\Delta E = \gamma h B_0/2\pi$) and the difference in populations of spin states n_α, n_β according to Boltzman law ($n_\alpha/n_\beta = e^{\Delta E/kBT}$) and therefore increases the *sensitivity of the NMR*

experiment and *spectral resolution* (increases separation of signals in Hz but it has no effect on coupling constants). Both these effects are very important since they can shorten measurement times and allow the study of smaller amounts of samples and the analysis of spectra that are too complex at lower magnetic fields B_0.

The NMR spectrum in solution is influenced by physical and chemical interactions between molecules of substrate as well as by interactions between molecules of substrate and solvent. These interactions (H-bonds, molecular associations, electric fields of polar molecules, Van der Waals forces etc.) lead to the dependence of chemical shifts (and to a smaller extent also coupling constants) on the substrate concentration, temperature, and solution pH.

The use of dilute solutions can reduce the effect of the substrate–substrate interactions while inert solvents minimise the substrate–solvent interactions. The change of dielectric constant of solvent mainly influences the chemical shifts of nuclei in the neighbourhood of substrate polar groups. Solvents capable of forming intermolecular hydrogen bonds (e.g. acetone, DMSO, etc.) have significant effects on the signals of labile hydrogen atoms (OH, NH, SH) of the substrate. Suppression of intermolecular exchange allows the observation of coupling constants of these protons which are not observed in other solvents. *Aromatic solvents* (benzene or pyridine) are strongly magnetically anisotropic. They can form dynamic collision complexes with a substrate containing a polar group and thus induce characteristic changes of chemical shifts in comparison with those observed in $CDCl_3$ (ASIS = aromatic solvent induced shifts). Some physical properties and chemical shifts of common NMR solvents are summarised in Table 2.11.

Temperature dependence of NMR spectra can be observed in such cases when in a given temperature range, there are some dynamic processes with a rate comparable with the time-scale of NMR spectra (e.g. conformation interconversion).

2.4.5 NMR Spectra of Nuclides Potentially Usable in Steroids

In the structure analysis of steroids the ^{1}H and ^{13}C NMR spectra play the most important role. In some special cases valuable information can be obtained also from ^{2}H, ^{3}H, ^{17}O and/or ^{19}F NMR spectra. The NMR properties of these nuclei together with some characteristic features of their NMR spectra will shortly be discussed in the following paragraphs.

2.4.5.1 Proton NMR Spectra

Hydrogen ^{1}H has an exceptional position in NMR spectroscopy of organic compounds due to its extensive occurrence and advantageous NMR properties (high natural abundance 99.98%, spin $I = 1/2$ and high sensitivity).

Table 2.11 Some physical properties and chemical shifts of common NMR solvents

Solvent	m.p. (°C)	b.p. (°C)	Dielectric constant	Nondeuterated			Deuterated
				δ (H)	δ (HOD)[a]	δ (C)	δ (C)
Acetic acid	16.7	117.9	6.1	2.10	11.6	20.9; 178.8	20.0; 178.4
Acetone	−94.7	56.3	20.7	2.09	2.0	30.7; 206.7	29.8; 206.5
Acetonitrile	−44.0	81.6	37.5	2.00	2.1	1.7; 118.2	1.3; 118.2
Benzene	5.5	80.1	2.3	7.27	0.4	128.5	128.0
Chloroform	−63.5	61.1	4.8	7.25	1.5	78.0	77.0
Cyclohexane	6.6	80.7	2.0	1.40	–	27.8	26.4
Dichlormethane	−95.1	39.8	8.9	5.30	1.5	54.2	53.7
Dimethylsulphoxide	20.2	189.0	46.7	2.50	3.3	40.9	39.7
1,4-Dioxane	11.8	101.3	2.2	3.57	2.4	67.6	66.5
Methanol	−97.7	64.7	32.7	3.30	5.0	49.9	49.0
Pyridine	−41.6	115.3	12.4	7.12; 7.50; 8.61	5.0	124.2; 136.2; 150.2	123.5; 135.5; 149.2
Tetrahydrofurane	−108.0	64.0	7.6	1.73; 3.58	2.5	26.7; 68.6	25.2; 67.4
Toluene	−94.9	110.6	2.4	2.30; 7.20	0.4	21.3; 125–138	20.4; 125–138
Trifluoroacetic acid	−15.2	72.4	39.5	–	11.5	113.6; 161.4	113.5; 161.1
Water	0	100.0	78.5	4.80	4.8	–	–

[a] Signal of water present in a given solvent.

Chemical Shifts

The chemical shifts of protons are sensitive to a number of structural factors (see Fig. 2.33). The small range of proton chemical shifts (<20 ppm) leads to overlap

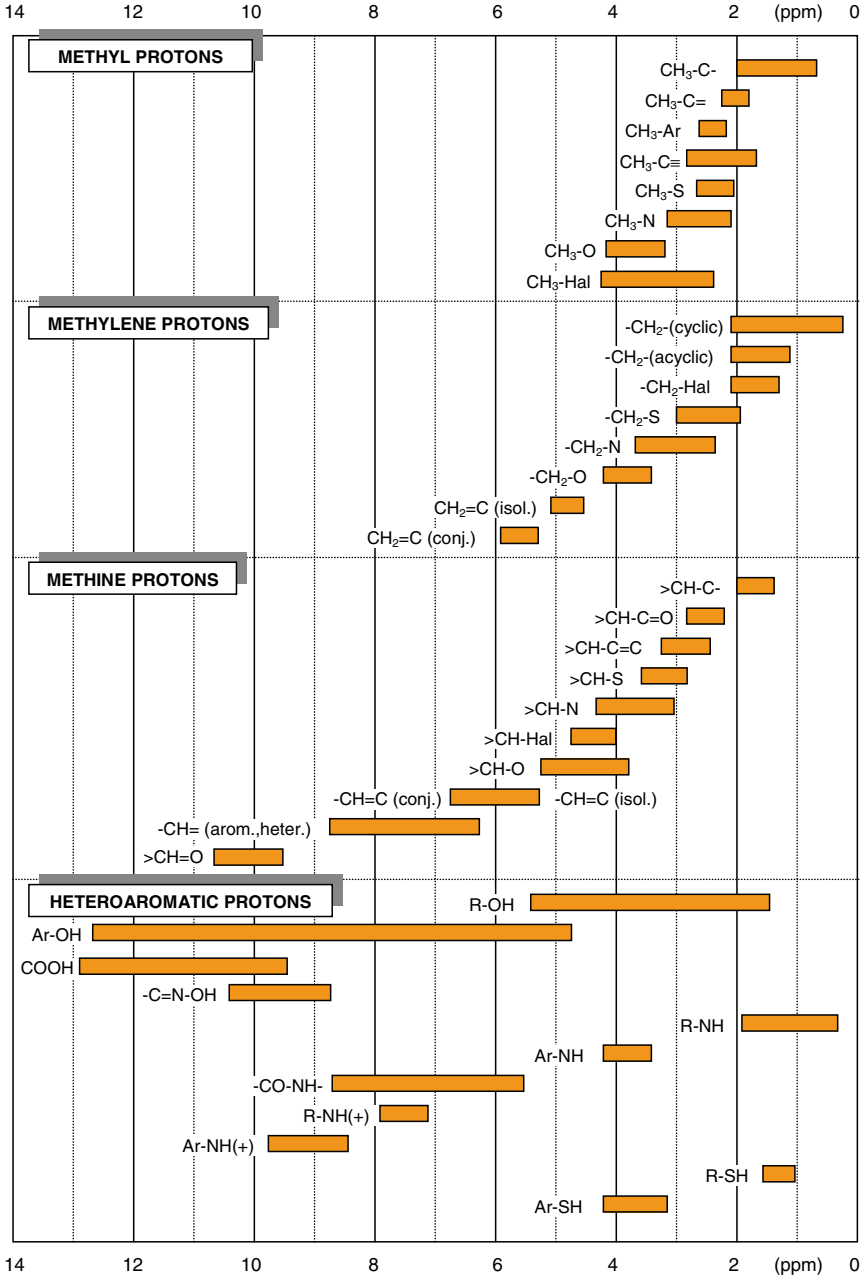


Fig. 2.33 Proton chemical shifts in organic compounds

of signals in spectra of medium size molecules (like steroids) measured even with high-field spectrometers. Protons in saturated linear or cyclic hydrocarbons generally resonate in the region δ 0.5–2.5. Methyl groups give strong signals (singlets, doublets or triplets) which are readily observed in the high-field part of this region (δ 0.5–1.5).

Larger chemical shifts arise for protons linked directly to unsaturated carbon atoms. This is due to the greater electronegativity of sp^2 compared with sp^3 hybridised carbon atoms and magnetic anisotropy (see below). Olefinic protons usually resonate in the range δ 5–6. A terminal hydrogen atom in alkynes gives a sharp peak at δ 2–3. Protons on aromatic rings resonate in the region δ 6.5–8.5.

Proximity to electronegative substituents is the second factor that leads to quite large downfield chemical shifts. Protons attached to carbon atoms substituted with hydroxyl, halogen, or other electronegative group give signals in the range δ 3–5, the precise value depending upon the functional group responsible. Two or more electronegative atoms on the same carbon atom as the proton cause larger downfield chemical shifts. The combined effects of electronegativity and unsaturation are seen in aldehydes, where the *CH=O* proton resonates at an exceptional chemical shift around δ 9.5–10. At the opposite end of the scale, the shielding effect of silicon, which puts the TMS signal at highest field, is due to the low electronegativity of silicon in comparison with carbon.

The third structural factor that strongly influences chemical shifts arises from the anisotropy of local magnetic fields produced by the motions of electrons in bonds. In simple terms, these magnetic fields have different strengths in different directions. The effect of a particular bond on any magnetic nucleus in its vicinity will therefore depend upon the orientation of the bond in relation to the affected atom.

Hydroxyl Protons–Deuterium Exchange

In simple alcohols, unless highly purified to exclude traces of acidic or basic impurities, proton exchange between hydroxyl groups is so rapid that the O*H* proton signal appears as a sharp or broad singlet, representing the time-averaged environments of the O*H* protons.

Low temperatures, or strongly associating solvents, especially C_5D_5N and d_6-DMSO, may slow proton exchange sufficiently to allow observation of hydroxyl proton couplings (doublet for CHO*H*; triplet for CH_2O*H*).

The signals of OH protons can be identified in the ^{1}H NMR spectrum by *deuterium exchange*. After the addition of a small amount of D_2O or CD_3OD the signals of XH protons disappear:

$$ROH + D_2O \Leftrightarrow ROD + DHO$$

Other easily exchangeable protons (N*H* or S*H*) produce the same effects, but they are rare in steroids. Deuterium exchange is also responsible for the absence of signals of exchangeable protons if D_2O and/or CD_3OD is used as a solvent.

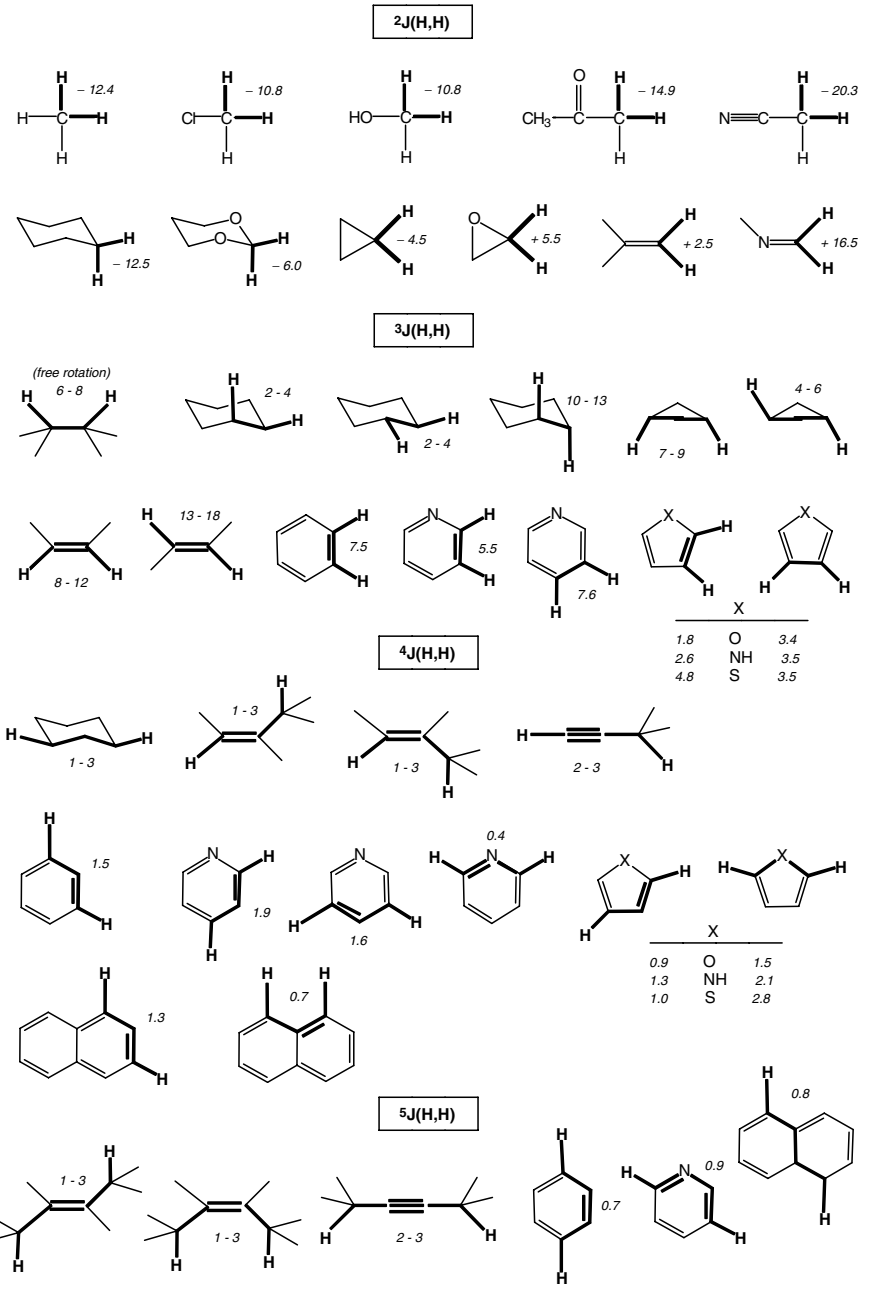


Fig. 2.34 Coupling constants *J*(H,H) in organic compounds

Coupling Constants *J*(H,H)

In ^{1}H NMR spectra commonly observed couplings are over two and three bonds.

Geminal coupling ^2J(H,H) depends on the number and type of substituents, bond angle H–C–H and the number and orientation of neighbouring multiple bonds as illustrated in Fig. 2.34.

Vicinal coupling ^3J(H,H) strongly depends on the torsion angle of the coupled protons (ϕ). Whereas in a freely-rotating alkyl chain the ^3J value is 6–7 Hz, in rigid ring systems the observed ^3J allows us to distinguish between protons in axial and equatorial conformations and estimate the approximate value of the torsion angle between protons. The so called generalised Karplus relationship, which takes into account the number, electronegativities and orientation of substituents (see Fig. 2.35) has found broad application. The relationship was derived by Haasnoot et al. (1980) and parametrised from large series of experimental NMR data and geometry parameters (mainly obtained from X-ray analysis).

A pair of vicinal axial protons ((ϕ) ~ 180°) in a six-membered ring with a chair conformation shows large values (^3J = 10–14 Hz), while if one or both of the protons

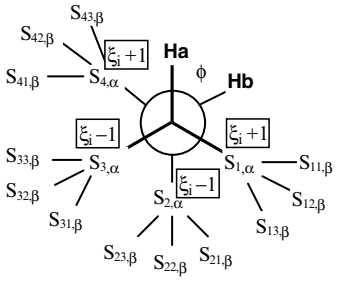
Generalized Karplus equation:

$$^3J(\mathrm{Ha,Hb}) = P_1\cos^2\phi + P_2\cos\phi + P_3 + \Sigma\Delta\chi_i\,(P_4 + P_5\cos^2(\xi_i\,\phi + P_6|\Delta\chi_i|))$$

$\Delta\chi_i = \Delta\chi_{i,\alpha} - P_7\,\Sigma\Delta\chi_{ij,\beta}$

$\Delta\chi_{i,\alpha}$, $\Delta\chi_{ij,\beta}$ Huggins electronegativity difference between α or β substituent and hydrogen with $\Delta\chi_{i,\alpha}$, $\Delta\chi_{ij,\beta}$ = 0.4 (C), 1.3 (O), S (0.4), 0.85 (N), –0.05 (P), 1.7 (F), 0.45 (I)

ξ_i is relative orientation factor of substituent in Newman projection
(has a value ξ_i = +1 if angle Hα-C-$S_{i,\alpha}$ = +120°
has a value ξ_i = –1 if angle Hα-C-$S_{i,\alpha}$ = –120°)



Numerical values of P_1 to P_7 depending on number of substituents in α-position:

Number of substituents	P_1	P_2	P_3	P_4	P_5	P_6	P_7
2	13.89	–0.96	0	1.02	–3.40	14.9	0.24
3	13.22	–0.99	0	0.87	–2.46	19.9	0.00
4	13.24	–0.91	0	0.53	–2.41	15.5	0.19
overall	13.70	–0.73	0	0.56	–2.47	16.9	0.14

Fig. 2.35 Generalised Kaplus relationship and definition of its parameters

are equatorial ($\phi \sim 60°$) the values are much smaller (^3J = 2–5 Hz). This allows a clear distinction between axial and equatorial secondary alcohols, halides, and similar derivatives, from the width of the signal of the **H**–C–X proton (where X is OH, OAc, Br, F, etc.). An axial group X implies equatorial **H**, with a narrow signal, whereas equatorial X leaves **H** axial and results in a broad signal.

Long-range couplings (over four and five bonds) are usually observed only in rigid (mostly cyclic) systems with the proper geometry of single bonds and/or over multiple bonds.

The so-called "W" coupling (in a near-planar H–C–C–C–H zig-zag arrangement) appears as a fine splitting 1–3 Hz (e.g. between 1,3-diequatorial hydrogen atoms).

The size of *allylic couplings* (0–3 Hz) over four bonds in an H_a–C–C=C–H_b fragment depends on the angle θ between the double bond plane and the C–C–H_a plane. The largest values are observed for $\theta \sim 90°$, with a maximum overlap of the π-electrons with the σ-electrons of the C–H_a bond, and the smallest values for $\theta \sim 0°$.

Homoallylic couplings over five bonds (0–4 Hz) in an H_a–C–C=C–C–H_b fragment reach their largest values when both angles θ_1 and θ_2 (between the C–C–H_a and C–C–H_b planes and the plane of the double bond) are close to 90°.

In aromatic rings characteristic values of ^3J(*ortho*-) = 5.5–8.5 Hz, ^4J(*meta*-) = 1–3 Hz and ^5J(*para*-) = 0–1 Hz can be used to determine the positions of ring substituents.

2.4.5.2 Carbon-13 NMR Spectra

^{13}C NMR spectra are the best source of direct information about the molecular framework. The ^{13}C nucleus gives intrinsically weaker signals than ^{1}H, and has low natural abundance (~1.1%). As a result, ^{13}C NMR is less sensitive than ^{1}H NMR by a factor of some 6,000. Nevertheless, modern FT NMR spectrometers have made ^{13}C spectra routinely available for about 3 decades.

The range of chemical shifts for ^{13}C nuclei is more than 200 ppm. TMS provides the usual reference signal at δ 0.0; all other ^{13}C signals appear at lower field. Figure 2.36 shows that many carbon-containing functional groups can be identified by characteristic chemical shifts.

^{13}C nuclei interact with the spins of any attached protons, so most ^{13}C signals are multiplets, unless they are deliberately decoupled from protons (see below). The ^1J(C,H) coupling constant varies with structure, between 120 and 250 Hz; values for saturated ring compounds are at the lower end of the range. The n +1 rule applies (CH_3, quartet; CH_2, triplet; CH, doublet). Only those carbon atoms with no attached protons (e.g. quaternary and carbonyl carbons) give singlet signals. Multiplets in the proton-coupled ^{13}C spectrum of a steroid invariably overlap. It is virtually impossible to analyse the raw spectrum by inspection. To overcome this difficulty, *broadband decoupling* is used to remove all the signal splitting due to protons. Each ^{13}C nucleus then resonates as a single sharp line. The number of carbon atoms in the molecule can be counted directly from the number of lines, except in the rare instance where two signals coincide.

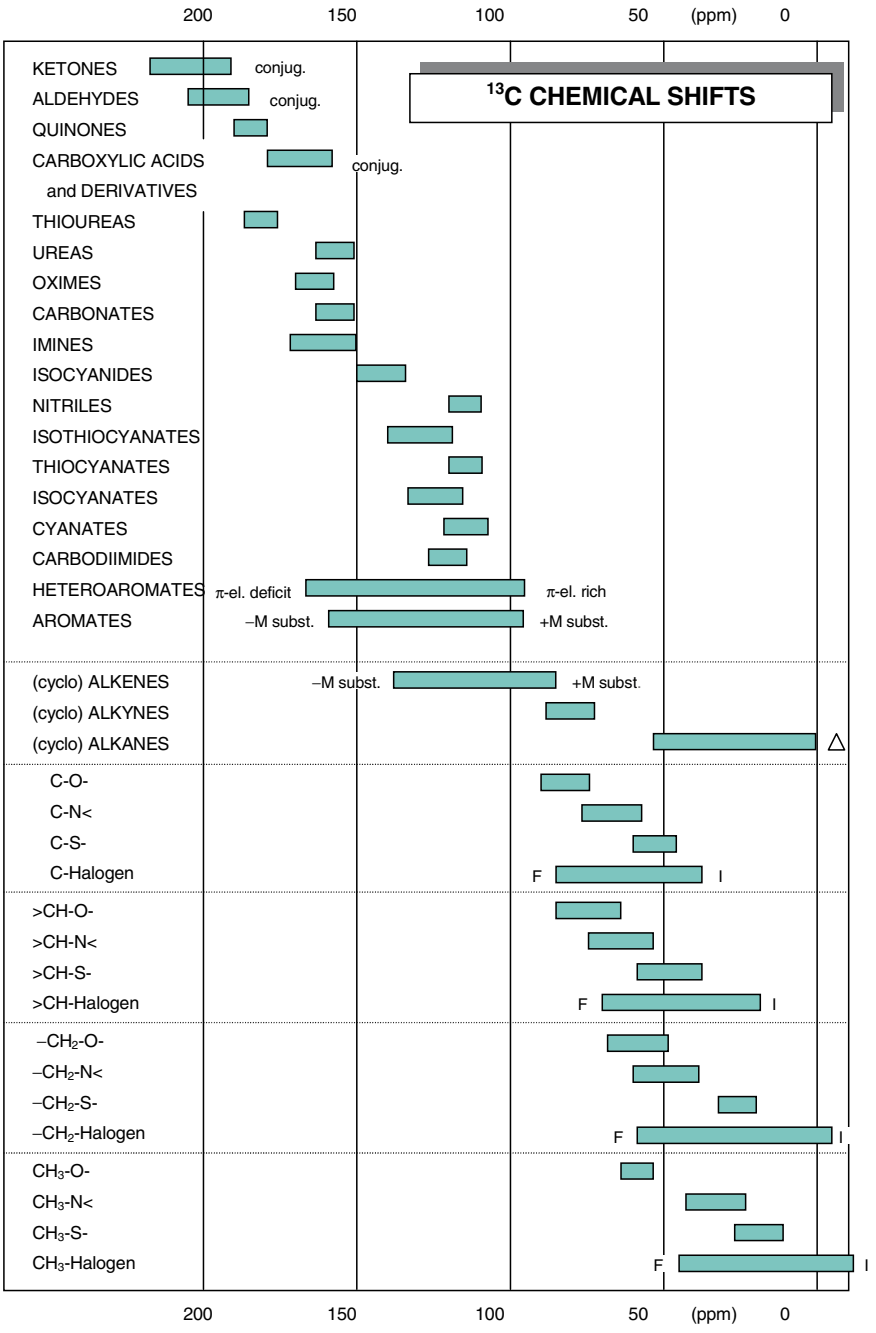


Fig. 2.36 Carbon-13 chemical shifts in organic compounds

The J(C,C) couplings are normally not observed, because the low natural abundance makes it extremely unlikely that two ^{13}C atoms are present in the same molecule.

Unlike ^{1}H spectra, signal intensities in routine ^{13}C NMR spectra do not correspond to the number of nuclei. In particular, carbon atoms with no attached protons give much weaker signals than proton-bearing carbons, due to long relaxation times and weaker NOEs. The quantitative ^{13}C NMR spectra are time-consuming since they require long-relaxation delays between pulses and suppression of NOE enhancement.

2.4.5.3 NMR Spectra of Other Nuclei

(a) Deuterium (^{2}H)

Deuterium has a spin $I = 1$, a quadrupole moment (leading to broad lines) and very low natural abundance (0.015%) and therefore also low sensitivity. Nevertheless, it is possible to measure proton-decoupled ^{2}H NMR spectra at natural abundance. The ^{2}H chemical shifts (on the δ-scale) are identical with ^{1}H shifts. In fully deuterated samples the $J(^{2}H,^{2}H)$ are very small (ca 40× smaller than $J(^{1}H,^{1}H)$) and not observable in broad deuterium signals. The couplings $J(^{2}H,^{1}H)$ ~1–2 Hz are observed in ^{1}H NMR spectra at the residual proton signals of deuterated solvents (e.g. pentets of CHD_2OD or CHD_2SOCD_3 in deuterated methanol and/or dimethyl sulphoxide solution). Similarly in ^{13}C NMR spectra the couplings $J(^{13}C,^{2}H)$ are responsible for the splitting of signals of the common deuterated solvents (triplet of $CDCl_3$ with $J \approx 32$ Hz or septets of CD_3OD and CD_3SOCD_3 with $J \approx 21$ Hz).

The presence and location of deuterium in a molecule is often inferred from the absence of signals in the ^{1}H spectrum. Replacement of a proton by deuterium causes an upfield shift of the methylene carbon by as much as 0.3–0.5 ppm (Eggert and Djerassi, 1973). Such isotope shifts have found use as an aid to ^{13}C spectroscopic assignment. The presence of deuterium may also result in very small isotope shifts of signals from neighbouring nuclei.

Applications of ^{2}H NMR have included the study of the mobility and motion of deuterium-labelled cholesterol in membranes (Dufourc and Smith, 1985) and in solution (Murari et al., 1985), and the determination of the sites of labelling resulting from steroid reactions (Hanson and Reese, 1985).

(b) Tritium (^{3}H)

Tritium is a radioactive isotope (half-time 12.3 years) with zero natural abundance. It has spin $I = 1/2$ and very high sensitivity (1.2× higher than ^{1}H). Due to the sharp lines and high sensitivity, tritium labeling even at a low level of ^{3}H enrichment is sufficient. Chemical shifts of ^{3}H are (in δ-scale) identical with ^{1}H shifts and coupling constants $J(^{3}H,^{1}H)$ in ^{3}H NMR spectra are usually eliminated by proton broadband decoupling.

^{3}H NMR spectra at high isotopic incorporation have been used to determine the distribution of the radioactive label in some natural and synthetic steroid hormones (Al-Rawi et al., 1976; Altman and Silberman, 1977; Funke et al., 1983).

(c) Oxygen (^{17}O)

Physical properties of the only magnetically active isotope ^{17}O – a very low natural abundance (0.037%), spin $I = 5/2$ and quadrupole moment – lead to broad signals, low sensitivity and technical difficulties of ^{17}O NMR measurement. The sensitivity can be enhanced with ^{17}O enrichment by synthesis with costly isotope. However, it has been shown that even at natural abundance of ^{17}O it is possible under proper experimental conditions (line narrowing at higher temperature, accumulation of the large number of spectra with short repetition time and using linear prediction to eliminate unwanted acoustic ringing) to detect different oxygen containing groups in ^{17}O NMR spectra. The range of chemical shifts for ^{17}O nuclei is more than 700 ppm and typical shift regions for selected oxygen substituents in steroids are shown in Fig. 2.37.

The ^{17}O NMR spectra of cholesterol and 31 other steroid alcohols, esters, ketones and acids enriched with ^{17}O have been described by Smith et al. (1993). Natural abundance ^{17}O NMR data of 74 steroids, including androstanes, estranes, pregnanes, cholanes and cholestane, have been described Kahlig and Robien (1994).

(d) Fluorine (^{19}F)

The ^{19}F nucleus has virtually 100% natural abundance, and with $I = 1/2$ gives easy measurable spectra with sharp lines. ^{19}F chemical shifts may be spread over some 250 ppm downfield from $CFC1_3$ as the reference compound. ^{19}F NMR spectra have been the subject of a number of studies with steroids, prompted particularly by the high physiological activities of some 9α-fluoro derivatives of the steroid hormones (Joseph-Nathan et al., 1984; Wong et al., 1984).

The NMR spectra of fluorinated steroids show splittings of proton signals as a result of heteronuclear spin–spin coupling. Multiplicities are as for ^1H–^1H coupling, but geminal H–^{19}F coupling ($^2J_{H,F}$) is larger (40–80 Hz). Vicinal coupling ($^3J_{H,F}$) shows torsion angle dependence, as for interproton coupling, with values from about 1 to 45 Hz. ^{13}C signals are also strongly split by fluorine (Rozen and Ben-Shushan, 1985). Typical coupling constants are of the order: $^1J_{C,F}$ (for C–F bonds), 160–175 Hz; $^2J_{C,F}$ (C–C–F), 15–30 Hz. Long-range C–F coupling can also be significant, and shows torsion-angle dependence (Joseph-Nathan et al., 1984).

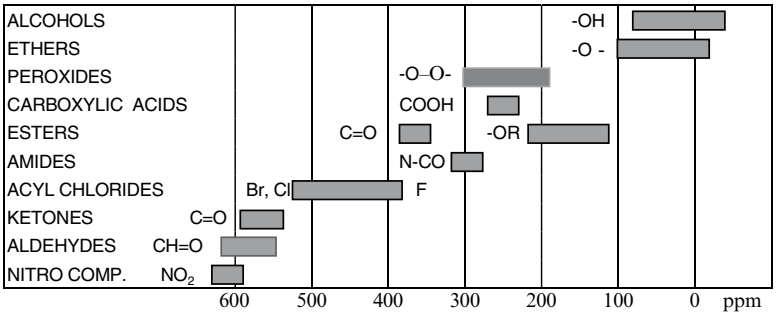
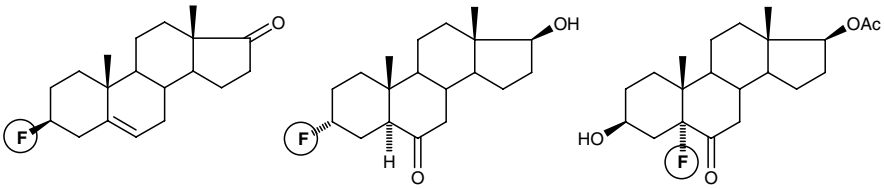


Fig. 2.37 Chemical shifts of ^{17}O in some oxygen substituents (referenced to H_2O)

While *J*(C,F) couplings are easily detected in ^{13}C NMR spectra by splitting of signals of corresponding carbon atoms into doublets the observation of *J*(H,F) couplings in 1D ^{1}H NMR spectra of steroids is more difficult and often limited to the downfield shifted protons out of steroid envelope. Long-range *J*(H,F) couplings can be detected using the heteronuclear 2D-^{1}H,^{19}F-COSY experiment (Hughes et al., 1991).

The *J*(H,F) and *J*(C,F) couplings observed in 1D NMR spectra of three fluorinated steroids are shown in Fig. 2.38.



^{2}J(F,H-3) = 49.5	^{2}J(F,H-3) = 48.4	^{3}J(F,H-4α) = 7.5
		^{3}J(F,H-4β) = 44.4
^{1}J(F,C-3) = 174.3	^{1}J(F,C-3) = 166.3	^{1}J(F,C-5) = 175.2
^{2}J(F,C-2) = 17.7	^{2}J(F,C-2) = 20.4	^{2}J(F,C-4) = 21.9
^{2}J(F,C4) = 19.5	^{2}J(F,C4) = 21.6	^{2}J(F,C-6) = 26.6
^{3}J(F,C-1) = 10.9	^{3}J(F,C-1) = < 1	^{2}J(F,C-10) = 19.3
^{3}J(F,C-5) = 12.7	^{3}J(F,C-5) = < 1	^{3}J(F,C-1) = < 1
^{4}J(F,C-6) = 1.2	^{5}J(F,C-19) = 2.3	^{3}J(F,C-3) = 2.4
^{4}J(F,C-10) = 1.2		^{3}J(F,C-7) = 1.8
^{5}J(F,C-7) = 1.1		^{3}J(F,C-9) = 4.1
^{5}J(F,C-9) = 2.0		^{3}J(F,C-19) = 5.7

Fig. 2.38 The observed *J*(H,F) and *J*(C,F) couplings in three fluorinated steroids

2.4.6 Complete NMR Structure Analysis of Steroids

In 1D proton NMR spectra of steroids overlapping signals of CH_2 and CH groups appear as a characteristic hump in the region 0.5–2.5 ppm that is difficult to analyse. Most of the structure information from low-frequency (<200 MHz) ^{1}H NMR data was therefore traditionally obtained from easily visible strong signals of methyl groups and from rare signals of protons shifted from the steroid hump either by hybridisation (olefinic protons) or by substituent effects (mainly CH–O protons). The much larger range of carbon-13 chemical shifts eliminates this problem with resolution of signals in ^{13}C NMR spectra of steroids.

High-field spectrometers with frequency 400–900 MHz for observation of protons, new 2D NMR methods (homo- and heteronuclear) and high-sensitive inversion techniques based on indirect detection of heteronuclei via protons brought fundamental progress in detailed NMR analysis of steroids in the sense of complete assignment of ^{1}H and ^{13}C signals (determination of chemical shifts and many coupling constants *J*(H,H) even for minute amounts of steroid samples brought.

The choice of proper NMR strategy depends on the type of problem to be solved, complexity of spectra, amount of sample and accessible NMR facilities. In case of the structural modification of a known starting steroid by chemical reaction, evidence for the presence of a functional group in a certain position can be simply obtained from 1D and/or the 2D-H,H-COSY spectrum. On the other hand identification of a new steroid compound isolated from natural material will require a complete analysis of the steroid skeleton combining a set of homo- and heteronuclear 2D-NMR experiments. The biochemical study of the structure-activity may require a complete 3D-structure of the steroid molecule in solution based on a combination of the observed NOE contacts, coupling constants *J*(H,H) and molecular modeling.

Table 2.12 gives an overview of the NMR parameters and methods used in the NMR structural study of steroids and the corresponding structural information. The detailed analysis of ^{1}H NMR spectra of steroids is achievable only with high-field NMR spectrometers (400 MHz and higher). An excellent review of the application of 2D-NMR methods in structural analysis of steroids has been published by Croasmun et al. (1994).

2.4.6.1 Dispersion of Signals in Steroid NMR Spectrum

NMR spectra of steroids are usually measured in $CDCl_3$. Dispersion of signals can be to a certain extent influenced by a change of solvent (mainly benzene or pyridine). Even small induced shifts can in some cases significantly simplify the analysis of spectra and enable the extraction of *J*(H,H) values in the region of strongly overlapping signals. The shifts induced with aromatic solvent (ASIS) are largest in steroids containing polar substituents and for protons in their neighbourhood. The observed changes of chemical shifts induced by benzene in the ^{1}H and ^{13}C NMR spectra of dehydroepiandrostanone are shown in Fig. 2.39.

Much larger shifts can be induced by lanthanide shift reagents (LSR). Paramagnetic lanthanide ion brings about dramatic changes in chemical shifts of nuclei in the substrate molecule. The size of lanthanide induced shifts (LIS) depends on the ratio of LSR and substrate. The relative LIS values for individual protons depend on the distance and orientation of a given proton and the lanthanide in the dynamic complex of LSR with a steroid. The disadvantage of LSR application is the line-broadening of the proton signal that increases with the LSR/substrate ratio and also with the increasing magnetic field of the spectrometer.

2.4.6.2 Connectivity Diagrams

NMR structure analysis is based on the detection of chemical bonds and spatially close atoms.

Heteronuclear 2D-methods (2D-H,C-HMQC, 2D-H,C-HSQC) correlate signals of directly bonded carbon and hydrogen atoms (C–H bonds).

Table 2.12 NMR parameters and methods used in NMR structural analysis of steroids

NMR parameter	NMR experiment	Data interpretation	Structural information
δ (^{1}H)	1D	CH_3-18,19 (Zürcher rules)	Substitution side and stereochemistry for steroids with known skeleton
	2D-H,H-COSY	Side-chain methyl groups	
	2D-H,H-*J*-res	CH_2, CH – substituent effects	
	2D-H,C-HMQC		
δ (^{13}C)	1D (APT, DEPT)	Correlation with steroid δ(^{13}C) data bases	Structural fragments or complete steroid structure
	2D-H,C-HMQC	Calculation of δ(^{13}C) – substituent effects	
$^n J$ (H,H)	1D	Multiplet patterns	Tentative assignment of skeletal protons
	2D-H,H-*J*-res	Determination of *J*(H,H) values	
	2D-H,H-COSY	Detection of proton spin systems	Isolated structural fragments
		Use of Karplus-type relation for ^3J(H,H) = $f(\theta)$	Dihedral angles of protons
^1J (H,C)	1D (APT, DEPT)	Multiplicity of carbon signals	Number and types of carbon atoms (C, CH, CH_2, CH_3)
	2D-H,C-HMQC	Detection of directly bonded H–C	Determination of ^1J(C,H)
	2D-C,H-*J*-res		
$^n J$ (H,C)	2D-H,C-HMBC	Detection of H and C separated by two or three bonds	Determination of ^2J(C,H) and ^3J(C,H)
	1D (^{13}C–^{1}H coupled)		Dihedral angles H-C–C–C
	2D-C,H-*J*-res		
^1J (C,C)	1D-INADEQUATE	Detection of directly bonded C–C	Complete steroid carbon skeleton
	2D-INADEQUATE		
NOE (H,H)	1D difference NOE	Detection of NOE contacts (mainly Me-18,19)	Spatial proximity of protons
	2D-H,H-NOESY	Quantitative NOE enhancements	Ring annelation
	2D-H,H-ROESY		Stereochemical assignment of methylene protons
			Distances H…H
T_1 (^{1}H)	Inversion recovery	Signal intensities as a function of delay times between 180° and 90° RF-pulses	T_1 values of protons
			Distinguishing of CH and CH_2 carbons on rigid skeleton
T_1 (^{13}C)	Inversion recovery	Signal intensities as a function of delay times between 180° and 90° RF-pulses	T_1 values of carbons
			Dynamic behavior (relative mobility of molecular fragments)

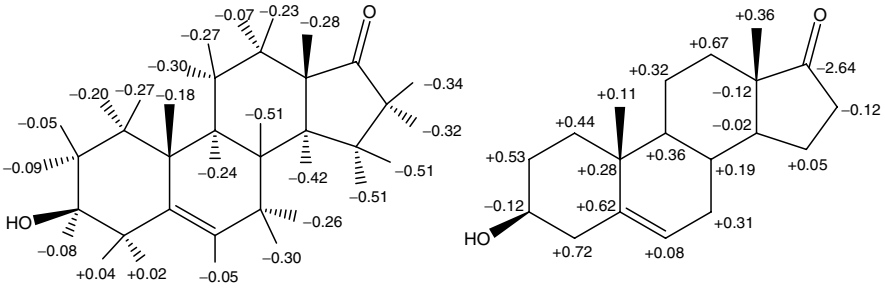


Fig. 2.39 Chemical shifts induced by benzene in ^{1}H and ^{13}C NMR spectrum of dehydroepiandrostanone

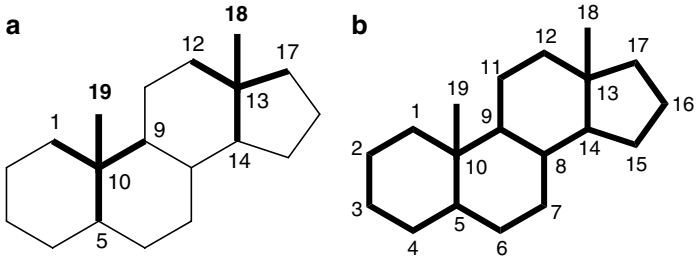


Fig. 2.40 Connectivity diagrams for 5α-androstane: (**a**) between methyl hydrogens and carbon atoms in the HMBC spectrum; (**b**) between directly bonded carbon atoms in 2D-INADEQUATE

Long-range heteronuclear methods (e.g. 2D-H,C-HMBC) correlate carbon atoms with hydrogens separated by two- and three-bonds. In the HMBC spectra the strong signals of methyl protons (Me-18 and Me-19) show strong characteristic cross-peaks with carbon atoms in positions 1,5,9,10 and 12,13,14, 17, respectively (see diagram in Fig. 2.40a).

The 2D-INADEQUATE spectrum allows, in principle, the detection of all C–C bonds and so the determination of the complete carbon skeleton (diagram – see Fig. 2.40b). Unfortunately, the low sensitivity of the experiment requires a large amount of sample, precise adjustment of experimental parameters and long measurement time.

Homonuclear 2D-H,H-COSY spectra detect connectivities between hydrogen atoms (J(H,H)) separated by two and three bonds. The connectivity diagram for androstane is shown in Fig. 2.41.

The spatial proximity of a given hydrogen atom to other hydrogens (up to ca 3.5 Å away) can be detected by difference 1D-NOE spectra. The more efficient 2D-H,H-NOESY or 2D-H,H-ROESY detect spatial proximity for all hydrogen atoms in the molecule. The contacts between the closest hydrogen atoms leading to the most intense NOEs (≤32% for geminal hydrogens and ≤6% for vicinal hydrogens in ax-eq positions) in androstane are shown in Fig. 2.42a. More distant

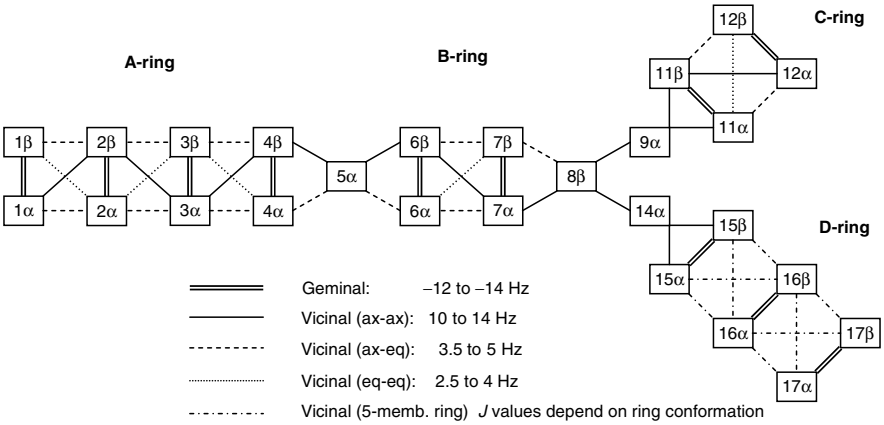


Fig. 2.41 Connectivity diagram between hydrogens from the 2D-H,H-COSY spectrum

contacts (weaker NOEs) are observable between hydrogen in 1,3-diaxial positions on both the α- and β-side of the steroid molecule (see Fig. 2.42b) and also between close hydrogens of neighbouring rings (Fig. 2.42c). The connectivity diagrams given above apply for all steroids with the 5α, 14α-configuration.

In most of the common steroids the A/B and C/D rings are *trans*-fused with hydrogens in the 5αH and 14αH configuration. Nevertheless, steroids with *cis*-fusion of the A/B or C/D rings are also known and the fusion has to be proven specifically for steroids isolated from natural material. The ring-fusion can be determined from NOE contacts that are characteristic for each configuration (5αH, 5βH, 14αH and 14βH) and these are shown in Fig. 2.43. Such an approach was used in the structure characterisation of some androgen hormones with inverted configuration at carbons 5, 9 and 10 (Kasal et al., 2002).

2.4.6.3 Chemical Shifts

The first step in the interpretation of an NMR spectrum is the determination of the number, intensities and chemical shifts of signals. The singlet for each carbon atom in the proton-decoupled ^{13}C NMR spectrum usually allows the determination of the number of carbon atoms in the molecule and chemical shifts directly from such spectrum. The overlap of signals is rather rare and can usually be recognised by the increased intensity. Figure 2.44 shows the ^{13}C NMR spectra of testosterone obtained by three different methods: (a) Proton-coupled spectrum with NOE (gated proton decoupling); (b) proton decoupled spectrum with suppressed NOE (inverse gated decoupling); (c) "attached proton test" (*J*-modulated spectrum with proton decoupling showing negative signals for $–CH_3$ and >CH–carbons and positive signals for $–CH_2–$ and >C< carbons). Distinguishing between CH, CH_3 and C, CH_2 allows different intensities of these signals. Due to longer relaxation times the CH_3 signals are less intensive than CH and quaternary carbons much less intensive than CH_2 signals (see Fig. 2.44c).

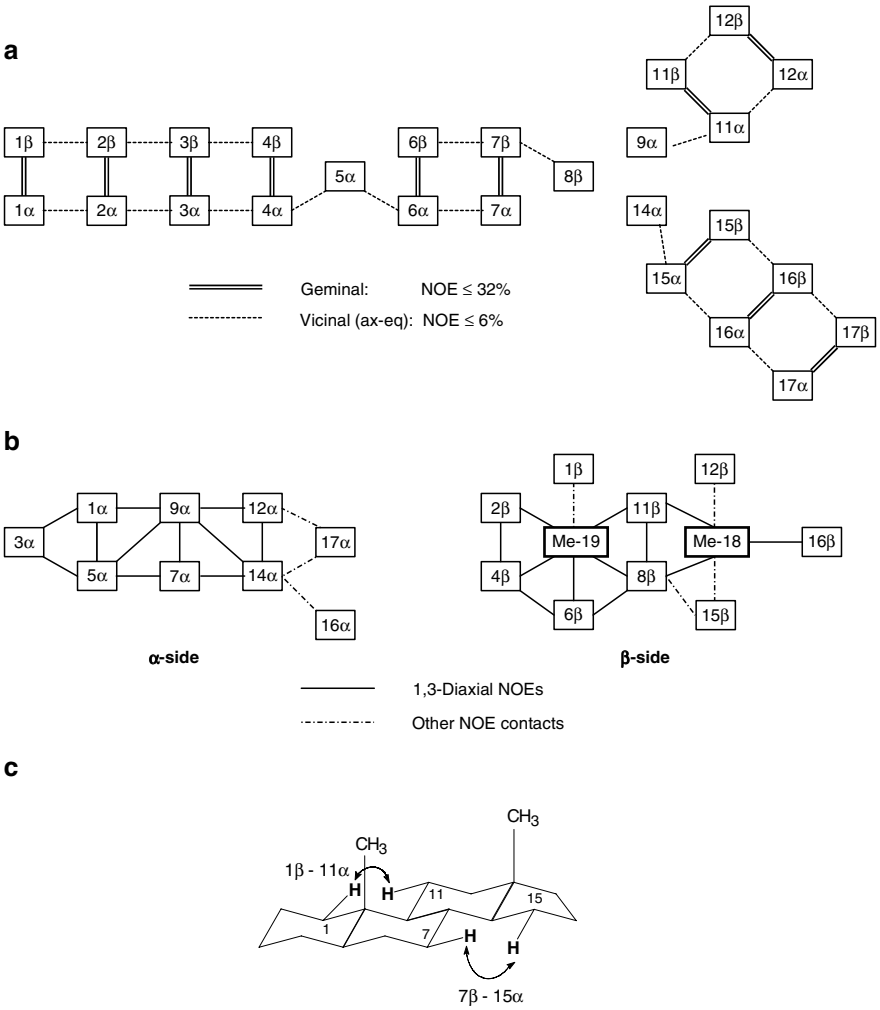


Fig. 2.42 Diagram of NOE contacts observable for 5α-androstane: (**a**) NOEs between geminal and vicinal hydrogens; (**b**) NOEs between hydrogens on the α- and β-side of the steroid; (**c**) NOEs between close hydrogens of neighbouring rings

On the other hand, the ^{1}H NMR spectra of steroids are much more complex and difficult to analyse. The only signals that are easily identified are the intense three-proton peaks of methyl signals and signals of the functional groups in the molecule (double bonds, CH–OH, etc.). The situation is well illustrated by the ^{1}H NMR spectrum of progesterone in Fig. 2.45. The chemical shifts of more or less overlapping signals of CH_2 and CH hydrogens in the region 0.5–2.0 ppm require at least the application of homonuclear 2D-NMR methods.

The homonuclear 2D-*J*-resolved spectrum separates chemical shifts and coupling constants into two axes and, in an ideal case, can show a separate multiplet for each

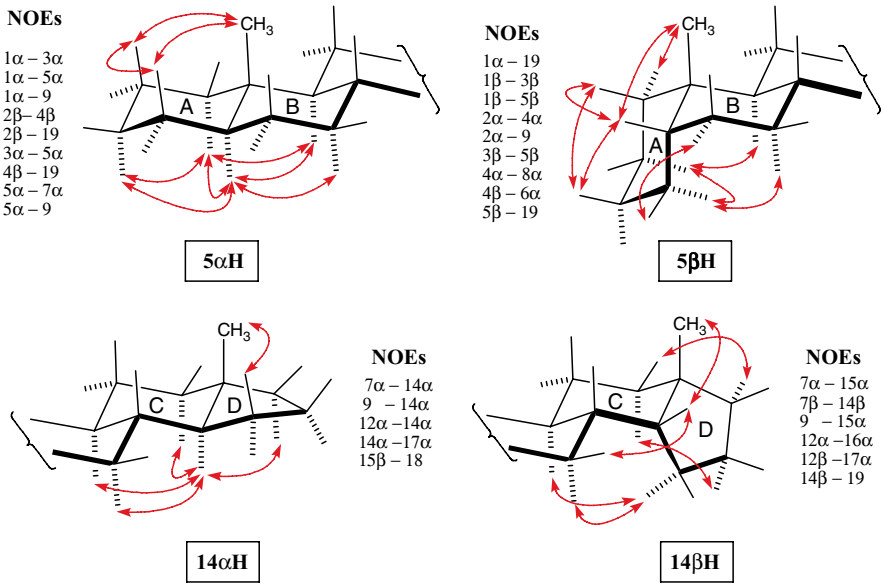


Fig. 2.43 Characteristic NOE contacts for steroids with configurations 5αH, 5βH, 14αH and 14βH

hydrogen atom. Unfortunately, artifacts appear for strongly coupled protons and 2D-*J*-resolved spectra found only limited practical application. The 2D-*J*-resolved spectrum of testosterone in Fig. 2.46 where all proton mltiplets can be identified, illustrates an efficient application of this method.

The 2D-H,H-COSY spectra correlate mutually coupled hydrogen atoms. The intensity of the cross-peaks depends on the *J*(H,H) value and on the signal intensities of both coupled protons in 1D spectrum. The special technique – long-range 2D-H,H-COSY – can be applied for identification of small couplings (<2 Hz). Problems can appear in the interpretation of COSY spectra of strongly coupled systems of protons (cross-peaks close to diagonal) and in the regions with many overlapping proton signals. The 2D-H,H-COSY spectrum of 5α-androstane is shown in Fig. 2.47. Since all the proton signals of androstane appear at only ~1 ppm range, spectrum was taken with a high spectral resolution and shows, therefore, fine structure for the individual cross-peaks that reflects the number and size of involved *J*(H,H)s.

The heteronuclear spectra (2D-H,C-HMQC, 2D-H,C-HSQC) correlate signals of directly bonded hydrogen and carbon atoms and thus allow the determination of the chemical shifts of both hydrogen and carbon atoms. The larger scale of carbon chemical shifts (~20 times) allows the discrimination of hydrogen atoms that are difficult to separate in 2D-H,H-COSY spectra. The 2D-H,C-HSQC spectrum also allows a clear distinction between protons belonging to methylene and methine groups and so simplifies the interpretation of the ^{1}H NMR spectrum. Figure 2.48 shows the 2D-H,C-HSQC spectrum of testosterone.

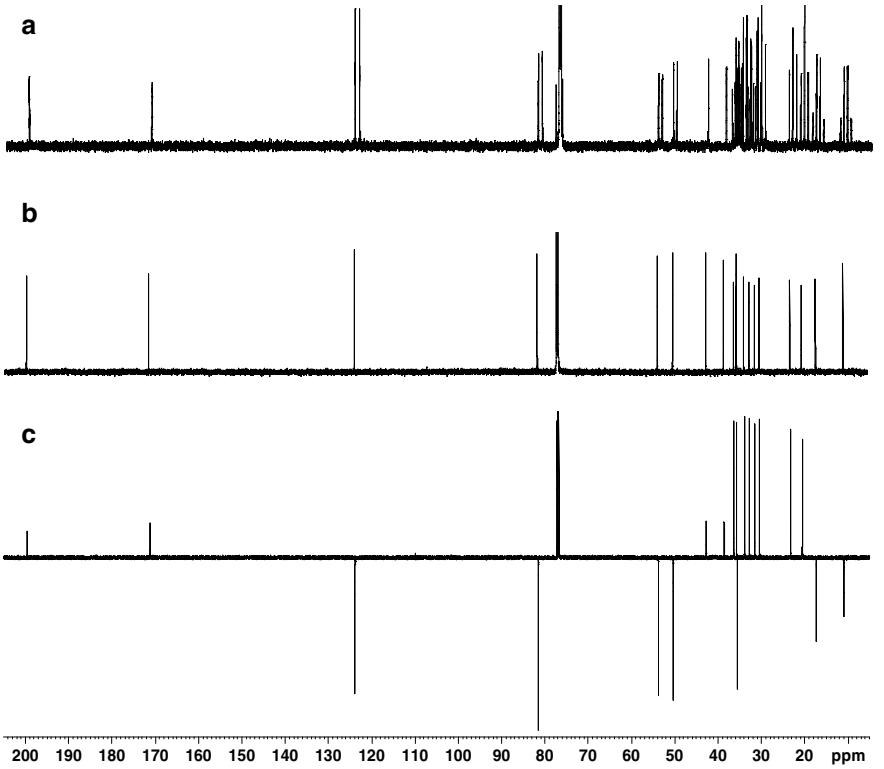


Fig. 2.44 Carbon-13 NMR spectra of testosterone: (**a**) Proton-coupled spectrum with NOE (gated proton decoupling); (**b**) proton decoupled spectrum with suppressed NOE (inverse gated decoupling); (**c**) "attached proton test" (*J*-modulated spectrum with proton decoupling)

After determination of proton and carbon chemical shifts, the number of hydrogen and carbon atoms in the steroid molecule should be known as well as the numbers of CH_3, CH_2, CH groups and quaternary carbons. The total number of carbon atoms together with the number and type of methyl groups (e.g. only angular or additional side-chain methyl groups) may indicate the type of steroid skeleton and make possible the presence of a conjugate (e.g. glucose).

Substituent effects on the ^1H chemical shifts of angular methyl groups (Zürcher rules – see Table 2.13) can be used to determine the configuration of substituents in steroids with known fusion of the A/B and CD rings. The approximate additivity of substitution effects fails for substituents in vicinal positions.

The proton signals out of the region δ 0.5–2.0 ppm and carbon signals with δ > 60 ppm indicate the presence of substituents or double bonds. For methylene hydrogens in the rigid steroid skeleton the equatorial hydrogen usually appears at about 0.5 ppm to lower field than the axial one. Nowadays there are some hundreds of completely assigned proton NMR spectra described in the literature (e.g. Kirk et al., 1990). Proton chemical shifts for a selected set of steroids **1–18** (see Fig. 2.49)

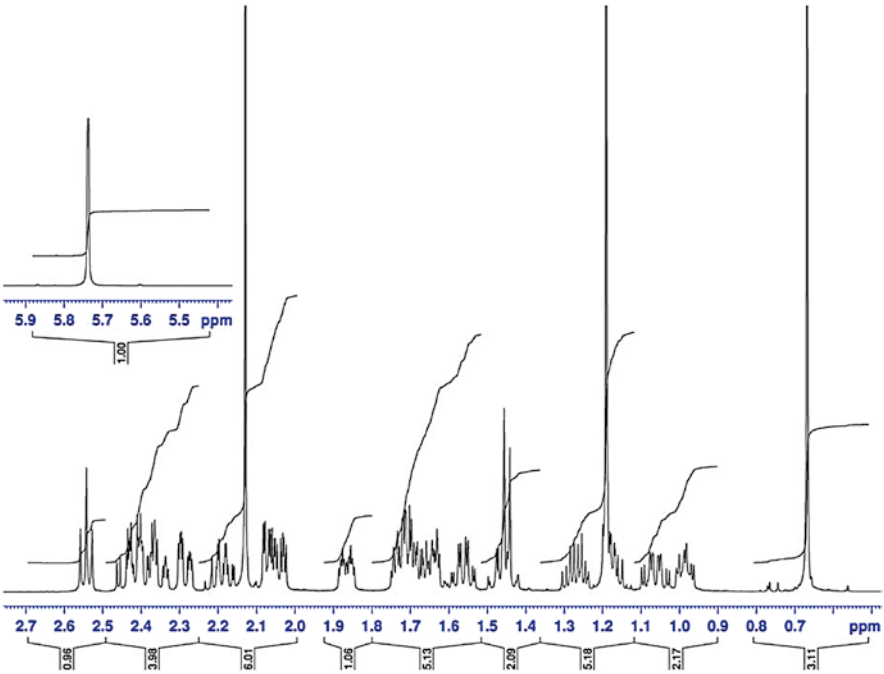


Fig. 2.45 Proton NMR spectrum in progesterone (in CDCl3 at 600 MHz)

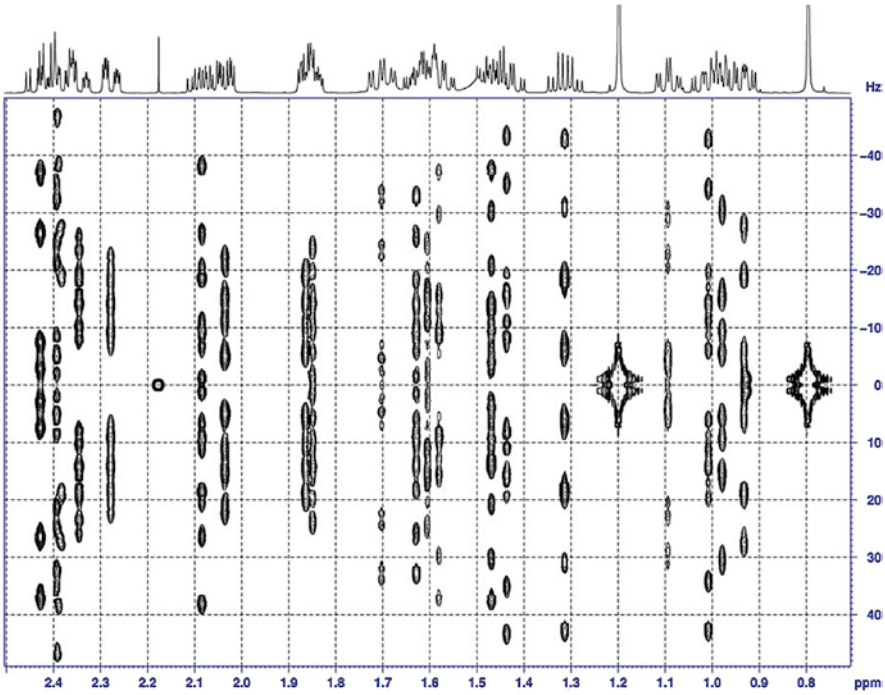


Fig. 2.46 The 2D-*J*-resolved spectrum (in $CDCl_3$ at 600 MHz) of testosterone (the upfield part of the spectrum without the well separated H-4 and H-17 is shown)

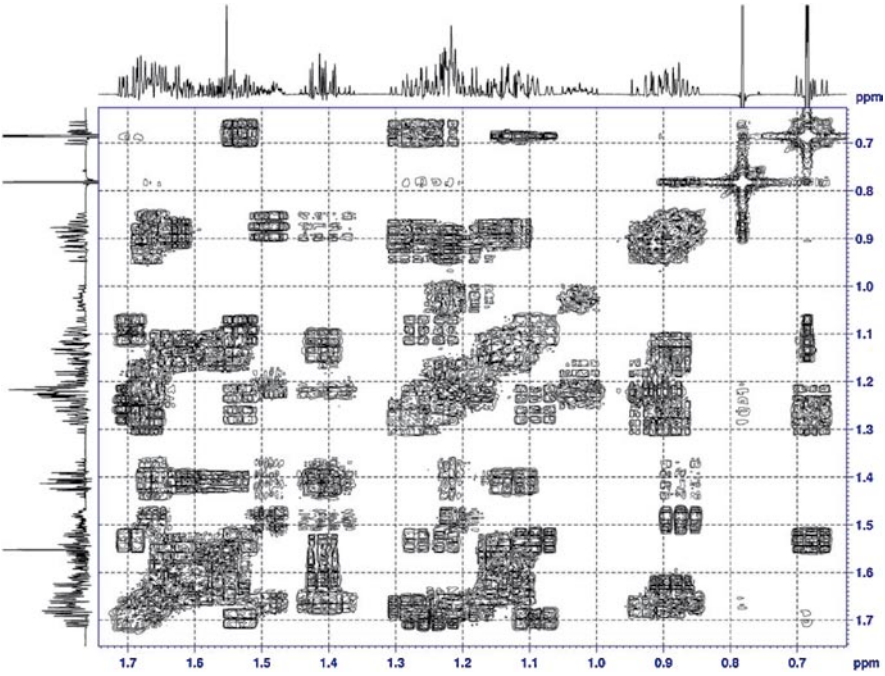


Fig. 2.47 The 2D-H,H-COSY spectrum of 5α-androstane (in $CDCl_3$ at 600 MHz)

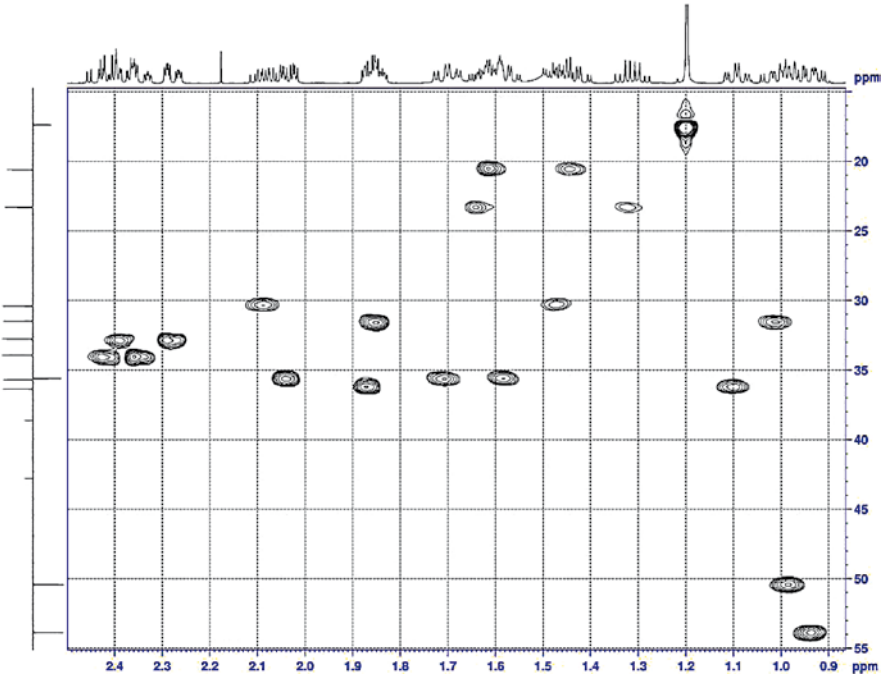


Fig. 2.48 The 2D–H,C–HSQC spectrum of testosterone (the upfield part of spectrum without the well separated cross-peaks of H-4/C-4 and H–17/C-17 is shown)

Table 2.13 Chemical shift increments for protons at C-18 and C-19[a]

	19-H	18-H		19-H	18-H
Parent steroid	**5α-series**			**5β-series**	
Androstane	0.79	0.69		0.93	0.69
Pregnane	0.78	0.55		0.92	0.55
Cholan-24-oic acid	0.78	0.65		0.92	0.65
Cholestane	0.78	0.64		0.91	0.64
Ring A substituents	**5α-series, 4-ene, or 5-ene**			**5β-series**	
1-oxo	0.38	0.02		0.20	–0.02
1-ene	0.05	0.02		[d]	[d]
1α-OH	±0.02	0.00		[d]	[d]
1β-OH	0.05	0.01		0.12	0.00
2-oxo	–0.03	0.01		[d]	[d]
2-ene	0.00	0.04		[d]	[d]
2α-OH	0.02 or 0.10[b]	0.00		[d]	[d]
2β-OH	0.25	0.01		[d]	[d]
3-oxo	0.24	0.04		0.12	0.04
3α-OH	0.00	0.01		0.01	0.01
3β-OH	0.03	0.01		0.05	0.01
4-oxo	–0.03	0.02		0.20	0.00
4-ene	0.25	0.04		–	–
4-en-3-one	0.42	0.08		–	–
4α-OH	0.02	0.00		0.01	0.01
4β-OH	0.24	0.00		[d]	[d]
5-OH	0.18	0.00		[d]	[d]
Ring B, C and D substituents	**(applicable to 5α- and 5β-series)**				
5-ene	0.23	0.03	11-oxo	0.22	–0.03
5,7-diene	0.14	–0.03	11-ene	–0.03	0.08
5-en-7-one	0.39	0.04	11α-OH	0.12	0.03
6-oxo	–0.09	0.03	11β-OH	0.26	0.24
6-ene	–0.03	0.05	12-oxo	0.10	0.38
6α-OH	0.03	0.00	12α-OH	–0.01	0.04
6β-OH (5α)	0.23	0.04	12β-OH	0.01	0.07
6β-OH (5β or 4-ene)	0.19	0.04	14-ene	0.01	0.25
6α-CH_3	0.00	0.00	14α-OH	0.00	0.12
6β-CH_3	0.08	0.00	15-oxo	0.01	0.08
7-oxo	0.28	0.01	15α-OH	0.01	0.03
7-ene	–0.01	–0.12	15β-OH	0.03	0.27
7α-OH	–0.01	0.01	16-oxo	0.04	0.17
7β-OH	0.03	0.03	16-ene[c]	0.03	0.07
8-ene	0.13	–0.08	16α-OH	–0.01	0.01
8(14)-ene	–0.12	0.18	16β-OH	0.02	0.25
8β-OH	0.18	0.18	17-oxo	0.02	0.17
9(11)-ene	0.14	–0.07	17α-OH	–0.02	–0.06
9α-OH	0.13	0.03	17β-OH	0.00	0.03

[a]Data selected from Bhacca and Williams (1964), from Bridgeman et al. (1970), or from D.N. Kirk's own collection. For more detailed listings Bhacca and Williams (1964) or Zürcher (1961, 1963).

[b]The increment 0.10 applies for 2α-OH in 4-en-3-ones.

[c]For androst-16-enes.

[d]Not available values.

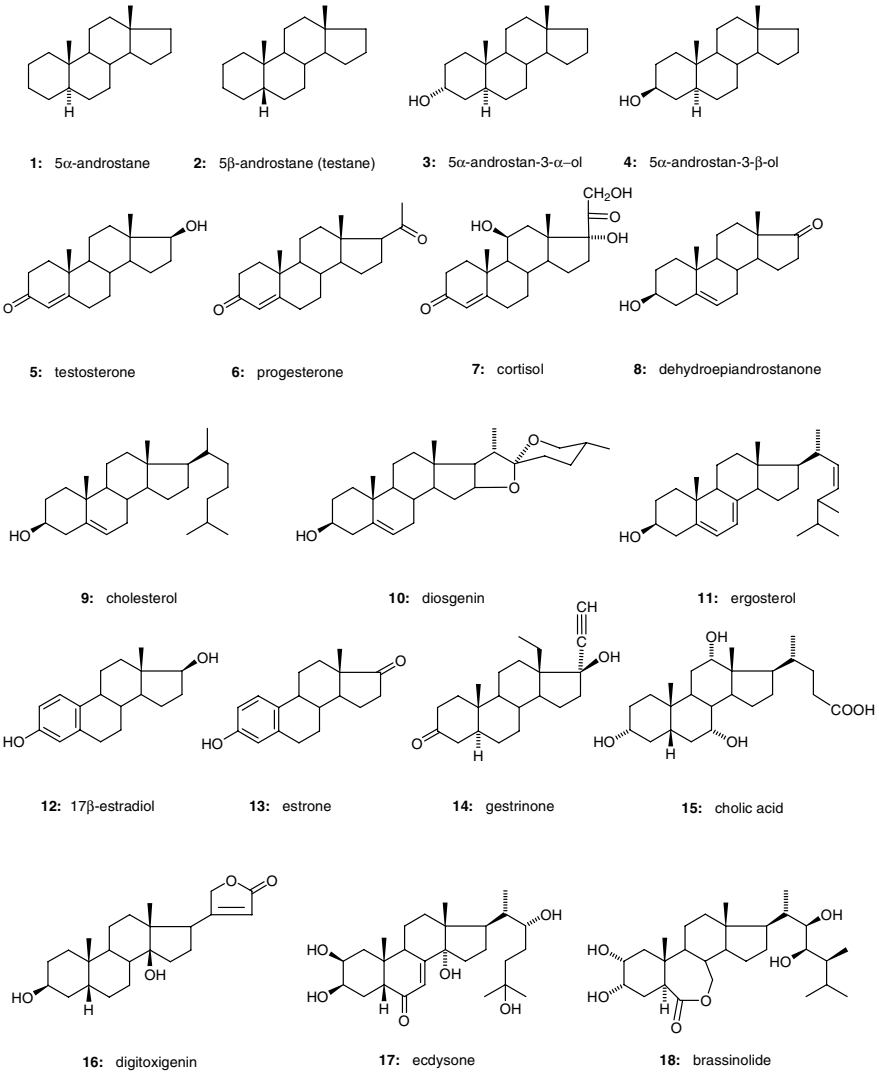


Fig. 2.49 The structures of the selected series of steroids **1–18**

containing representatives of simple unsubstituted steroids (**1**,**2**), hydroxy derivatives (**3**,**4**), steroid hormones (**5**,**6**,**11–13**), anabolic steroids (**14**), cholic acids (**15**), ecdysteroids (**17**) and brassinolides (**18**) are presented in Table 2.14. These data can be successfully used for NMR analysis of structurally related steroids.

The complete ^{13}C NMR data of steroids in the literature are much more extensive. Fully assigned spectra have been reported for hundreds of steroids (Blunt and Stothers, 1977; Smith, 1978; Hickey et al., 1980; Ciuffreda et al., 2004). Table 2.15 shows carbon chemical shifts for the same selected set of steroids **1–18**.

Table 2.14 Proton chemical shifts of selected steroids 1–18 (our data at 600 MHz)

	Compound (solvent)[a]																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Proton	(C)	(C)	(C)	(C)	(C)	(C)	(M)	(C)	(C)	(C)	(C)	(C)	(C)	(C)	(M)	(C)	(M)	(C)
1α	0.88	1.76	1.30	0.98	1.70	1.72	1.88	1.10	1.07	1.08	1.30	7.16	7.16	2.81	1.81	1.47	1.79	1.56
1β	1.67	0.88	1.48	1.72	2.03	2.04	2.22	1.86	1.84	1.84	1.90			2.89	0.98	1.51	1.43	1.87
2α	1.49	1.30	1.62	1.80	2.34	2.35	2.32	1.85	1.83	1.84	1.88	6.63	6.64	2.49	1.43	1.50	3.84	–
2β	1.40	1.34	1.68	1.40	2.43	2.43	2.50	1.51	1.50	1.50	1.50			2.49	1.60	1.56	–	3.72
3α	1.22	1.74	–	3.59	–	–	–	3.54	3.53	3.52	3.64	–	–	–	–	4.13	3.95	–
3β	1.66	1.20	4.04	–	–	–	–	–	–	–	–	–	–	–	3.37	–	–	4.02
4α	1.22	1.24	1.38	1.56	5.73	5.74	5.66	2.33	2.29	2.30	2.47	6.56	6.59	5.79	2.29	1.90	1.70	1.96
4β	1.22	1.74	1.52	1.29				2.25	2.24	2.23	2.28				1.66	1.37	1.76	2.15
5α	1.03	–	1.52	1.10	–	–	–	–	–	–	–	–	–	–	–	–	–	3.11
5β	–	1.27	–	–	–	–	–	–	–	–	–	–	–	–	1.39	1.77	2.38	–
6α	1.22	1.21	1.20	1.27	2.27	2.29	2.27	5.38	5.35	5.35	5.57	2.80	2.85	2.62	1.53	1.27	–	–
6β	1.22	1.87	1.20	1.27	2.39	2.40	2.56					2.85	2.89	2.57	1.96	1.88	–	–
7α	0.91	1.12	0.96	0.91	1.01	1.06	1.12	1.65	1.53	1.57	5.39	1.32	1.43	1.33	–	1.25	5.82	4.07
7β	1.67	1.42	1.70	1.69	1.85	1.87	2.05	2.12	1.97	2.01		1.87	2.00	1.88	3.80	1.70		
8	1.27	1.34	1.29	1.28	1.57	1.56	2.06	1.85	1.46	1.64	–	1.43	1.58	2.52	1.55	1.56	–	1.72
9	0.68	1.44	0.77	0.65	0.93	0.99	1.00	1.01	0.93	0.96	1.97	2.17	2.24	–	2.25	1.62	3.16	1.24
11α	1.53	1.43	1.55	1.52	1.60	1.64	4.40	1.68	1.48	1.53	1.74	2.30	2.38	6.63	1.58	1.43	1.81	1.77
11β	1.25	1.23	1.26	1.30	1.42	1.46	–	1.48	1.48	1.47	1.60	1.49	1.50		1.58	1.25	1.67	1.42
12α	1.09	1.13	1.11	1.10	1.09	1.46	2.02	1.28	1.15	1.18	1.26	1.29	1.49	6.48	–	1.39	2.11	1.25
12β	1.69	1.71	1.71	1.70	1.86	2.07	1.60	1.67	2.01	1.74	2.06	1.94	1.96		3.96	1.52	1.77	1.98
14	0.89	0.96	0.91	0.89	0.97	1.17	1.76	1.29	0.99	1.11	1.89	1.19	1.51	2.05	2.00	–	–	1.20
15α	1.64	1.64	1.66	1.64	1.63	1.72	1.79	1.95	1.06	1.99	1.75	1.70	2.06	1.74	1.12	2.14	1.96	1.66
15β	1.14	1.15	1.14	1.14	1.31	1.27	1.40	1.55	1.58	1.29	1.32	1.37	1.63	1.51	1.75	1.71	1.60	1.24
16α	1.57	1.57	1.58	1.57	2.08	1.68	1.47	2.09	1.82	4.41	1.66	2.12	2.15	2.15	1.90	1.87	1.96	1.97
16β	1.62	1.63	1.63	1.63	1.47	2.19	2.73	2.47	1.25	–	1.36	1.48	2.51	2.41	1.31	2.14	1.50	1.34
17α	1.13	1.15	1.13	1.13	3.65	2.54	–	–	1.09	1.78	1.27	3.73	–	–	1.86	2.79	2.03	1.56
17β	1.41	1.42	1.41	1.42	–	–	–	–	–	–	–	–	–	–	–	–		

18	0.68	0.68	0.69	0.69	0.80	0.67	0.89	0.89	0.68	0.79	0.63	0.78	0.91	1.49 1.73	0.72	0.88	0.73	0.71
19	0.78	0.92	0.78	0.81	1.20	1.19	1.47	1.04	1.01	1.03	0.95	–	–	1.04	0.92	0.96	0.97	0.92
20	–	–	–	–	–	–	–	–	1.37	1.87	2.03	–	–	–	1.43	–	1.75	1.51
21	–	–	–	–	–	2.13	4.63 4.27	–	0.91	0.97	1.04	–	–	2.53	1.02	5.00 4.81	0.95	0.90
22	–	–	–	–	–	–	–	–	1.33 1.00	–	5.18	–	–	–	1.79 1.35	5.88	3.59	3.54
23	–	–	–	–	–	–	–	–	1.34 1.14	1.61 1.61	5.22	–	–	–	2.35 2.22	–	1.32 1.54	3.70
24	–	–	–	–	–	–	–	–	1.12 1.12	1.63 1.46	1.86	–	–	–	–	–	1.78 1.41	1.21
25	–	–	–	–	–	–	–	–	1.52	1.63	1.47	–	–	–	–	–	–	1.63
26	–	–	–	–	–	–	–	–	0.87	3.47 3.38	0.84	–	–	–	–	–	1.19	0.96
27	–	–	–	–	–	–	–	–	0.86	0.79	0.82	–	–	–	–	–	1.20	0.94
28	–	–	–	–	–	–	–	–	–	–	0.92	–	–	–	–	–	–	0.84

[a]C = chloroform, M = methanol.

Table 2.15 Carbon-13 chemical shifts of selected steroids 1–18 (our data at 150.9 MHz)

	Compound (solvent)[a]																	
Carbon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
	(C)	(C)	(C)	(C)	(C)	(C)	(M)	(C)	(C)	(C)	(C)	(C)	(C)	(C)	(M)	(C)	(M)	(C)
C-1	38.76	37.73	32.23	37.06	35.69	35.64	35.84	37.11	37.20	37.18	38.33	126.54	126.54	24.35	36.48	29.58	37.33	41.49
C-2	22.21	21.33	29.03	31.53	33.93	33.88	33.29	31.47	31.57	31.57	31.75	112.61	112.79	36.64	31.17	27.85	68.69	68.06
C-3	26.84	27.04	66.61	71.34	199.60	199.48	202.50	71.49	71.78	71.69	69.98	153.23	153.44	199.09	72.88	66.78	68.50	68.15
C-4	29.12	27.26	35.89	38.21	123.84	123.86	122.51	42.10	42.20	42.23	40.54	115.20	115.25	123.71	40.45	33.26	32.89	31.02
C-5	47.04	43.75	39.14	44.84	171.27	170.99	176.60	140.96	140.69	140.76	139.32	138.30	138.05	156.22	43.19	35.92	51.79	40.87
C-6	29.07	27.54	28.59	28.74	32.76	32.72	34.30	120.85	121.70	121.40	115.87	29.60	29.46	31.47	35.90	26.41	206.55	176.60
C-7	32.56	26.98	32.37	32.43	31.49	31.81	34.60	30.71	31.87	32.01	115.87	27.15	26.46	26.99	69.05	21.32	122.01	70.41
C-8	35.87	36.25	35.83	35.83	35.61	35.46	32.88	31.42	31.85	31.39	140.67	38.78	38.30	37.72	41.01	41.77	167.63	39.22
C-9	55.02	40.81	54.57	54.60	53.86	53.55	57.61	50.14	50.06	49.99	46.21	43.90	43.92	141.45	27.86	35.43	35.23	58.16
C-10	36.36	35.51	36.19	35.56	38.62	38.50	40.70	36.57	36.46	36.60	37.00	132.73	132.08	127.99	35.83	35.36	39.24	38.32
C-11	20.81	20.82	20.77	21.24	20.60	20.94	68.68	20.29	21.04	20.83	21.14	26.30	25.90	124.76	29.57	21.12	21.57	22.25
C-12	38.97	39.16	38.88	38.88	36.37	38.58	40.75	31.35	39.73	39.74	39.06	36.66	31.52	139.78	74.02	39.98	32.03	39.60
C-13	40.82	40.95	40.80	40.81	42.78	43.86	48.28	47.50	42.27	40.22	42.74	43.23	48.02	51.56	47.48	49.57	48.11	42.44
C-14	54.65	54.66	54.56	54.51	50.42	55.94	53.40	51.68	56.71	56.47	54.42	49.98	50.36	49.09	42.98	85.57	85.07	51.28
C-15	25.49	25.58	25.46	25.50	23.30	24.29	24.66	21.82	24.26	31.81	22.99	23.11	21.57	22.29	24.22	33.11	32.07	24.69
C-16	20.49	20.59	20.47	20.48	30.40	22.74	34.60	35.80	28.21	80.79	28.19	30.58	35.88	39.62	28.66	26.83	27.00	27.59
C-17	40.45	40.54	40.39	40.40	81.59	63.43	90.29	221.32	56.09	62.02	55.67	81.92	221.21	73.07	48.03	50.86	48.79	52.27
C-18	17.54	17.5t1	17.53	17.51	11.02	13.28	17.85	13.49	11.83	16.27	12.06	11.04	13.84	22.86	12.98	15.75	16.18	11.84
C-19	12.26	24.31	11.20	12.34	17.38	17.30	21.40	19.37	19.37	19.40	16.27	–	–	11.02	23.16	23.69	24.46	15.48
C-20	–	–	–	–	–	208.33	212.95	–	35.76	41.56	40.24	–	–	87.97	36.77	174.52	43.46	36.85
C-21	–	–	–	–	–	31.47	67.66	–	18.68	14.51	21.07	–	–	78.70	17.60	73.42	13.22	11.84
C-22	–	–	–	–	–	–	–	–	36.15	109.27	135.03	–	–	–	32.32	117.66	75.24	74.57
C-23	–	–	–	–	–	–	–	–	23.79	31.34	131.51	–	–	–	31.98	174.55	25.30	73.57
C-24	–	–	–	–	–	–	–	–	39.48	28.75	42.68	–	–	–	178.25	–	42.25	40.06
C-25	–	–	–	–	–	–	–	–	27.99	30.26	33.04	–	–	–	–	–	71.41	30.78
C-26	–	–	–	–	–	–	–	–	22.54	66.81	19.61	–	–	–	–	–	29.05	20.51
C-27	–	–	–	–	–	–	–	–	22.81	17.12	19.89	–	–	–	–	–	29.61	20.86
C-28	–	–	–	–	–	–	–	–	–	–	17.57	–	–	–	–	–	–	10.07

[a]C = chloroform, M = methanol.

The effects of substituents on ^{13}C chemical shifts in steroids have been extensively investigated: data are available for the effects of keto groups (Eggert and Djerassi, 1973), hydroxyl groups (Eggert et al., 1976), and unsaturation (Eggert and Djerassi, 1981). These, and a variety of other functional groups and side chains, are included in the review by Blunt and Stothers (1977). Substituent effects are reasonably additive, and they can be applied to a suitably chosen 'parent' steroid structure to predict chemical shifts in a derivative. The effects of common substituents on the chemical shifts of carbon atoms in rings A–D in the steroids with 5α- and 5β-configuration are summarised in Table 2.16. Substitution on the tetracyclic carbon skeleton has little influence on the chemical shifts of side-chain carbons. Carbon chemical shifts, calculated using published substituent effects, are usually in good agreement with experimental data (the average deviation is ca 0.5 ppm), unless substituents are too close. For the prediction of ^{13}C chemical shifts in polysubstituted steroids the known data of structurally most similar compounds should be used as reference.

Although in principle it is possible to make a correct structural assignment empirically (on the basis of chemical shifts only) there are known examples of reinterpretations and corrections in the literature. Experimental structural assignments using correlation 2D-H,C-NMR methods are always more reliable and should be preferred.

2.4.6.4 Coupling Constants *J*(H,H)

The values of *J*(H,H) can be determined: (a) from well recognised multiplets observed in the 1D spectrum, (b) from weakly coupled regions of the 2D-*J*-resolved spectrum, (c) from the fine pattern of cross peaks observed in 2D-H,H-COSY spectrum measured with high resolution. In rigid steroid molecules *J*(H,H) values have a high diagnostic value. Typical geminal couplings are 12–14 Hz, but in the presence of a neighbouring double bond or carbonyl group they increase up to 15–20 Hz (depending on the angles between the geminal hydrogens and the π-bond). Vicinal coupling depends on the dihedral angle between the coupled protons and on the number, type and orientation of substituents (generalised Karplus relation see p. 79). The typical values observed for protons on the six-membered A–C rings are: *J*(ax,ax) = 10.5–13.5 Hz, *J*(ax,eq) = 3.5–5.0 Hz and *J*(eq,eq) = 2.5–4.0 Hz. Vicinal couplings in the five-membered ring D depend on its conformation which can be influenced significantly by ring-subtitution.

The regularity of coupling constants in 5α,14α-steroids leads to a characteristic multiplet pattern of >CH–X hydrogen atoms in individual positions (given by the number and orientation of coupled partners) which appears in many different steroid molecules. Characteristic multiplet patterns for C<u>H</u>–OH hydrogens in different positions and configurations in 5α-steroids are shown in Fig. 2.50.

Some long-range couplings can be usually observed in the ^{1}H NMR spectra of steroids:

Table 2.16 Substituent effects at saturated carbon atoms in 5α-steroids (from Blunt and Stothers, 1977)

	Substituent effect (ppm) in position																		
Substituent	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
4-Oxo	−1.0	0.4	14.3		12.2	−8.7	−1.7	−0.5	0.3	6.2	0.9	−0.1	0.0	−0.2	0.0	0.0	−0.1	0.0	1.5
6-Oxo	−0.5	−0.8	−1.6	−8.8	11.7		14.5	2.3	0.0	5.4	0.2	−0.5	0.4	0.0	−0.2	0.0	−0.3	−0.1	0.8
7-Oxo	0.1	−0.5	−0.4	0.0	2.0	17.4		14.5	−0.2	0.4	0.4	−1.9	−0.1	−7.6	0.8	0.2	−0.2	−0.1	−0.6
11-Oxo	−1.0	−0.4	−0.1	−0.6	−0.2	−0.7	0.6	1.4	9.8	−0.4		15.9	4.1	−0.5	−0.6	0.4	−1.2	0.6	−0.2
12-Oxo	−0.5	−0.4	−0.3	−0.4	−0.1	−0.4	−0.9	−1.0	1.4	0.5	16.6		14.5	−0.1	−0.7	−1.0	−8.6	0.1	−0.4
15-Oxo	−0.1	−0.1	−0.1	−0.2	0.2	−0.6	−1.8	−3.5	−0.1	0.1	−0.5	0.4	−1.6	8.7		14.6	−5.1	0.7	−0.1
16-Oxo	−0.4	−0.2	−0.1	−0.4	−0.1	−0.2	−0.2	−1.0	−0.4	0.1	−0.5	−0.6	−1.6	−2.8	13.8		15.4	0.5	0.0
17-Oxo	−0.2	−0.2	−0.2	−0.4	−0.1	−0.2	−1.6	−0.9	−0.2	0.0	−0.8	−7.3	6.9	−3.1	−3.8	15.2		−3.8	−0.1
1α-OH	32.7	6.7	−6.6	−0.6	−8.1	−0.2	−0.4	−0.1	−7.6	3.8	−0.8	−0.2	0.0	−0.2	0.1	0.0	0.0	−0.1	0.6
1β-OH	40.0	11.1	−2.2	−0.5	−0.8	−0.3	−0.1	0.3	0.3	6.2	3.6	0.3	−0.6	−0.1	0.3	−0.1	0.1	−0.2	−5.6
2α-OH	9.4	45.7	9.2	−1.5	−0.7	−1.0	−0.2	−0.7	−0.1	1.2	0.2	0.0	0.1	−0.1	0.1	0.0	0.0	0.0	1.1
2β-OH	6.5	45.8	7.0	−5.3	0.3	−0.3	−0.1	−0.6	0.8	−0.3	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.1	2.5
3α-OH	−6.4	6.8	39.7	6.8	−7.9	−0.5	−0.1	0.0	−0.4	−0.1	0.0	0.0	0.1	0.0	0.1	0.1	0.0	0.0	−1.1
3β-OH	−1.7	9.3	44.3	9.0	−2.2	−0.4	−0.1	−0.1	−0.4	−0.8	0.4	−0.1	−0.2	0.0	0.0	0.0	−0.1	0.0	0.1
4α-OH	−0.7	−1.8	9.5	41.3	7.2	−6.4	−0.5	−0.4	0.0	1.3	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.3
4β-OH	−0.1	−5.2	7.0	43.3	3.1	−3.1	0.3	0.0	0.8	−0.1	−0.6	0.0	0.1	0.1	0.1	0.0	0.0	0.0	2.4
5α-OH	−7.2	−1.4	−6.2	5.2	26.0	5.4	−5.9	−0.7	−8.7	3.0	0.0	−0.1	0.0	−0.5	−0.1	−0.1	−0.2	0.0	3.9
6α-OH	0.1	−0.5	−0.7	−6.4	6.7	40.8	9.5	−1.3	−0.7	0.6	−0.1	−0.3	0.0	−0.4	0.0	−0.1	−0.1	−0.1	1.1
6β-OH	1.7	−0.1	0.2	−3.1	2.7	43.3	7.4	−5.3	−0.1	0.0	−0.2	0.0	0.1	−0.3	0.0	0.0	0.0	0.0	3.5
7α-OH	−0.3	−0.1	−0.1	−0.5	−7.9	7.6	36.0	4.1	−8.5	0.1	−0.2	−0.5	0.1	−5.9	−0.5	0.0	−0.2	−0.3	−1.0
7β-OH	−0.1	−0.2	−0.3	−0.5	−3.0	9.5	43.0	8.0	−1.8	−0.6	0.0	−0.1	1.0	−0.8	2.8	0.4	−1.1	0.0	0.1
11α-OH	2.0	0.3	−0.2	0.5	−0.1	0.5	0.2	−0.6	6.1	2.0	48.3	11.5	0.4	−1.0	0.1	0.1	−0.3	0.8	0.5
11β-OH	0.1	−0.3	−0.3	−0.6	0.7	−0.6	0.3	−4.3	3.9	0.1	47.7	8.8	−0.9	1.7	−0.1	−0.4	0.3	2.4	3.2

12α-OH	0.0	0.0	0.0	0.0	0.1	0.0	−0.2	0.1	−6.8	−0.3	7.5	33.7	4.1	−8.3	−0.3	−0.3	−7.5	1.1	−0.1
12β-OH	−0.1	−0.1	−0.1	−0.2	0.0	−0.2	−0.4	−1.1	−1.2	−0.1	9.0	40.7	5.5	−1.4	−0.3	0.2	−2.3	−5.8	−0.1
15α-OH	0.0	−0.1	−0.2	−0.2	−0.2	−0.3	−0.1	−0.5	−0.1	0.0	−0.2	0.4	0.9	7.2	50.2	12.4	−2.2	1.2	0.0
15β-OH	0.0	0.0	0.0	−0.1	0.3	−0.1	−0.7	−4.1	0.6	0.2	−0.1	1.6	−0.2	4.9	45.0	13.5	−0.1	2.4	0.0
16α-OH	0.0	−0.1	0.0	−0.1	0.1	−0.1	−0.1	−0.5	0.0	0.0	−0.4	0.0	1.1	−2.4	11.8	51.3	11.7	1.2	0.0
16β-OH	0.0	0.0	0.0	−0.1	0.1	−0.1	−0.1	−0.5	−0.1	0.1	−0.3	0.3	−0.5	0.4	11.8	51.4	11.0	1.5	0.0
17α-OH	0.0	−0.1	−0.1	−0.1	−0.1	−0.1	−0.1	−0.2	−0.5	0.0	−0.6	−7.4	4.5	−5.8	−0.9	12.0	39.5	−0.4	0.0
17β-OH	0.0	−0.1	−0.1	−0.1	0.1	−0.1	−0.8	−0.2	−0.1	0.0	−0.4	−2.1	2.3	−3.4	−2.1	10.1	41.6	−6.4	0.0
17β-C_8H_{17}	0.0	0.0	0.0	0.0	0.0	0.0	−0.4	−0.4	−0.2	−0.1	0.0	1.2	1.8	2.0	−1.3	7.8	15.9	−5.4	−0.1
Δ^2	1.4			1.2	−5.5	−0.3	−0.3	0.1	−0.6	−1.6	0.1	−0.3	0.0	−0.1	0.1	0.0	0.0	−0.2	−0.5
Δ^4	−0.9	−2.9	−1.1			4.2	0.5	0.5	−0.4	−0.8	−0.6	−0.1	0.0	−0.3	0.1	0.0	0.0	−0.2	7.2
Δ^5	1.2	0.3	1.2	3.8			−0.3	−3.7	−4.2	1.3	0.0	−0.2	−0.2	0.3	0.1	0.0	−0.1	−0.3	7.3
Δ^7	0.1	−0.1	−0.6	−0.3	−4.7	0.8			−5.0	−1.4	0.2	−0.7	−0.5	−1.5	v1.4	−0.2	−0.5	0.0	0.7

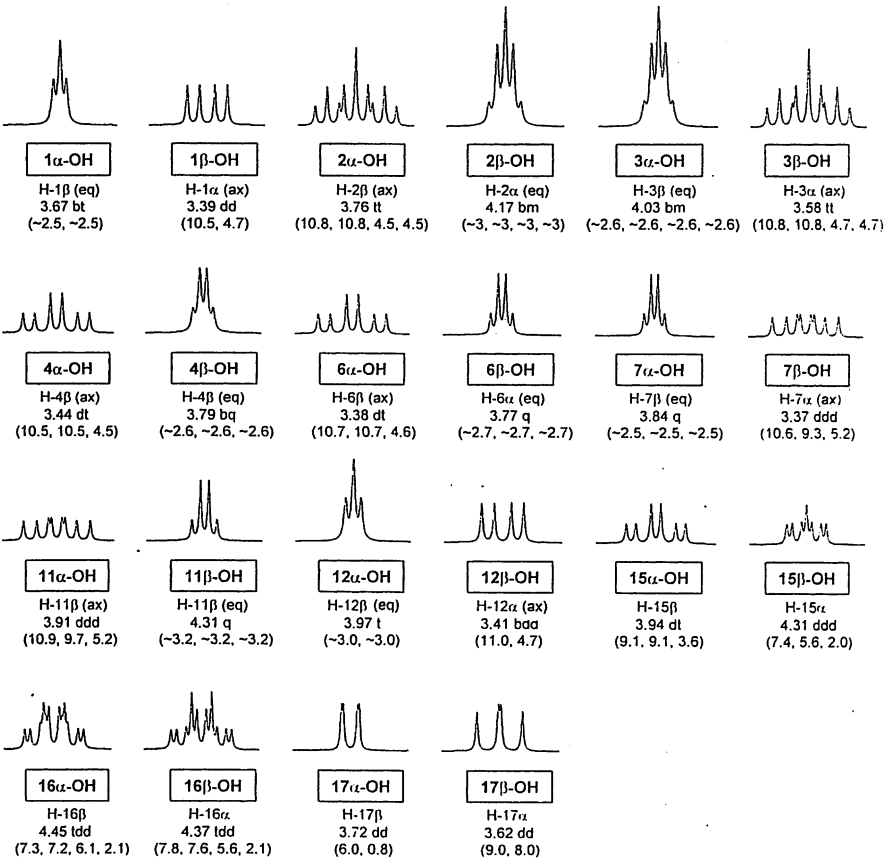


Fig. 2.50 Characteristic multiplet pattern of CH–OH hydrogens in 5α-steroid alcohols

a. In 5α,14α-steroids small couplings (<1 Hz) between angular methyl groups and neighbouring axial protons (*J*(H-19,H-1α) and *J*(H-18,H-12α) eventually *J*(H-18,H-17α)
b. Four bound couplings (<2 Hz) in a planar "zig-zag" arrangement between equatorial protons (e.g. *J*(H-2α,H-4α)
c. Allylic and homoallylic couplings (<3 Hz) in steroids with double bonds or aromatic ring

2.4.6.5 Coupling Constants *J*(C,H)

The couplings between carbon atoms and directly bonded hydrogens are used to distinguish CH_3, CH_2, CH and C types of carbon in ^{13}C NMR spectra (APT, DEPT) and to determine chemical shifts of directly bonded C and H atoms (2D-H,C-HMQC,

2D-H,C-HSQC). Values of ^{1}J(C,H) can be obtained either from 1D-^{13}C-proton coupled spectra (which are for steroids very difficult to analyse due to the strong overlap of complex multiplets) or from 2D-C,H-*J*-resolved spectra (easily analysable but demanding on measurement time). Figure 2.51 shows as an example the 2D-C,H-*J*-resolved spectrum of testosterone. The ^{1}J(C,H)s strongly depend on the hybridisation of the carbon atom and the electronegativity of substituents. Their practical use in the structure analysis of steroids is very limited.

Couplings *J*(C,H) over two and three bonds are qualitatively detected in hetero-correlated 2D-H,C-experiments (e.g. 2D-H,C-HMBC). They allow us "to see over quaternary carbons" and so to connect the molecular fragments determined from 2D-H,H-COSY and 2D-H,C-HMQC experiments. Figure 2.52 shows the 2D-H,C-HMBC spectrum of testosterone. The values of ^{2}J(C,H) and ^{3}J(C,H) are difficult to obtain from complex multiplet patterns in the 1D-^{13}C-proton coupled spectra or 2D-C,H-*J*-resolved spectra. Geminal ^{2}J(C,H) values in steroids are usually small (3–7 Hz) and have a little structural importance. Vicinal ^{3}J(C,H) are, like ^{3}J(H,H), dependent on the dihedral angle of the coupled nuclei (Karplus-like relation). Although they could provide some useful stereochemical information their use in the steroid field is very limited due to difficulties in their measurement.

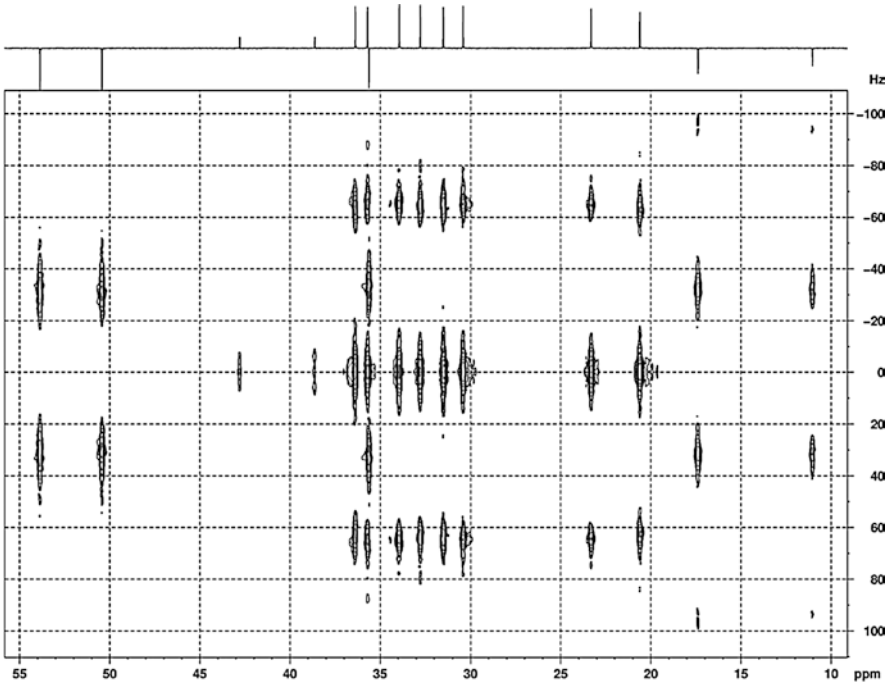


Fig. 2.51 The 2D-C,H-*J*-resolved spectrum of testosterone (for obtaining *J*(C,H) values from this spectrum the numbers on a vertical scale have to be multiplied by two)

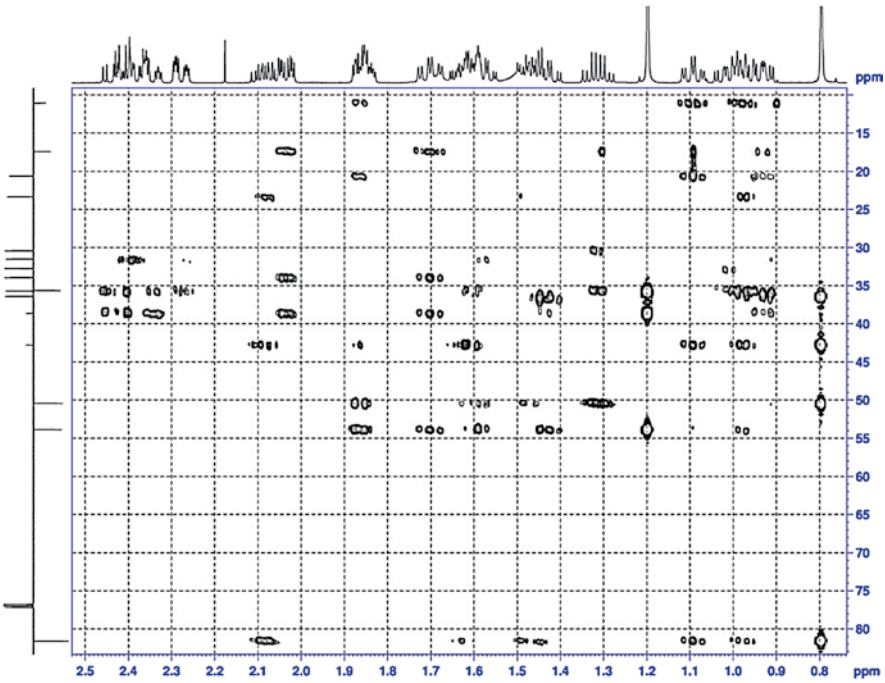


Fig. 2.52 The 2D-H,C–HMBC spectrum of testosterone (**5**)

2.4.6.6 Coupling Constants *J*(C,C)

One bond couplings ^{1}J(C,C) operate in 1D- and/or 2D-INADEQUATE experiments and in principle they allow the complete derivation of a steroid skeleton. The extremely low sensitivity of these experiments (due to extremely low population of molecules with two neighbouring ^{13}C atoms in steroids at natural abundance) makes them very demanding on the amount of sample, experimental time and proper adjustment of experimental parameters. The diagram of the 2D-INADEQUATE spectrum of 5α-androstane is shown in Fig. 2.53.

2.4.6.7 Relaxation Times T_1

Steroid hydrogen atoms relax dominantly by a dipole–dipole mechanism. In the rigid steroid skeleton therefore >CH– hydrogens relax more slowly than $–CH_2–$ hydrogens. This different relaxation rate can be used for distinguishing methine and methylene hydrogen atoms in a partially relaxed ^{1}H NMR spectrum (1D inversion-recovery experiment).

The relaxation times T_1 of carbon atoms depend on the number of directly bonded hydrogens and the correlation time of a given carbon atom. For skeletal carbons T_1 values of CH are about two-times longer than T_1 values of CH_2 carbons. Quaternary

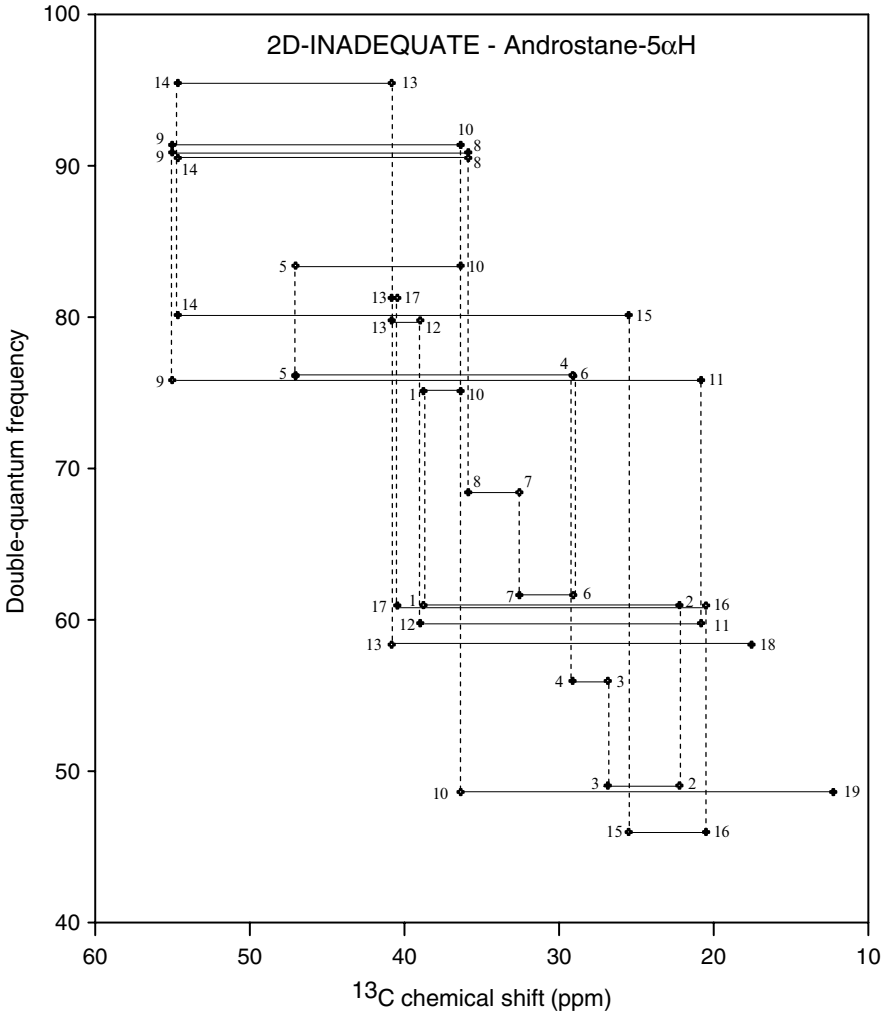


Fig. 2.53 The diagram of 2D-INADEQUATE spectrum of 5α-androstane (**1**)

carbon atoms have much longer T_1 values. The fast rotation of methyl groups and higher flexibility of side-chains leads to an increase in T_1 values of the corresponding carbon atoms (see T_1 values for 5α-cholestan-3β-ol in Fig. 2.54).

2.4.6.8 Nuclear Overhauser Effect

The 1D- or 2D-measurement of NOE can be used for detection of spatially close hydrogen atoms, and from the relative intensities of the NOEs can provide a measure of the distances between corresponding hydrogen atoms. Figure 2.55

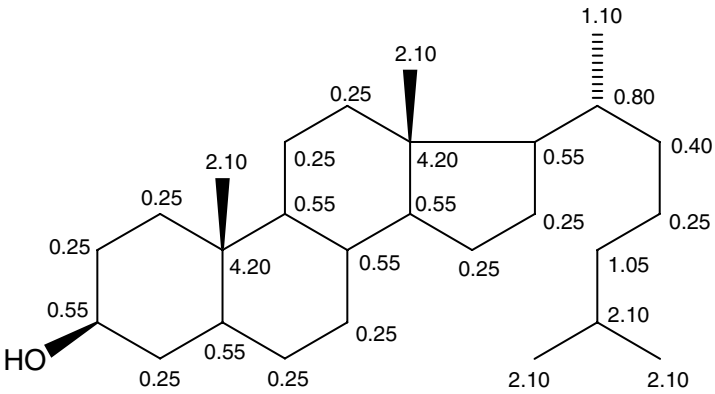


Fig. 2.54 Relaxation times T_1 [s] of carbon atoms in cholestan-3β-ol

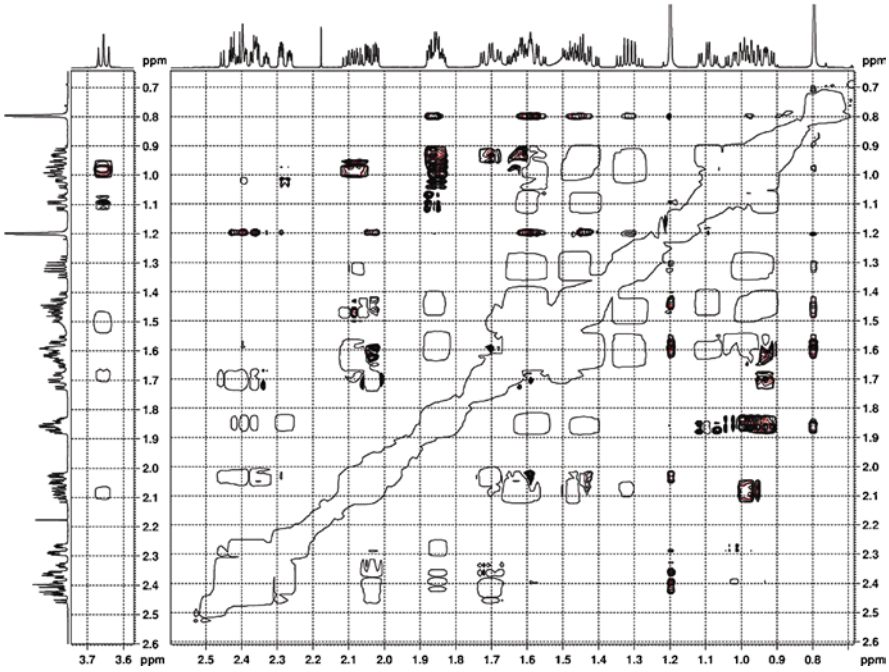


Fig. 2.55 The 2D-H,H-ROESY spectrum of testosterone (**5**) (in $CDCl_3$ at 600 MHz)

shows the 2D-ROESY spectrum of testosterone. The series of NOE cross-peaks of the 18- and 19-methyl groups is clearly visible and it can be used for the stereospecific assignment of protons on the β-side of the steroid skeleton. The application of NOE's for determination of the A/B and C/D ring-fusion was discussed in paragraph 2.4.6.3.

2.4.6.9 Derivatisation

The assignment of signals and structure interpretation of NMR spectra may require in some cases, a chemical modification of the compound. Acetylation, methylation and trimethylsilylation of hydroxy compounds are the most frequently used *derivatisation reactions*. The NMR spectra of such derivatives contain a characteristic signal of the introduced substituent and changes in chemical shifts of nearby nuclei, induced by substitution. For example in the ^{13}C NMR spectra of 3β-hydroxysteroids acetylation induced shifts of about +3 ppm at the α-carbon, about –4 ppm at the β-carbon and about –0.3 ppm at the γ-carbon are observed.

In situ reactions in the NMR tube without isolation of products represent an efficient way of derivatisation. Such reactions should be fast and quantitative, yielding one product and the signals of reagent should not complicate the interpretation of the spectrum. *In situ* reactions of trichloroacetyl isocyante (TAI) (Goodlett, 1965; Trehan et al., 1968; Samek and Buděšínský, 1979), that reacts spontaneously with alcohols, amines and thiols to form trichloroacetyl carbamoyl derivatives has found broad application.

$$\mathrm{R{-}XH + Cl_3C{-}CO{-}NCO \rightarrow R{-}X{-}CO{-}NH{-}CO{-}CCl_3\ (X = O, N, S)}$$

In the ^{1}H NMR spectra the number of NH signals in the product (at δ 8–11) gives the number of –XH groups and the induced acylation shifts of protons in the α- or β-positions allow the distinction between primary, secondary and tertiary functional groups. The characteristic ranges of TAI-acylation shifts in the ^{1}H NMR spectra of alcohols are shown in Table 2.17. The TAI-acylation shifts of >CH–OR hydrogens on six-membered rings A–C are about +1.1 to +1.4 ppm and on the five-membered ring D about +0.9 to +1.2 ppm. Smaller induced shifts reflecting the mutual orientation of the OR group and the hydrogen atom are also observed on protons at the β- and γ-positions.

Significantly higher TAI-acylation shifts are observed for carbon atoms in ^{13}C NMR spectra (see Table 2.18). The TAI-induced shifts of α-carbons are always positive (+4.5 to +8.5 ppm for secondary and more than +15 ppm for tertiary alcohols) and at β-carbons are always negative (−0.2 to −6.1 ppm).

Table 2.17 Characteristic TAI-acylation shifts ($\Delta\delta = \delta$(ROTAC)-δ(ROH)) in ^{1}H NMR spectra of alcohols[a]

Type	$\Delta\delta$ (α)	Type	$\Delta\delta$ (β)	Type	$\Delta\delta$ (γ)
–C**H**$_2$–OH	0.5–0.9	C**H**$_3$–C–OH	0.3–0.5	C**H**$_3$–C–C–OH	0.05–0.25
>C**H**–OH	0.9–1.7	–C**H**$_2$–C–OH	0–0.2 (*trans*)	–C**H**$_2$–C–C–OH	–0.3 (*ax*,*ax*)
		–C**H**$_2$–C–OH	0.2–1.2 (*gauche*)	–C**H**$_2$–C–C–OH	0–0.1 (*ax*,*eq*)
		=C**H**–C–OH	–0.05	–C**H**=C–C–OH	0.15–0.20

[a]Signals of NH protons at δ 8–11.

Table 2.18 TAI-induced acylation shifts in ^{13}C NMR spectra of 5α-steroid alcohols

	TAI-acylation shifts: δ(R-OTAC) – δ(ROH) (ppm)			
Substituent	α-Carbon	β-Carbons		
1α-OH (ax)	7.73	–2.94 (C-2)	–0.41 (C-10)	
1β-OH (eq)	6.40	–4.97 (C-2)	–0.90 (C-10)	
2α-OH (eq)	7.63	–4.44 (C-1)	–4.42 (C-3)	
3α-OH (ax)	8.29	–3.00 (C-2)	–3.26 (C-4)	
3β-OH (eq)	6.12	–4.25 (C-2)	–4.47 (C-4)	
4α-OH (eq)	7.87	–3.61 (C-3)	–3.30 (C-5)	
4β-OH (ax)	6.74	–3.26 (C-3)	–1.01 (C-5)	
5α-OH (ax)	16.07	–5.95 (C-4)	–6.10 (C-6)	–1.11 (C-10)
6α-OH (eq)	7.92	–3.18 (C-5)	–5.94 (C-7)	
6β-OH (ax)	6.54	–1.01 (C-5)	–3.35 (C-7)	
7α-OH (ax)	8.50	–3.28 (C-6)	–1.18 (C-8)	
7β-OH (eq)	5.85	–3.94 (C-6)	–3.77 (C-8)	
11α-OH (eq)	7.07	–4.41 (C-9)	–5.14 (C-12)	
11β-OH (ax)	6.36	–1.82 (C-9)	–4.06 (C-12)	
15α-OH	6.36	–4.58 (C-14)	–4.14 (C-16)	
16α-OH	7.02	–3.43 (C-15)	–4.04 (C-17)	
16β-OH	6.67	–3.11 (C-15)	–3.70 (C-17)	
17α-OH	6.17	–0.34 (C-13)	–2.60 (C-16)	
17β-OH	4.56	–0.22 (C-13)	–3.21 (C-16)	

2.4.6.10 Structure Determination of Steroid Side-Chain

A great variety of side-chain types appear in natural sterols. They may differ in size (from C_2 to C_{11}), branching and configuration at certain positions. Although high-field ^{1}H NMR spectra can provide important information about the type of side chain (number and type of methyl groups), ^{13}C chemical shifts have proved to be more useful and even allow distinction of side-chain epimers. Figure 2.56 shows the ^{13}C chemical shifts for selected types of C_8–C_{10} side-chains (a–z), most of which were taken from NMR data of Δ^5-4-desmethylsterols (Goad and Akihisa, 1997). These data can be very helpful for the determination of side-chain structures since structural changes in rings A, B and C have a little or no effect on the chemical shift of side-chain carbons.

2.4.6.11 Molecular Modeling and Calculation of NMR Parameters

Theoretical calculation of energy minimised structures of steroids, using molecular mechanics (MM) and molecular dynamics (MD) methods are very useful for estimation of distances between protons which could give observable NOE contacts and for comparison of calculated torsion angles with the observed vicinal coupling constants of protons. The calculated energy minimised structures are compared with available NMR data to find the best fit. Poor correlation of some NMR data to a single energy minimised structure may indicate the flexibility in a certain part of

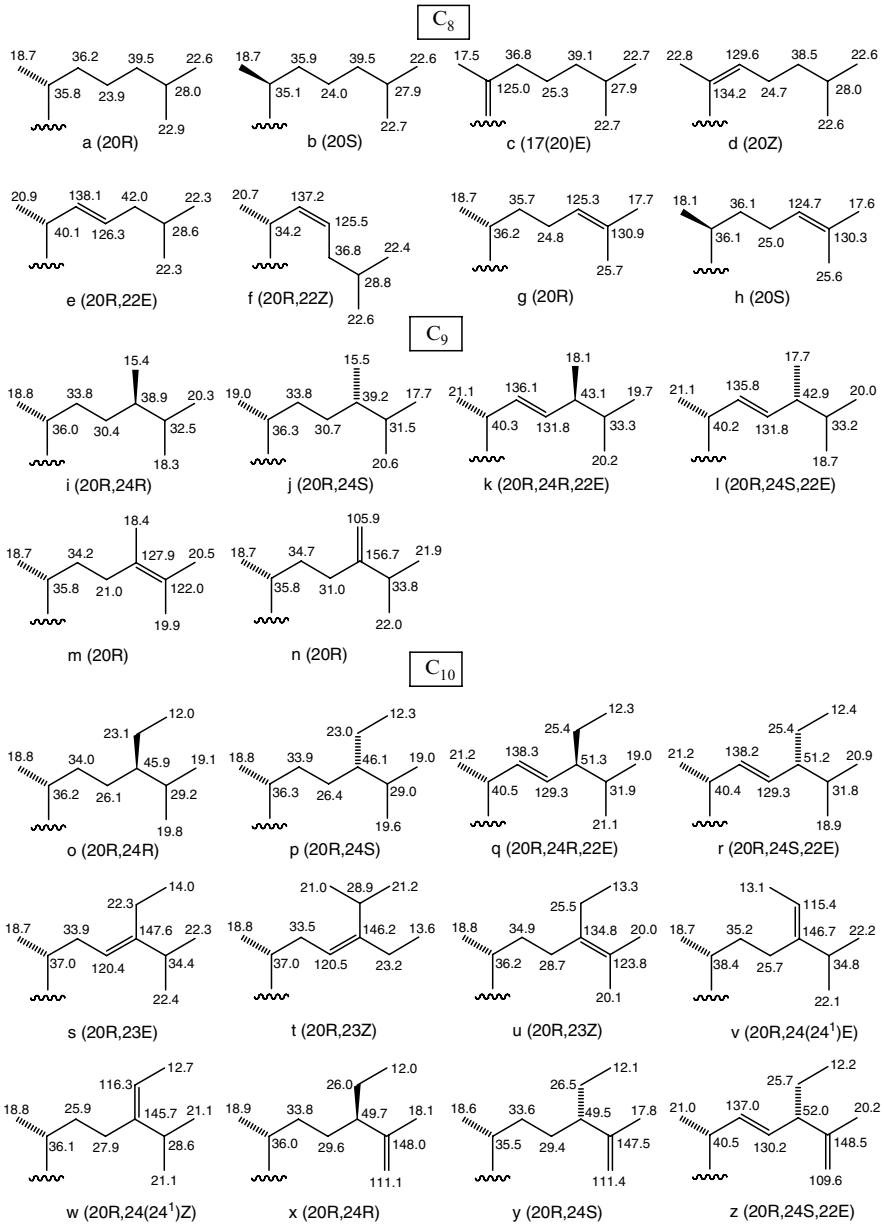


Fig. 2.56 Chemical shifts of carbons in selected types of steroid side-chains

a steroid molecule and the presence of more than one steroid conformation in solution. The observed NMR parameters then correspond to the average values of individual conformers. Multiple solution conformations are known to appear for the flexible D-ring and for the A-ring in some Δ^4-3-keto-steroids.

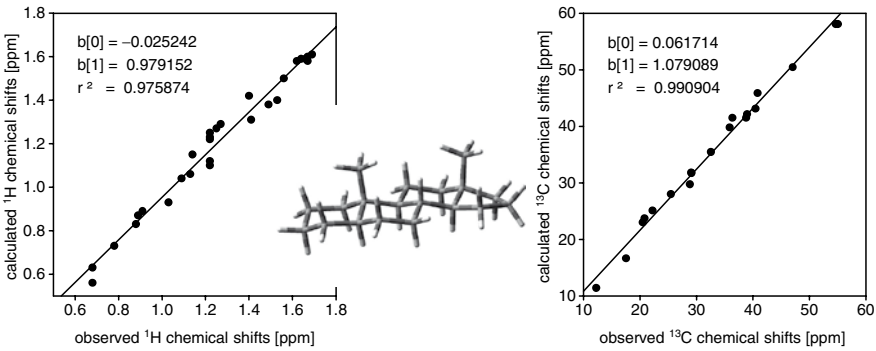


Fig. 2.57 Comparison of the observed and calculated ^{1}H and ^{13}C chemical shifts for 5α-androstane (**1**). Its energy minimised conformation is also shown

Nowadays, the calculation of 3D-structures and NMR parameters of steroids with quantum chemical *ab initio* methods can be performed even on modern PC in a reasonable time. The commercial programme packages (like e.g. *Gaussian03*, Frisch et al., 2004) offer a variety of methods with different levels of accuracy for these purposes. The density functional theory (DFT) has proved to be the most efficient and most widely used approach. Calculation of realistic and accurate geometry of the molecule is the necessary first step for following calculation of NMR parameters. We have applied the DFT methodology (B3LYP, basis set 6-311G(d,p)) for the estimation of energy minimised geometry as well as for the calculation of proton and carbon chemical shifts of a series of ten steroids (**1–6**, **8**, **11–13)** and compared the calculated shifts with experimental data. In general a good linear correlation between calculated and observed chemical shifts was found although the individual values show some differences. The results obtained for 5α-androstane (**1**) are shown in Fig. 2.57. The average deviation between observed and calculated chemical shifts is 0.06 ppm for protons and 2.85 ppm for carbon atoms. This type of calculation can be used nowadays either for chemical shift prediction of steroids with expected structure and/or for checking of correct chemical shift assignment done from experimental NMR spectra.

2.4.6.12 Identification of Trace Impurities in NMR Spectra

In the use of NMR spectroscopy for structural determination a common problem is the identification of signals of minor contaminants (mainly solvents used during sample preparation and isolation). Since their chemical shifts are dependent on the NMR solvent used (and to a lesser extent also on the concentration) it is very practical to have on hand a list of data obtained under defined conditions. Such data in various deuterated solvents (selected from Gottlieb et al. (1997), Crews et al. (1998) and completed with our own unpublished data) are presented in Tables 2.19 and 2.20.

Table 2.19 ^{1}H NMR shifts of common impurities in various deuterated solvents

Δ	Proton	Mult., *J*	$CDCl_3$	CD_3COCD_3	DMSO-d_6	C_6D_6	CD_3OD	D_2O	Pyridine-d_5
Solvent residual peak			7.26	2.05	2.50	7.16	3.31	4.79	8.72
									7.60
									7.23
Acetic acid	CH_3	s	2.10	1.96	1.91	1.55	1.99	2.08	2.13
Acetone	CH_3	s	2.17	2.09	2.09	1.55	2.15	2.22	2.00
Acetonitrile	CH_3	s	2.10	2.05	2.07	1.55	2.03	2.06	1.85
Benzene	CH	s	7.36	7.36	7.37	7.15	7.33		7.33
t-Butanol	CH_3	s	1.28	1.18	1.11	1.05	1.40	1.24	1.37
Chloroform	CH	s	7.26	8.02	8.32	6.15	7.90		8.41
Cyclohexane	CH_2	s	1.43	1.43	1.40	1.40	1.45		1.38
Dichlormethane	CH_2	s	5.30	5.63	5.76	4.27	5.49		5.62
Diethyl ether	CH_3	t, 7	1.21	1.11	1.09	1.11	1.18	1.17	1.12
	CH_2	Q, 7	3.48	3.41	3.38	3.26	3.49	3.56	3.38
Dimethyl formamide	CH	s	8.02	7.96	7.95	7.63	7.97	7.92	
	CH_3	s	2.96	2.94	2.89	2.36	2.99	3.01	2.72
	CH_3	s	2.88	2.78	2.73	1.86	2.86	2.85	2.66
Dimethyl sulphoxide	CH_3	s	2.62	2.52	2.54	1.68	2.65	2.71	2.49
1,4-Dioxane	CH_2	s	3.71	3.59	3.57	3.35	3.66	3.75	3.61
Ethanol	CH_3	T, 7	1.25	1.12	1.06	0.96	1.19	1.17	1.29
	CH_2	q, 7	3.72	3.57	3.44	3.34	3.60	3.65	3.86
Ethyl acetate	CH_3CO	s	2.05	1.97	1.99	1.65	2.01	2.07	1.94
	CH_2	q, 7	4.12	4.05	4.03	3.89	4.09	4.14	4.06
	CH_3	t, 7	1.26	1.20	1.17	0.92	1.24	1.24	1.10
Methanol	CH_3	s	3.49	3.31	3.16	3.07	3.34	3.34	3.57
Isopropanol	CH_3	d, 6	1.22	1.10	1.04	0.95	1.50	1.17	1.29

(continued)

Table 2.19 (continued)

Δ	Proton	Mult., *J*	$CDCl_3$	CD_3COCD_3	DMSO-d_6	C_6D_6	CD_3OD	D_2O	Pyridine-d_5
	CH	h, 6	4.04	3.90	3.78	3.67	3.92	4.02	4.16
Pyridine	CH(2)	m	8.62	8.58	8.58	8.53	8.53	8.52	8.71
	CH(3)	m	7.29	7.35	7.39	6.66	7.44	7.45	7.21
	CH(4)	m	7.68	7.76	7.79	6.98	7.85	7.87	7.58
Tetrahydrofurane	CH_2	m	1.85	1.79	1.76	1.40	1.87	1.88	1.64
	CH_2O	m	3.76	3.63	3.60	3.57	3.71	3.74	3.67
Toluene	CH_3	s	2.36	2.32	2.30	2.11	2.32		2.22
	CH(*o*,*p*)	m	7.17	7.1–7.2	7.18	7.02	7.16		7.22
	CH(*m*)	m	7.25	7.1–7.2	7.25	7.13	7.16		7.22
Triethylamine	CH_3	t, 7	1.03	0.96	0.93	0.96	1.05	0.99	0.96
	CH_2	q, 7	2.53	2.45	2.43	2.40	2.58	2.57	2.43

Table 2.20 ^{13}C NMR shifts of common impurities in various deuterated solvents

Compound	Carbon	$CDCl_3$	CD_3COCD_3	DMSO-d_6	C_6D_6	CD_3OD	D_2O	Pyridine-d_5
Solvent signal		77.16 ± 0.06	29.84 ± 0.01 206.26 ± 0.13	39.52 ± 0.06	128.06 ± 0.02	49.00 ± 0.01	–	149.2 ± 0.2 135.5 ± 0.2 123.5 ± 0.2
Acetic acid	CO	175.99	172.31	171.93	175.82	175.11	177.21	173.45
	CH_3	20.81	20.51	20.95	20.37	20.56	21.03	21.41
Acetone	CO	207.07	205.87	206.31	204.43	209.67	215.94	205.83
	CH_3	30.92	30.60	30.56	30.14	30.67	30.89	30.53
Acetonitrile	CN	116.43	117.60	117.91	116.02	118.06	119.68	117.50
	CH_3	1.89	1.12	1.03	0.20	0.85	1.47	1.08
Benzene	CH	128.37	129.15	128.30	128.62	129.34		128.76
t-Butanol	C	69.15	68.13	66.88	68.19	69.40	70.36	67.58
	CH_3	31.25	30.72	30.38	30.47	30.91	30.29	31.86
Chloroform	CH	77.36	79.19	79.16	77.79	79.44		79.80
Cyclohexane	CH_2	26.94	27.51	26.33	27.23	27.96		27.07
Dichlormethane	CH_2	53.52	54.95	54.84	53.46	54.78		55.06
Diethyl ether	CH_3	15.20	15.78	15.12	15.46	15.46	14.77	15.55
	CH_2	65.91	66.12	62.05	65.94	66.88	66.42	65.83
Dimethyl formamide	CH	162.62	162.79	162.29	162.13	164.73	165.53	162.48
	CH_3	36.50	36.15	35.73	35.25	36.89	37.54	35.74
	CH_3	31.45	31.03	30.73	30.72	31.61	32.03	30.87
Dimethyl sulphoxide	CH_3	40.76	41.23	40.45	40.03	40.45	39.39	41.04
1,4-Dioxane	CH_2	67.14	67.60	66.36	67.16	68.11	67.19	67.23
Ethanol	CH_3	18.41	18.89	18.51	18.72	18.40	17.47	19.22
	CH_2	58.28	57.72	56.07	57.86	58.26	58.05	57.37
Ethyl acetate	CH_3CO	21.04	20.83	20.68	20.56	20.88	21.15	20.84
	CO	171.36	170.96	170.31	170.44	172.89	175.26	170.68
	CH_2	60.49	60.56	59.74	60.21	61.50	62.32	60.32
	CH_3	14.19	14.50	14.40	14.19	14.49	13.92	14.26
Methanol	CH_3	50.41	49.77	48.59	49.97	49.86	49.50	49.70

(continued)

Table 2.20 (continued)

Compound	Carbon	$CDCl_3$	CD_3COCD_3	DMSO-d_6	C_6D_6	CD_3OD	D_2O	Pyridine-d_5
Isopropanol	CH_3	25.14	25.67	25.43	25.18	25.27	24.38	26.08
	CH	64.50	63.85	64.92	64.23	64.71	64.88	63.22
Pyridine	CH(2)	149.90	150.67	149.58	150.27	150.07	149.18	150.31
	CH(3)	123.75	124.57	123.84	123.58	125.53	125.12	124.08
	CH(4)	135.96	136.56	136.05	135.28	138.35	138.27	136.05
Tetrahydrofurane	CH_2	25.62	26.15	25.14	25.72	26.48	25.67	25.82
	CH_2O	67.97	68.07	67.03	67.80	68.83	68.68	67.84
Toluene	CH_3	21.46	21.46	20.99	21.10	21.50		21.34
	CH(*i*)	137.89	138.48	137.35	137.91	138.85		138.05
	CH(*o*)	129.07	129.76	128.88	129.33	129.91		129.43
	CH(*m*)	128.26	129.03	128.18	128.56	129.20		128.69
	CH(*p*)	125.33	126.12	125.29	125.68	126.29		125.78
Triethylamine	CH_3	11.61	12.49	11.74	12.35	11.09	9.07	12.28
	CH_2	46.25	7.07	45.74	46.77	46.96	47.19	46.61

2.4.7 *Pulse Sequences of 1D and 2D NMR Spectra*

An NMR experiment is defined by the corresponding pulse sequence. A pulse sequence is a time description of the actions in the observed and decoupled channels (event in a pulse field gradient module) of the spectrometer. The elements of

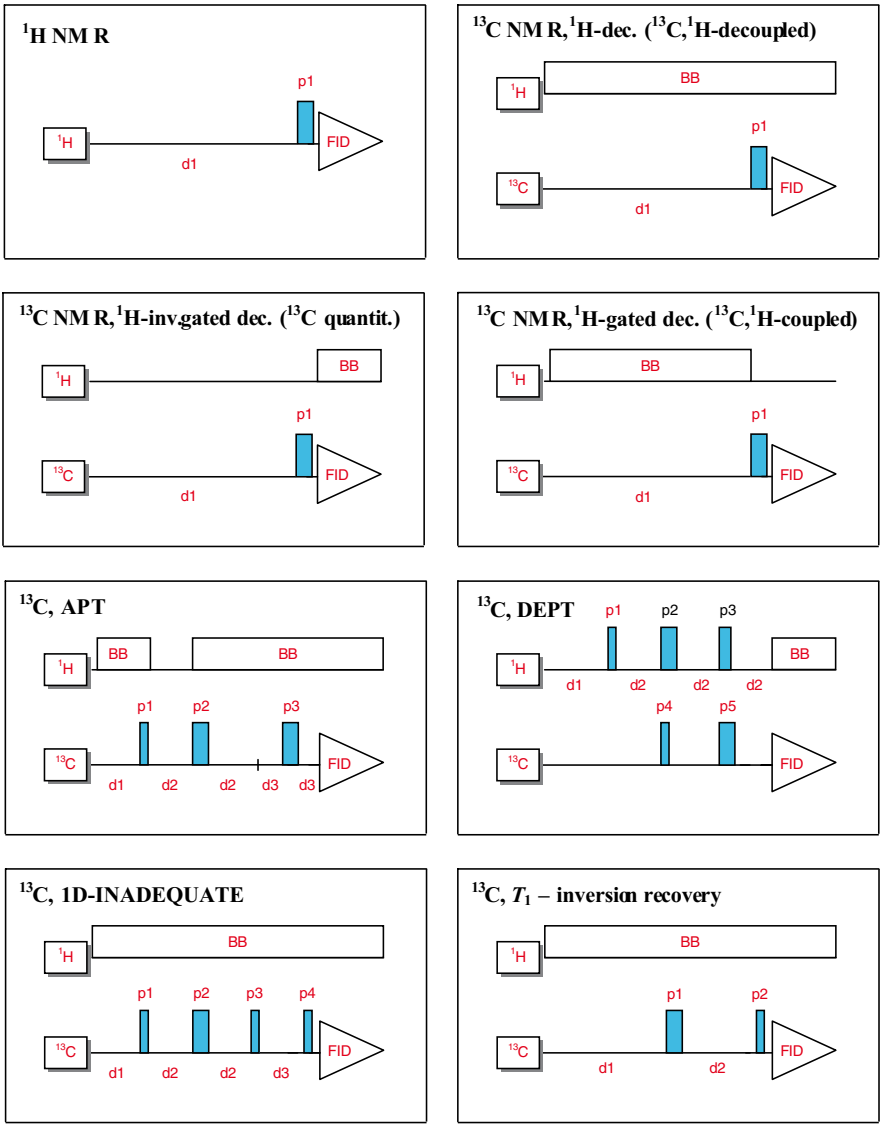


Fig. 2.58 The pulse sequence diagrams of the 1D-NMR experiments commonly used in the structure analysis of steroids

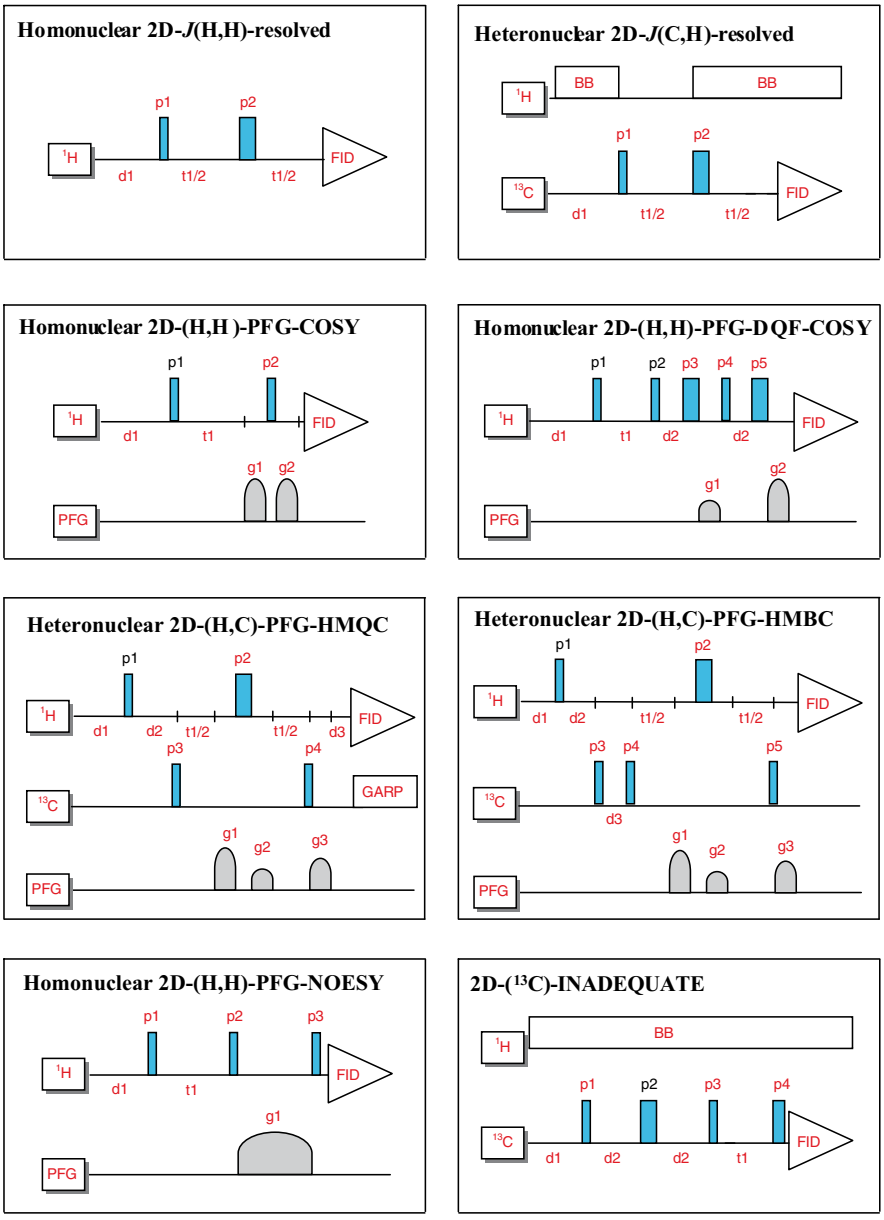


Fig. 2.59 The pulse sequence diagrams of the 2D-NMR experiments commonly used in the structure analysis of steroids

pulse sequences are the time periods of relaxation delays, evolution, mixing and data acquisition (FID), the RF pulses, decoupling, and pulse field gradients (PFG). Pulse sequences can be graphically visualised by time diagrams and they are often designated by acronyms (e.g. APT, COSY, etc.).

The effect of the *RF pulse* depends on its amplitude (power), length (in μs) and phase (along x, –*x*, *y* or –*y* axis). The RF pulse turns a macroscopic magnetisation (in the plane perpendicular to the pulse-axis) about an angle which depends on the pulse length and power.

Time periods (delays) serve for the relaxation of the spin-system, the achievement of requested relations between magnetisation components or the modulation of phase and/or amplitude 2D NMR spectra.

Different types of *decoupling* (homo- or heteronuclear, selective or broadband) have been already discussed.

Gradient pulses of magnetic field (PFG) are used either for selection of certain nuclei in a given NMR experiment or for removing of RF pulse phase-cycling, that is otherwise (without PFG) needed in most of the advanced NMR experiments. Modern NMR spectrometers use PFG also for mapping the static field of the magnet and following *gradient shimming* (automatic optimisation of field homogeneity).

The pulse sequences of the 1D- and 2D-NMR experiments commonly used in the structure analysis of steroids are shown in Figs. 2.58 and 2.59.

2.5 Spectroscopic Methods of Steroid Analysis: Mass Spectrometry

2.5.1 Introduction

Simplistically a mass spectrometer consists of an "ion-source", a "mass analyser", a "detector" and a "data system". Sample molecules are admitted to the "ion-source" where they are vaporised and ionised; the ions are separated according to their mass-to-charge ratio (*m/z*) in the "mass analyser" and are then detected. The resulting signals are transmitted to the "data system" and a plot of ion-abundance against *m/z* corresponds to a mass spectrum. In many cases, a "separating inlet" device precedes the ion-source, so that complex mixtures can be separated prior to admission to the mass spectrometer. Today, the "separating inlet" device is usually either a capillary gas chromatography (GC) column or a high performance liquid chromatography (HPLC) column, although capillary electrophoresis or thin layer chromatography can also be interfaced with mass spectrometry.

For steroid analysis a number of different types of ionisation methods are used to generate gas-phase ions and include; electron ionisation (EI), chemical ionisation (CI), electrospray (ES), atmospheric pressure chemical ionisation (APCI), atmospheric pressure photoinisation (APPI), and the recently introduced, desorption electrospray ionisation (DESI) technique. Other ionisation techniques used, but to a lesser extent are: fast atom bombardment (FAB), liquid secondary ion mass spectrometry (LSIMS), matrix-assisted laser desorsption/ionsation (MALDI) and desorption ionisation on-silicon (DIOS). The selection of the appropriate ionisation mode is one of the key decisions for the analyst to make, and thus we discuss the most important ionisation modes in some detail below.

In the early days of mass spectrometry, when EI was the dominant mode of ion formation, organic chemists were primarily interested in finding molecular ions as a simple means of structure determination. Realising that identification of the origin of individual fragment-ions might offer further valuable clues to structure; they postulated how these were formed. Soon they discovered that their traditional chemical intuition often failed. For instance finding $[M-18]^+$ ions suggested the loss of water from the molecular-ion from which the presence in the molecule of a hydroxyl group might be inferred. However, many non-hydroxylated compounds still produced $[M-18]^+$ ions in their spectra and a new chemistry of steroid fragmentation had to be proposed. Using known compounds, selectively labelled with deuterium, proved that some oxo derivatives also give $[M-18]^+$ fragments, and water loss originated from quite unexpected positions (Trka and Kasal, 1980).

Enormous technological progress, in both instruments and computers, now enables spectroscopists to interpret mass spectra directly without the need to speculate. Exact mass measurements made at high resolution on all fragments provide information about their elemental composition, and tandem mass spectrometry (MS/MS) techniques make it possible to follow the fate of each fragment. Thus, the structure of unknown compounds can now often be found merely by using mass spectrometry.

The reader of this text, although a user of mass spectrometry technology, is likely not to be a mass spectrometry expert. However, he/she will need to know the scope and limitation of individual techniques. It will become evident that vacuum ionisation techniques i.e. EI, are most useful for volatile compounds; while atmospheric ionisation methods such as ES and APCI are more suitable for less volatile compounds.

2.5.2 Ionisation Under Vacuum

Electron Ionisation (*EI*) *and Chemical Ionisation* (*CI*) The sample can be introduced into the EI or CI source via a direct inlet probe, or as the effluent from a GC column thereby providing the basis for GC–MS. EI involves the bombardment of gas-phase sample molecules (M) with high-energy electrons ($\underline{e}^-$), usually of 70 eV energy; the result is the generation of molecular ions ($M^{+\cdot}$), which are usually radical cations, and free electrons (e^-) (Eq. 2.2).

$$M_{(g)} + \underline{e}^- \rightarrow M^{+\cdot}{}_{(g)} + 2e^- \tag{2.2}$$

In many cases the molecular ions ($M^{+\cdot}$) are unstable and fragment to generate more stable products (Eq. 2.3).

$$M^{+\cdot}{}_{(g)} \rightarrow A^+_{(g)} + B_{(g)} \tag{2.3}$$

A pre-requisite of EI is that the sample to be ionised must be in the gas-phase, this is also true for GC, and has lead to the extensive development of derivatisation chemistry to allow the vaporisation of steroids and bile acids without their thermal decomposition (Blau and King, 1977; Blau and Halket, 1993).

CI is a close relative of EI. It differs in that, analyte ionisation is achieved via proton attachment to generate protonated molecules, i.e. $[M+H]^+$ ions, rather than electron ejection to generate radical cations ($M^{+\cdot}$). In CI the ion-source contains a reagent gas, often methane, which becomes ionised by an EI event and acts as a proton donor to the analyte (Eq. 2.4).

$$CH_{4(g)} + \underline{e}^- \rightarrow CH^{+\cdot}_{4\ (g)} + 2e^- \tag{2.4a}$$

$$CH^{+\cdot}_{4\ (g)} + CH_{4(g)} \rightarrow CH^{+}_{5(g)} + CH_{3(g)} \tag{2.4b}$$

$$CH^{+}_{5(g)} + M_{(g)} \rightarrow MH^{+}_{(g)} + CH_{4(g)} \tag{2.4c}$$

The analyte ion, $[M+H]^+$, is an even-electron protonated molecule, which is more stable than the equivalent odd-electron molecular-ion ($M^{+\cdot}$), formed by EI, and thus fragments to only a minor extent in the ion-source.

Electron-capture negative ionisation (ECNI), also called electron-capture negative chemical ionisation (EC–NCI), exploits the electron capturing properties of groups with high electron affinities (Hunt et al., 1976). The method often utilises fluorinated agents in the preparation of volatile derivatives with high electron affinities. For example, trifluoroacetic, pentafluoropropionic or heptafluorobutyric anhydrides can be used to prepare acyl derivatives of amines and hydroxyl groups (Liere et al., 2004), perfluorinated alcohols can be used to generate esters of carboxylic acids, while carbonyl groups can be converted to oximes which can then be converted to e.g. pentafluorobenzyl oximes (Vallée et al., 2000) or pentafluorobenzylcarboxymethoximes (Kim et al., 2000). Ionisation proceeds with the capture of a secondary low-energy electron generated under CI conditions, by the high electron affinity fluorinated groups. Ionisation may lead to the formation of stable $M^{-\cdot}$ ions (Liere et al., 2004), or it may be dissociative (Kim et al., 2000; Vallée et al., 2000; Liere et al., 2004) depending on the analyte and the derivative used (Eq. 2.5). The major advantages of EC–NCI are that ionisation is specific to compounds containing the electron capturing tag, and it provides excellent sensitivity in terms of signal-to-noise ratio when either a stable $M^{-\cdot}$ ion or a negatively charged fragment-ion is monitored.

$$M_{(g)} + e^- + M^{-\cdot}_{(g)} \tag{2.5a}$$

$$M^{-\cdot}_{(g)} \rightarrow A^{-}_{(g)} + B^{\cdot}_{(g)} \tag{2.5b}$$

$$M^{-\cdot}_{(g)} \rightarrow A^{-\cdot}_{(g)} + B_{(g)} \tag{2.5c}$$

It is of historical interest that HPLC has been combined with EI and CI, but as both these ionisation modes require high vacuum, the necessary removal of HPLC solvent has made these combinations difficult. The natural marriage for HPLC is with atmospheric pressure ionisation (API) methods which are discussed in Section 2.5.3.

Fast Atom Bombardment (*FAB*) *Ionisation and Liquid Secondary Ion Mass Spectrometry* (*LSIMS*) It can be argued that the introduction of the FAB method of ionisation by Barber and colleagues in 1981 initiated the revolution in biological mass spectrometry (Barber et al., 1981). Although less popular today (Setchell et al., 1998;

Bove et al., 2004), FAB was widely used for bile acid (Whitney et al., 1981; Ballatore et al., 1983; Egestad et al., 1985; Clayton et al., 1987; Wahlén et al., 1989) and steroid (Shackleton and Straub, 1982; Shackleton et al., 1983; Shackleton, 1983) analysis throughout the 1980s and into the early 1990s, and protocols developed for bile acid and steroid conjugate analysis during this era are easily incorporated into analytical procedures using API methods (Meng et al., 1996, 1997; Yang et al., 1997).

FAB is most suitable for the ionisation of polar or ionic molecules e.g. bile acids, steroid glucuronides and sulphates. FAB ionisation is achieved by the generation of a fast beam of neutral atoms (6–8 keV kinetic energy, usually Ar or Xe atoms) in the "FAB gun" by a process of ionisation, acceleration, and neutralisation, which impinge on a viscous solution of sample dissolved in a matrix, usually glycerol. In the positive-ion mode proton transfer reactions result in the formation of protonated molecules, i.e. $[M+H]^+$ ions, while in the negative-ion mode, deprotonated molecules are formed, i.e. $[M-H]^-$ ions. Both protonated, and deprotonated molecules are stable and little fragmentation occurs in the ion-source. LSIMS is very similar to FAB; however a beam of Cs^+ ions (20–30 keV) rather than a beam of neutral atoms is used to bombard the sample-containing matrix. LSIMS spectra are essentially identical to those generated by FAB, and in this chapter, for simplicity, both ionisation modes will be referred to as FAB. Negative-ion FAB was found to be particularly suitable for the ionisation of bile acids and steroid sulphates, alleviating the need for hydrolysis, solvolysis and derivatisation reactions required for GC–MS analysis (Whitney et al., 1981; Ballatore et al., 1983; Shackleton and Straub 1982; Shackleton et al., 1983; Shackleton 1983). FAB is a vacuum ionisation technique and is not suitable for combination with conventional flow-rate HPLC. However, capillary column HPLC has been successfully interfaced with FAB (Yang et al., 1997).

Matrix-Assisted Laser Desorption/Ionisation (*MALDI*) is not extensively used for steroid and bile acid analysis at present, although some applications have been published (Schiller et al., 2000, 2001; Rujoi et al., 2003; Mims and Hercules, 2003, 2004). MALDI is usually combined with time-of-flight (TOF) analysers, and its major advantage is robustness and ease of use. The sample is mixed with a solution of matrix, often α-cyano-4-hydroxy-cinnamic acid in aqueous acetonitrile containing 0.1% trifluoroacetic acid, spotted on a stainless steel target plate and allowed to co-crystallise in air. The MALDI plate is then admitted to the high vacuum system of the mass spectrometer, and irradiated with laser light, usually of 337 nm from a N_2 laser. The matrix absorbs the light energy with the result that the sample and matrix become ionised and vaporised. In the positive-ion mode $[M+H]^+$ ions are usually formed, while $[M-H]^-$ ions are formed in the negative-ion mode. For steroid analysis, the most promising MALDI studies have been performed on steroids which have been derivatised so as to possess a preformed charge (Griffiths et al., 2003; Khan et al., 2006; Wang et al., 2006).

A relative of MALDI is DIOS which stands for desorption ionisation on silicon, and as the name suggests, a silicon support is used as an alternative to matrix. Siuzdak and colleagues have demonstrated the use of DIOS for the analysis of steroids in plasma (Shen et al., 2001).

2.5.3 Atmospheric Pressure Ionisation

Electrospray (*ES*) Like FAB, ES is suitable for the analysis of polar and ionic biomolecules. ES occurs at atmospheric pressure and can be used with direct infusion of sample, or is readily coupled with HPLC. Fenn and colleagues were the first practitioners of ES mass spectrometry (Yamashita and Fenn, 1984a, b), and by the turn of the last century, ES had largely replaced FAB for the analysis of bile acids and steroid conjugates (Griffiths et al., 2005).

In ES, the analyte is dissolved in a solvent, very often methanol or ethanol, or an aqueous solution of methanol, ethanol, or acetonitrile, and sprayed from a metal or fused silica capillary (needle) of 20–100 μm i.d. at a flow-rate of 1–500 μL/min (Fig. 2.60, upper panel). An ES is achieved by raising the potential on the spray capillary to ~4 kV (+4 kV in the positive-ion mode, and −4 kV in the negative-ion mode) and applying a back-pressure to the contents of the capillary, e.g. via a syringe pump or HPLC pump. The resulting spray of charged droplets is directed toward a counter electrode which is at lower potential. As the spray of fine droplets travels towards the counter electrode, the droplets lose solvent, shrink and break up into smaller droplets (Fig. 2.60, lower panel). The small offspring droplets are derived from the surface of their predecessors, which contains the highest concentration of charge, and hence the offspring droplets are generated with an enhanced charge-to-mass ratio. Eventually, the droplets become so small that the charge density on the droplets exceeds the surface tension and gas-phase ions are desorbed (ion evaporation model (Iribarne and Thomson, 1976)), or alternatively very small droplets containing a single charged species completely lose solvent leaving the residual charged species free (charge residue model (Dole et al., 1968)). Surface active compounds tend to be enhanced in the small droplets, and hence are preferentially brought into the gas-phase. The counter electrode contains a circular orifice through which ions are transmitted into the vacuum chamber of the mass spectrometer. By traversing differentially pumped regions via skimmer lenses the ions are transmitted to the high vacuum region of the mass spectrometer for subsequent analysis. Early ES experiments were performed at flow rates of 5–50 μL/min, compatible with narrow-bore and micro-bore HPLC columns Weidolf et al., 1988; Wong et al., 1922, although today ES interfaces are compatible with 4.2 mm columns operating at 500 μL/min flow-rates. It should, however, be noted that ES is a concentration rather than a mass dependent process, which effectively means that maximum sensitivity is achieved with high-concentration, low-volume samples, rather than high-volume dilute samples. So theoretically a combination of low-flow-rate HPLC with ES should provide better sensitivity than conventional flow-rate HPLC with ES.

Low-flow-rate (<1 μL/min) ES provides a gain in sensitivity when compared to conventional flow-rate ES on account of the concentration dependence of the ES process, and the better ion formation and sampling characteristics of the low-flow-rate interface. Today, the terms micro-ES and nano-ES are both used to describe low-flow-rate ES and are interchangeable, although the terms were originally

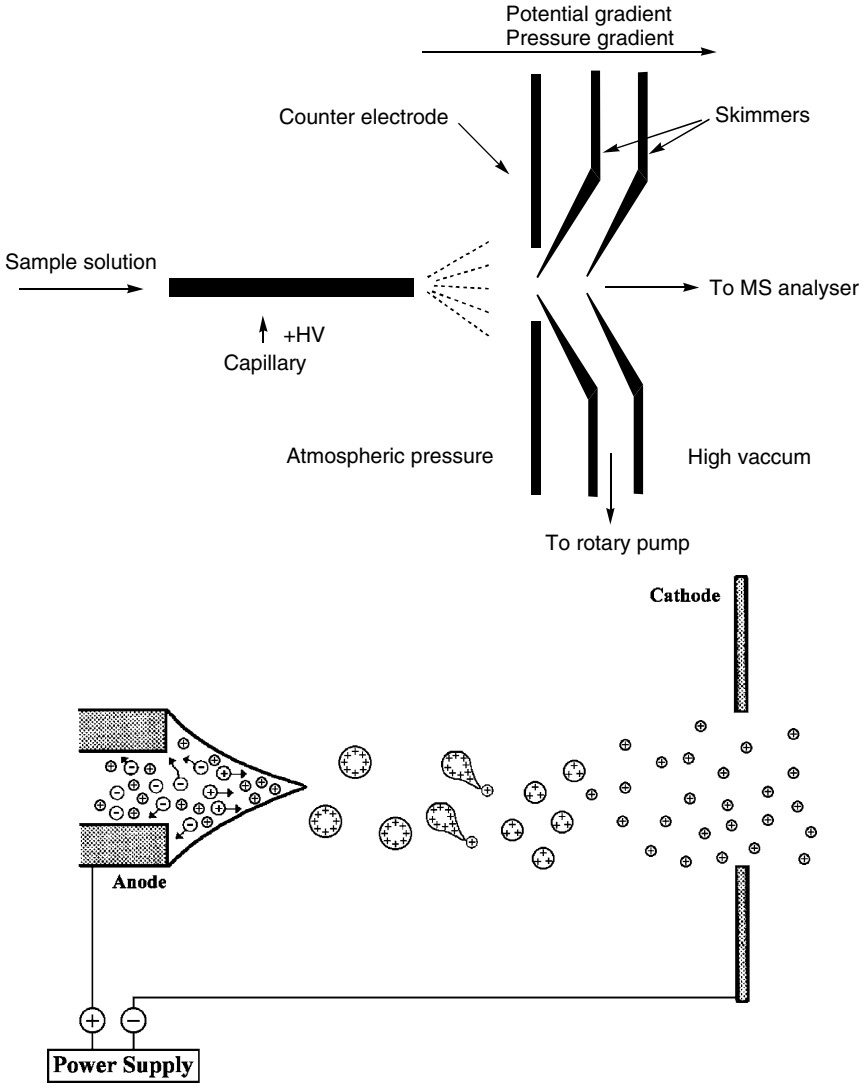


Fig. 2.60 (*Upper panel*) Features of the ES interface, and (*Lower panel*) schematic representation of the ES process. In the positive-ion mode a high positive potential is applied to the capillary (anode), causing positive ions in solution to drift towards the meniscus. Destabilisation of the meniscus occurs, leading to the formation of a cone (Taylor, 1964) and a fine jet, emitting droplets with excess positive charge. Gas-phase ions are formed from charged droplets in a series of solvent evaporation-Coulomb fission cycles. With the continual emission of positively charged droplets from the capillary, to maintain charge balance, oxidation occurs within the capillary (Kebarle and Ho, 1997). If the capillary is metal, oxidation of the metal may occur at the liquid/metal interface:

$$M_{(s)} \rightarrow M^{n+}_{(aq)} + ne^-_{(in\ metal)}$$

or, alternatively negative ions may be removed from the solution by electrochemical oxidation:

$$4OH^-_{(aq)} \rightarrow O_{2(g)} + 2H_2O_{(l)} + 4e^-_{(in\ metal)}$$

invented to discriminate between two slightly different forms of ES. The term micro-ES was initially coined by Emmett and Caprioli (1994), and used to refer to a miniaturised form of pressure driven ES (i.e. pumped flow) operated at sub microlitre per minute flow-rate. Alternatively, nano-ES was invented by Wilm and Mann (1994) and differs from micro-ES in that it is a pure form of ES, where sample flow is at nl/min rates and initiated by the electrical potential between the capillary tip and counter electrode, rather than being pressure driven. We prefer the term low-flow-rate ES to either micro-ES or nano-ES, and use it here to refer to ES operated at flow-rates of 1 μL/min and below.

Thermospray (TS) mass spectrometry predates ES (Blakeley et al., 1978). TS, like ES, is a technique which involves the spraying of analyte dissolved in solvent from a capillary into an ion-source at atmospheric pressure. But TS differs from ES in that vaporisation and ionisation are a result of thermally heating the spray, as opposed to raising the sprayer to a high potential. TS performs best with buffered aqueous mobile phase (0.1–0.01 M ammonium acetate). Droplets become charged by an uneven distribution of cations and anions between droplets, with the result that gaseous $[M+H]^+$, $[M+NH_4]^+$ or $[M+Na]^+$ ions are formed in the positive-ion mode in an ion-evaporation process similar to that occurring in ES. In the negative-ion mode $[M-H]^-$ ions are formed, and this mode is preferably used for the analysis of acidic compounds, e.g. bile acids, steroid sulphates and glucuronides. TS can be operated in either a "filament-on" or "filament-off" (direct ion-evaporation) mode. By the incorporation of a filament in the ion-source, analytes which are not readily ionised by direct ion-evaporation can be ionised in the "filament-on" mode via a CI process. Spectra of steroids and bile acids generated by ES and TS are similar; however, dehydration of the protonated or deprotonated molecule tends to occur to a much greater extent in TS than in ES (Eckers et al., 1991).

Atmospheric Pressure Chemical Ionisation (*APCI*) is a technique that has become popular for the ionisation of neutral steroids. It is very similar to TS described above (Covey et al., 1986). Analyte dissolved in solvent is sprayed into an atmospheric pressure ion-source. Vaporisation of sample and solvent is achieved by the application of heat, and ionisation of analyte is achieved by a CI event. The APCI source differs from the ES source in that it additionally contains a corona discharge needle. Analyte ionisation can be achieved by two processes:

(i) The first is a primary CI process. The nebulised spray results in small droplets of differing charge formed as a result of statistical random sampling of buffer ions

When the interface is operated in the negative-ion mode, potentials are reversed, and to maintain charge balance cations or neutral molecules in solution may become reduced at the walls of the capillary.

$$C^{n+}_{(aq)} + ne^- \rightarrow C_{(g/s)}$$

$$H_2O_{(l)} + 2e^- \rightarrow H_{2(g)} + O^{2-}_{(aq)}$$

(from Griffiths et al., 2001, with permission)

(cf. TS). The charged droplets will shrink as in the ES process with the eventual formation of gas-phase buffer ions, analyte molecules (M), and solvent molecules (Sv). If ammonium acetate, ($NH_4^+ CH_3CO_2^-$), is the buffer, gas-phase $[NH_4]^+$ and $[CH_3CO_2]^-$ ions will be generated. The buffer ions will be free to react with analyte molecules in a CI event which will generate analyte ions (Eqs. 2.6 and 2.7).

$$NH^+_{4(g)} + M_{(g)} \rightarrow NH_{3(g)} + MH^+_{(g)} \tag{2.6a}$$

$$NH^+_{4(g)} + M_{(g)} \rightarrow M + NH^+_{4(g)} \tag{2.6b}$$

$$NH^+_{4(g)} + Sv_{(g)} \rightarrow NH_{3(g)} + SvH^+_{(g)} \tag{2.6c}$$

$$SvH^+_{(g)} + M_{(g)} \rightarrow M + SvH^+_{(g)} \tag{2.6d}$$

In the positive-ion mode the exact products of the CI event will depend on the gas-phase basicity of the analyte, solvent and buffer. Adduct ions can also be formed, e.g. $[M+NH_4]^+$ and $[M+CH_3CN+H]^+$ in aqueous acetonitrile buffered with ammonium acetate. When the ion-source is operated in the negative-ion mode the products of the CI event will depend on the gas-phase acidity of the sprayed components (Eq. 2.7).

$$CH_3CO^-_{2(g)} + M_{(g)} \rightarrow CH_3CO_2H_{(g)} + [M-H]^-_{(g)} \tag{2.7a}$$

$$CH_3CO^-_{2(g)} + M_{(g)} \rightarrow M + CH_3CO^-_{2(g)} \tag{2.7b}$$

(ii) APCI can also be achieved in a secondary process, in which electrons from the Corona discharge ionise nitrogen gas in the APCI source leading to the eventual CI of the analyte. In the positive-ion mode, again the eventual products depend on the proton affinity of the components (Eq. 2.8), while in the negative-ion mode the gas-phase acidity of the components will define which deprotonated molecules are generated.

$$N^{+\cdot}_{2\,(g)} + Sv_{(g)} \rightarrow Sv^{+\cdot}_{(g)} + N_{2(g)} \tag{2.8a}$$

$$Sv^{+\cdot}_{(g)} + Sv_{(g)} \rightarrow SvH^+_{(g)} + [Sv\text{-}H]^{\cdot}_{(g)} \tag{2.8b}$$

$$SvH^+_{(g)} + M_{(g)} \rightarrow MH^+_{(g)} + Sv_{(g)} \tag{2.8c}$$

$$SvH^+_{(g)} + M_{(g)} \rightarrow M + SvH^+_{(g)} \tag{2.8d}$$

Both primary and secondary processes can operate simultaneously, although by turning the corona discharge needle off, only the primary processes proceed.

APCI has been extensively used for the ionisation of neutral steroids (Ma and Kim, 1997; Shimada and Mukai, 1998; Rule and Henion, 1999; Draisci et al., 2000; Lagana et al., 2000; Mitamura et al., 2000a, b; Nassar et al., 2001; Leinonen et al.,

2002; Starcevic et al., 2003; Cristoni et al., 2004; Wang et al., 2004), oxysterols (Burkard et al., 2004), bile acid acyl glucosides and glucuronides (Goto et al., 1998, 2004), bile acids (Ikegawa et al., 1995) and steroid glucuronides (Kuuranne et al., 2000). It is compatible with HPLC operated at high-flow-rates (100–500 μL/min), and this makes it a favoured mode of ionisation for "high-throughput" analysis (Clarke and Goldman, 2005; Redor-Goldman et al., 2005a, b).

Electron Capture Atmospheric Pressure Chemical Ionisation (*ECAPCI*) is suitable for the ionisation of molecules which contain a group with high electron affinity, and it can be regarded as the atmospheric pressure equivalent of EC–NCI. For steroid analysis it is necessary to derivatise the steroid with an electron capturing group e.g. pentafluorobenzyl moiety, (Singh et al., 2000), 2-nitro-4-trifluoromethylphenylhydrazone moiety (Higashi et al., 2002). The resultant derivative is then introduced into a conventional APCI source operated in the negative-ion mode with the corona discharge needle "on". Low-energy secondary electrons are generated as a side-product of N_2 ionisation, and are captured by the electron capturing group tagged to the steroid to generate $M^{-\cdot}$ ions (Eq. 2.9). When the pentafluorobenzyl derivatising group is employed, dissociative electron capture occurs with the loss of the pentafluorobenzyl radical and formation of a negative ion (A^-) which reflects the structure of the original steroid. Alternatively, 2-nitro-4-trifluoromethylphenylhydrazones give stable $M^{-\cdot}$ ions which can be fragmented in a MS/MS experiment to give product ions (A^-) and neutral fragments ($B^{\cdot}$), which reflect the structure of the steroid or are characteristic of the derivatising agent.

$$N_{2(g)} + \underline{e}^- \rightarrow N^{+\cdot}_{2\,(g)} + 2e^- \tag{2.9a}$$

$$e^- + M_{(g)} \rightarrow M^{-\cdot}_{(g)} \tag{2.9b}$$

$$M^{-\cdot} \rightarrow A^-_{(g)} + B^{\cdot}_{(g)} \tag{2.9c}$$

ECAPCI is specific to molecules containing an electron capturing group, and hence provides high specificity, and has been shown to provide enhancement in sensitivity compared to APCI of 20- (Higashi et al., 2005) to 200-fold (Singh et al., 2000). ECAPCI is used extensively for neurosteroid analysis, particularly by Higashi and colleagues (Higashi et al., 2002, 2003).

Atmospheric Pressure Photoionisataion (*APPI*) was introduced in 2000 by Robb, Covey and Bruins (2000). Photons (10 eV, 10.6 eV) are provided by a krypton lamp, and dopant molecules e.g. toluene (BzH), introduced into the ion-source in combination with analyte (M) and mobile phase, are vaporised and photoionised. This results in the formation of radical cations e.g. BzH^+ (Eq. 2.10).

$$BzH_{(g)} + h\upsilon\,(10 \text{ or} 10.6 \text{ eV}) \rightarrow BzH^{+\cdot}_{(g)} + e^- \tag{2.10a}$$

$$BzH^{+\cdot}_{(g)} + Sv_{(g)} \rightarrow Bz^{\cdot}_{(g)} + SvH^+_{(g)} \tag{2.10b}$$

$$M_{(g)} + SvH^+_{(g)} \rightarrow MH^+_{(g)} + SV_{(g)} \tag{2.10c}$$

$$BzH^{+\cdot}_{(g)} + M_{(g)} \rightarrow M^{+\cdot}_{(g)} + BzH_{(g)} \quad (2.10d)$$

The radical cations, e.g. $BzH^{+\cdot}$, react with solvent molecules (Sv) producing protonated solvent molecules (SvH^+), if the proton affinity of the solvent molecule is higher than that of the benzyl radical ($Bz^{\cdot}$). The analyte is then ionised by proton transfer, if the proton affinity of the analyte is higher than that of the solvent molecule. Alternatively, if the proton affinity of the solvent molecule is lower than that of the benzyl radical ($Bz^{\cdot}$), a charge exchange reaction between the radical cation ($BzH^{+\cdot}$) and the analyte can take place. If the ionisation energy of the analyte is lower than that of the radical cation, the result is the formation of analyte radical cations ($M^{+\cdot}$) (Leinonen et al., 2002). APPI has been used for the analysis of steroids including, sterols (Trösken et al. 2004; Lembcke et al., 2005), corticosteroids (Greig et al., 2004), anabolic steroids (Leinonen et al., 2002), and estradiol derivatives (Shou et al., 2004). A disadvantage of the technique, as illustrated in the study of Greig et al. (2003) is the formation of overlapping $[M+1]^{+\cdot}$ (due to the ^{13}C isotope) and $[M+H]^+$ ion-peaks. This can cause confusion in molecular weight determination.

2.5.4 Mass Analysers

Once a sample has been ionised it is transported from the ion-source to the mass analyser. Trapping mass analysers can provide an exception in that ionisation and mass analysis can be achieved in the trap itself. Mass analysers operate by separating ions according to their *m/z*. Early mass analysers used in steroid and bile acid research were based on magnetic-sectors, but such analysers are less widely used for biological applications today. However, ion-cyclotron-resonance (ICR) analysers, which also use a magnetic field to separate ions, are currently gaining popularity. As an alternative to using a magnetic field to separate ions, ions can be separated according to their behaviour in electrical fields, and electric field analysers constitute the majority of mass analysers in current use.

Magnetic-Sector Analysers Like all other mass analysers, magnetic-sector analysers separate ions according to their *m/z*. Usually, the magnetic-sector (B) is arranged in series with an electric-sector (E) and the combination (either EB or BE) can give resolutions in excess of 100,000 (10% valley definition) for ions in the steroid mass range. Modern magnetic-sector instruments are most commonly interfaced with an EI source, with or without a GC inlet, although in the past they have been coupled to TS, FAB, MALDI and ES ion-sources. Magnetic-sectors are still used today for exact mass measurements (<5 ppm) made at high resolution (>10,000, 10% valley), and these instruments offer excellent dynamic range which is particularly important in isotope abundance measurements. Magnetic-sector analysers operate with keV ion-beams which can be a major advantage when the

instrument is operated in the MS/MS mode, allowing collision-induced dissociation (CID) reactions to occur at high collision-energy (keV).

Quadrupole Mass Filters The quadrupole mass filter provides a much smaller and lower cost analyser than the magnetic-sector. It consists of four parallel rods arranged equidistantly from a central axis. By the application of a combination of radio frequency (rf) alternating current (ac) and direct current (dc) voltage-components to the rods, ions of one particular *m/z* can be transmitted along the central axis between the rods, and conveyed to the detector. Others are deflected from the central axis and are not transmitted. By scanning the voltages applied to the rods an *m/z* range can be scanned (usually up to a maximum of 2,000 or 4,000 *m/z*). For steroid analysis, quadrupole mass filters are usually operated at unit mass resolution, i.e. 0.7 Da full width at half maximum height (FWHM), they are most often interfaced with EI/CI or API sources, and offer the advantages of fast scanning and stability. Quadrupole mass filters are additionally compatible with both GC- and LC- interfaces. Quadrupole mass filters can be arranged in series to give tandem quadrupole instruments, and have also been coupled in series with magnetic-sectors and orthogonal acceleration (OA) TOF analysers to give hybrid tandem mass spectrometers.

Quadrupole Ion-Trap A cylindrical, or three dimensional quadrupole ion-trap can be imagined as a quadrupole bent around on itself to form a closed loop. The inner rod is reduced to a point at the centre of the trap; the outer rod is a circular ring electrode, and the top and bottom rods become two end cap electrodes. Ions can be formed by EI within the ion-trap or can be introduced from an external source e.g. ES, APCI, MALDI. In the case of EI, electrons are admitted through a small central hole in one of the end caps; alternatively ions formed in an external source can be transported to the trap and similarly admitted through the end cap. Initially ions of all *m/z* values are confined in the trap, and are expelled and detected according to their *m/z* by ramping linearly the amplitude of the radio frequency potential applied to the ring electrode. Each ion species is ejected from the potential well at a specific radio frequency amplitude and, because the initial amplitude and ramping rate are known, the *m/z* can be determined for each ion species upon ejection. This method for measuring the *m/z* of confined ions was developed by Stafford et al. (1984) and is known as the "mass-selective axial instability scan-mode".

Commercial ion-traps have *m/z* ranges up to 6,000 depending on their application, they are fast scanning (1,000 *m/z* units/s), and are capable of enhanced resolution or "zoom" scans offering resolutions of 10,000 (FWHM) by scanning a short *m/z* range slowly. Unfortunately, as a result of space charging within the trap, mass accuracy is considerably lower than that achievable on beam instruments. Like the quadrupole mass filter the ion-trap can be used as a tandem mass spectrometer, but has the additional capability of multiple stages of fragmentation i.e. MS^n. The cylindrical ion-trap is now being replaced by a new generation of linear ion-traps (LIT), where ions are trapped within a quadrupole itself. This allows better mass accuracy as a result of reduced space charging provided by the greater cell volume. Linear ion-traps can be combined with quadrupole mass filters or ICR cells to give tandem instruments.

Orbitrap-Analyser A new type of electrostatic ion-trap is the Orbitrap analyser manufactured by Thermo Electron Corp as part of a LIT-Orbitrap hybride MS/MS instrument. This instrument provides resolution of up to 100,000 (FWHM) and mass accuracy of better than 5 ppm. MS/MS can be performed in the LIT and fragment ions formed, analysed by the Orbitrap. The instrument offers the sensitivity advantages of a LIT, with high performance mass analysis of the Orbitrap. Ions from the ion-source are initially stored in the LIT and analysed in either MS or MS^n modes. Ions can be detected at the LIT detector, or ejected axially and trapped in an intermediate C-trap from which they are "squeezed" into the Orbitrap. Trapped ions in the Orbitrap assume circular trajectories around the central electrode and perform axial oscillation. The oscillating ions induce an image current into the two halves of the Orbitrap, which can be detected. The axial oscillation frequency of an ion (ω) is proportional to the square root of the inverse of m/z ($\omega = (k/m/z)^{½}$), and the frequencies of complex signals derived from many ions can be determined using a Fourier transformation (FT) (Scigelova and Makarov, 2006). This instrument has already gained popularity for steroid and sterol analysis (Thevis et al., 2005; Griffiths et al., 2008).

Time-of-Flight Analysers (*TOF*) The first generation of TOF mass analysers were coupled to EI sources (Wiley and McLaren, 1955) and it was not until the advent of MALDI that the modern era of TOF mass spectrometry was initiated. The pulsed nature of the MALDI source compliments the necessity for time measurements in TOF analysis. ES, APCI and EI/CI sources are now found coupled to TOF analysers, but in an orthogonal arrangement. Ions generated by these continuous-beam ion-sources are pulsed in packets in an orthogonal direction into the TOF analyser, and the time taken for the ions to traverse the TOF drift tube to the detector is a measure of their m/z. The TOF analyser is based on the following:

Ions pulsed into the TOF, are accelerated through a potential V, and will gain a kinetic energy $mv^2/2$, so that

$$mv^2 / 2 = zeV \tag{2.11}$$

where m is the mass of the ion, z the number of charges on the ion, and e the charge of an electron, then

$$m / z = 2eV / v^2 \tag{2.12}$$

$$(m / z)^{1/2} = (2eV)^{1/2} \cdot t / d \tag{2.13}$$

where t is the time the ion takes to travel down the drift tube of length d and reach the detector. For a given instrument operated at constant accelerating potential, V and d are constants, then

$$t \propto (m / z)^{1/2} \tag{2.14}$$

For steroid analysis, MALDI is seldom used, but TOF analysers are interfaced with API and EI/CI sources. TOF analysers are fast "scanning", theoretically have unlimited mass range, and when combined with delayed extraction and a reflectron, or

ion-mirror, offer resolutions of up to 20,000 (FWHM). TOF analysers can be coupled to quadrupole mass filters to give quadrupole-TOF (Q-TOF) tandem instruments, or can be arranged in series to give TOF–TOF instruments.

Fourier Transform Ion Cyclotron Resonance (*FTICR*) *Mass Spectrometers* are ion trapping instruments, however, unlike the quadrupole ion-trap or Orbitrap, the trapping field is magnetic rather than electrostatic. Ionisation occurs in an external source and ions are transported into the high vacuum ICR cell. Within the cell ions move with cyclotron motion governed by their cyclotron frequency. Ions of differing *m/z* values have different cyclotron frequencies, which are detected as an induced- or image-current as ions pass the receiver plates, and can be converted into *m/z* values by application of Fourier transform. The FTICR mass spectrometer (and Orbitrap) is unlike other forms of mass spectrometer in that it is non-destructive and signal enhancement can be achieved by signal averaging multiple cycles of the same ion. FTICR provides exceptionally high resolution in the steroid mass range (>100,000 FWHM), and additionally provides high accuracy of mass measurement (<2 ppm) (Greig et al., 2003). Like quadrupole ion-traps, FTICR instruments can be used as tandem mass spectrometers, and have MS^n capability. The cost of the high performance of FTICR instruments is in time, as FTICR is based on frequency measurements. This can have significant implications when FTICR instruments are interfaced to LC (Schrader and Klein, 2004).

2.5.5 Tandem Mass Spectrometry

While bile acids and steroids fragment in the ion-source upon EI to give structurally informative fragment ions (see Section 2.5.7), in-source fragmentation with API is usually only minor. To obtain detailed fragmentation information on molecules ionised by API (and FAB or CI methods) it is usual to perform MS/MS. With respect to bile acid and steroid analysis, MS/MS is usually performed by incorporating a CID step, although other methods to induce fragmentation exist. CID is regarded to occur within two different collision-energy regimes, i.e. at high collision-energy (>1,000 eV), or at low collision-energy (<200 eV). The spectra recorded under these two regimes may be very different in appearance. When spectra are recorded at intermediate collision-energy i.e. 400–800 eV, the nature of the collision-gas dictates whether the spectra appear more like high or low collision-energy spectra. Heavy collision-gas atoms e.g. Xe, promote high collision-energy like CID, while light gas atoms e.g. He, promote low collision-energy like CID.

High collision-energy spectra are usually recorded on magnetic-sector instruments or on TOF-TOF instruments, while most other tandem mass spectrometers give low-energy CID spectra. Low-energy CID spectra recorded on beam instruments e.g. tandem quadrupole, Q-TOF instruments, differ in appearance to those recorded on trapping instruments i.e. quadrupole ion-trap, FTICR. With ion beam instruments fragmentation occurs in a multiple collision process and a broad distribution of energy is imparted into the fragmenting ion. Initially formed fragment ions may decompose further, resulting in a mix of different fragment ions eventually

reaching the detector. With an ion-trap, the ion of interest is selected while all others are expelled from the trap. In the conventional quadrupole ion-trap collisional activation involves a competition between ion excitation and ion ejection. Poor efficiencies are obtained at low trapping levels due to precursor ion ejection. This is avoided by using higher trapping levels during excitation, but this can result in the loss of information if some of the product ions fall below the low mass cut-off. The low mass cut-off corresponds to the bottom third of the MS/MS spectrum recorded on an ion-trap, and is perhaps the biggest disadvantage of the ion-trap. With a quadrupole ion-trap, the ion-current is usually concentrated into a single or just a few fragmentation channels, i.e. those with the lowest activation energy. This results in the formation of only a few different fragment ions, but those that are formed are of high abundance. The MS/MS spectrum is obtained by sequentially ejecting the product-ions out of the trap towards the detector. Alternatively, one fragment-ion can be maintained in the trap while the others are ejected, this fragment can then be activated to give a MS^3 spectrum. This process can be repeated to give up to MS^6.

FTICR instruments can also be used for MS/MS experiments, again the ion of interest is trapped while all others are expelled, the ion is then activated and the fragment ions detected. FTICR instruments offer the highest performance characteristics of all MS/MS instruments (except a capacity to perform high-energy CID). Precursor-ions can be selected at high-resolution, and fragment ions measured with high mass accuracy and at high resolution. Additionally, the non-destructive nature of the FTICR ion-detection system allows ion re-measurement with the accompanied gain in signal-to-noise ratio. The non-destructive nature of the FTICR also allows MS^n on a single population of ions, without the necessity of re-populating the trap between MS^n steps. The down side to the high performance of the FTICR is the cost in time, which introduces severe limitations when the mass spectrometer is combined with a chromatography inlet. To counter this problem, many of the current generation of FTICR instruments, are LIT–FTICR hybrids, where the FTICR can be used as a high-resolution, high mass accuracy analyser and detector, and the LIT can be used to perform the MS/MS and MS^n process.

Beam instruments offer a major advantage over trapping instruments in that they are able to perform many different types of scans. In addition to product-ion scans, magnetic-sector, tandem quadrupole, and to a lesser extent Q-TOF type instruments can perform precursor-ion and neutral loss-scans. A product-ion scan is recorded by setting MS_1 to transmit the ion of interest, fragmentation, usually by CID, occurs in the collision cell, and MS_2 is scanned to record the *m/z* of the product-ions. On a beam instrument these three events are separated in space, on a trapping instrument the events are separated in time. A precursor-ion scan is recorded by scanning MS_1, CID occurs in the collision cell, and MS_2 is set to transmit fragment ions of only one *m/z* value. In this way precursor-ions of a selected product-ion are identified. In a neutral-loss scan MS_1 and MS_2 are scanned in parallel, but MS_2 is offset, so that only fragment ions lighter than the precursor ions by an amount equal to the offset (neutral-loss) are transmitted. Tandem quadrupole instruments are particularly adept at performing the above scans, and in addition can be used in the multiple reaction monitoring (MRM) mode to achieve high specificity and sensitivity. In an MRM experi-

ment MS_1 is set to transmit a defined precursor-ion and MS_2 a known fragment of the precursor. The high-transmission of the quadrupole in this non-scanning mode results in high sensitivity, while the "parent-daughter" relationship provides specificity. In a given acquisition the quadrupoles can be set to monitor many such transitions.

2.5.6 Electron Impact Mass Spectra

While the early work using EI was performed mainly on natural steroids (see Section 2.5.8), increased structural information is usually obtained by preparation of derivatives which help to direct the localisation of charge and thus the fragmentation. More importantly, the analysis of steroid mixtures by GC/MS requires derivatisation because many naturally occurring steroids are polar and thermally labile even after removal of conjugating moieties. Pioneering work on methods to derivatise steroids was performed by Horning and co-workers (Horning, 1968).

Derivatives should be simple to prepare, result in single products, be thermally stable, and provide structurally informative mass spectra. General references can be found in books by Blau and King (1977), and Blau and Halket (1993), and information relating to steroid derivatisation for GC/MS can be found in these books and numerous reviews (Sjövall and Axelson, 1982; Shackleton et al., 1990; Wolthers and Kraan, 1999; Griffiths et al., 2005). Generally, the most useful derivatives of hydroxyl groups are trialkylsilyl ethers, of carboxyl groups methyl esters, and of carbonyl groups alkyl oximes. Trimethylsilyl (TMS) ethers are most commonly used because of their ease of preparation. Numerous methods and reagents have been described since the first report of their use in steroid analysis (Luukainen et al., 1961). References to reaction conditions useful for derivatisation of steroids are given in Table 2.22. Depending on their position and orientation, hydroxyl groups will react at different rates. This can be used to advantage in structure determinations, but normally conditions giving complete silylation are desired. Side reactions giving rise to artifacts are not common but should be kept in mind, especially when steroids containing an oxo group are reacted under forceful conditions. It is a drawback that the molecular ion of polyhydroxysteroid TMS ethers may not be seen because of extensive loss of trimethylsilanol giving $[M-90]^+$ ions. Ethyl-, propyl-, or t-butyldimethylsilyl (t-BDMS) ethers may then help to define the molecular ion since loss of the alkyl group increases with the size of the group with possible appearance of an M-alkyl ion. However, steric hindrance may prevent reaction of bulky alkylsilyl reagents with hydroxyl groups in many positions. This can be used to advantage for preparation of mixed TMS-alkyldimethylsilyl derivatives as an aid in the interpretation of spectra and in quantitative analytical work.

Depending on reagent and conditions, oxo groups may react to form enol ethers. This has found special applications since molecular ions of enol-TMS ethers are usually abundant and fragmentation may be characteristic (Chambaz et al., 1973). However, it is often difficult to obtain single derivatives quantitatively, which complicates analyses of mixtures. Oxo groups are therefore usually converted into methyl oximes (MO) when biological mixtures are analysed. Thenot and Horning

Table 2.21 Convenient methods for partial or complete conversion of hydroxyl and oxo groups into silyl derivatives. The possibility of side reactions under certain conditions should be considered (modified from Griffiths et al., 2005)

Hydroxyl/oxo groups	Reagent mixture[a]	References
Primary, equatorial secondary	HMDS/DMF	Eneroth et al. (1966)
Most secondary	HMDS/TMCS/Pyr	Makita and Wells (1963)
Some tertiary, ketones partially[b]	BSTFA/TMCS	Poole (1977)
All hydroxyls	TMSIM[c]	Evershed (1993)
As for TMSIM but slower	EDMSIM and *n*-PDMSIM[c]	Evershed (1993); Miyazaki et al. (1977)
All hydroxyls, ketones partially[b]	TMSIM/TMCS[c]	Evershed (1993)
Most ketones, all hydroxyls	MO HCl/Pyr-TMSIM[c,d]	Thenot and Horning (1972a, b)
Most hydroxyls and ketones[b]	MSTFA/NH_4I/ET	Evershed (1993); Donike (1969); Thevis et al. (2001)
Unhindered hydroxyls	TBDMCS/IM/DMF[c]	Evershed (1993); Kelly and Taylor (1976); Phillipou et al. (1975)
Unhindered hydroxyls	MTBSTFA/TBDMCS	Evershed (1993); Donike and Zimmerman (1980)

[a] Temperatures, heating times and reagent proportions determine extent of reaction. Unless indicated by [c], the reagents can be removed under a stream of nitrogen.
[b] Ketones form enol ethers.
[c] Following the reaction, the nonvolatile reagents and by-products are rapidly removed on a minicolumn of lipidex 5,000 in hexane (Axelson and Sjövall, 1977).
[d] The oxo groups are first converted into methyl oximes.
Abbreviations: HMDS, hexamethyldisilazane; TMCS, trimethylchlorosilane; BSTFA, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide; TMSIM, trimethylsilylimidazole; EDMSIM, ethyldimethylsilylimidazole; *n*-PDMSIM, *n*-propyldimethylsilylimidazole; MO HCl, methoxyammonium hydrochloride; MSTFA, *N*-methyl-*N*-trimethylsilyltrifluoroacetamide; TBDMCS, *t*-butyldimethylchlorosilane; MTBSTFA, *N*-methyl-*N*-*t*-butyldimethylsilyltrifluoroacetamide; ET, ethanethiol; DMF, dimethylformamide; Pyr, pyridine.

performed detailed studies of the preparation of MO-TMS derivatives of natural and synthetic steroid hormone metabolites (1972a, b). Except for the formation of *syn* and *anti* isomers, which may separate in GC/MS analyses depending on the stationary phase, their methods usually provide single derivatives. Unsubstituted oximes can be converted to trialkylsilyl ethers which can be an advantage in quantitative analyses by providing intense molecular ions or ions due to loss of the alkyl group.

For GC/MS analyses, carboxyl groups are commonly converted into methyl esters. Many methods have been described, but side reactions depending on the steroid structure are often neglected. The mildest methylation is achieved with diazomethane. By using the commercially available trimethylsilyldiazomethane as a reagent, the preparation of diazomethane from toxic precursors can be avoided. Artefactual formation of ethyl and/or trimethylsilyl esters is sometimes seen due to transesterification or incomplete methylation, respectively. Using these methods of

derivatisation it is also possible to analyse steroid conjugates with neutral and acidic sugars by GC/MS.

Derivatisation can also be a means of introducing a functional group with special mass spectrometric properties e.g. suitable for detection by chemical ionisation. Hydroxyl groups may be converted into perfluoroacyl esters and carbonyl groups into perfluorobenzyl oximes (see Section 2.5.2). These have found use in quantitative GC/MS analysis but are not suitable for polyfunctional steroids because of partial reactions. It is also a disadvantage (unless used for precursor-ion scanning) that the property on which detection is based is located in the derivatising moiety and thus not specific for the steroid. The reactions are also sensitive to water and side products may disturb the analyses.

2.5.7 General Patterns of EI Fragmentation of Derivatised Steroids

There is a vast literature on EI mass spectra and fragmentation mechanisms of steroids and their derivatives. Detailed information can be obtained in numerous books and reviews (Zaretskii, 1976; Brooks and Gaskell, 1980; Budzikiewicz, 1980; Elliott, 1980; Gerst et al., 1997; Griffiths et al., 2005). Chemical ionisation of steroids has been also been reviewed (Lin and Smith, 1984).

The four most general types of fragmentation are: loss of a methyl group (angular or from a TMS group), loss of hydroxyl groups as water (or its equivalent in derivatives), loss of the side-chain, and loss of part of or the entire D-ring. Loss of the A-ring can also give ions useful for the localisation of substituents. These losses are seen in different combinations and the intensities of the fragment ions vary depending on the positions and orientation of substituents and the type of derivative. Positional isomers can usually be distinguished when suitable reference spectra are available (except in the case of double bonds that may isomerise in the ion-source), and stereoisomers often give spectra in which relative intensities of common ions differ reproducibly.

TMS ethers of polyhydroxysteroids and steroids with vicinal hydroxyl groups often lose the last trimethylsiloxy group without a hydrogen {–(CH_3)SiO, –89 Da}. TMS ethers of steroids with a primary hydroxyl group (at C-18, C-19, or C-21) give ions at *m/z* 103 $[CH_2OTMS]^+$ and/or $[M-103]^+$. In most cases an ABCD-ring ion is seen at an *m/z* value determined by the number of double bonds after loss of all hydroxyl functions and the nature of remaining substituents on the ring system (e.g. at *m/z* 257, 255, 253 and 251 with 1–4 double bonds, respectively, and at 14 Da higher for each oxo or methyl group present). Free oxo groups are more clearly seen (giving discrete ABCD-ring ion peaks) and localised than their methyloxime derivatives. In the case of sterols with a double bond in the side-chain, the loss of the side-chain is often accompanied by loss of two nuclear hydrogens resulting in an ABCD-ring ion 2 Da lower than for a saturated analogue. In some cases two ABCD ions are formed with or without transfer of two nuclear hydrogens (Griffiths

et al., 2005). Steroids without a side-chain (androgens, estrogens) will give ABCD-ring ions 1 Da heavier than those from steroids with a saturated side-chain. Spectra of TMS ethers show typical peaks at *m/z* 73 and 75.

TMS residues tend to migrate more readily than other functional groups. This can be both an advantage and a disadvantage in structure determinations. Steroids with more than one TMSO group often give an ion at *m/z* 147 $[(CH_3)_3SiOSi(CH_3)_2]^+$ which is particularly prominent for vicinal OTMS structures (Sloan, et al., 1971). Estrogens and androgens with two TMSO groups in positions 15–18 give an ion at *m/z* 191 $[(CH_3)_3SiO{-}CH{=}O^+Si(CH_3)_3]$ due to migration of a TMSO group (Gustafsson, et al., 1969). For example, *m/z* 191 is the base peak in spectra of C_{18} and C_{19} steroids with three TMSO groups at positions 15α, 16α, and 17β. An ion at *m/z* 191 is seen in spectra of many steroids with closely located TMSO groups but the steric requirements for the formation of the ion remain to be determined.

TMS groups may migrate within the steroid skeleton or between the side-chain and the skeleton. An example is the loss of 56 Da from $[M]^+$ of TMS ethers of 3β-hydroxy-Δ^5-steroids also having a carbonyl group in the D-ring or side-chain. Isotope labelling indicated transfer of the TMS group, probably to C-6, with loss of C-1–C-3 with the oxygen (CH_2=CHCH=O) (Björkhem et al., 1973). TMS ethers of isomers of 20-hydroxy-5α-pregnan- and -5-pregnen-3-one steroids give fragment ions at $[M{-}44]^+$ and $[M{-}59]^+$ due to loss of CH_3CHO and $CH_3CHO{+}CH_3$ with migration of the TMS group to the charged fragment (Smith et al., 1976). A corresponding Δ^{16} steroid loses the entire side-chain and D-ring with migration of the TMS group to the charged ABC ring $[M{-}156{+}73]^+$. In addition to the *m/z* 103 $[CH_2OTMS]^+$ typical of TMS ethers of primary alcohols, the TMS ether of a 17-oxo-18-hydroxy-C_{19} steroid gives a peak at $[M\text{-}CH_2O]^+$ due to TMS migration probably to the oxo group (Smith et al., 1976). TMS ethers of 3-oxo-Δ^4-steroids with a hydroxyl group in a C_8 side-chain give an ion at *m/z* 196 which arises by a combination of the TMS group from the side-chain with a ring A fragment of 123 Da produced by cleavage through ring B. The importance of the carbonyl group is evident from the absence of migration in the analysis of the analogous methyl oximes. Table 2.22 lists common fragment ions and losses seen in mass spectra of TMS and MO-TMS derivatives of C_{19} and C_{21} steroids (Griffiths et al., 2005).

The effect of derivatisation can be seen in the spectra of testosterone and its trimethylsilyl derivatives: with the increasing number of TMSO groups, the stability of the molecular ion grows and its fragmentation declines thus making the spectrum clearer (Fig. 2.61).

2.5.8 Chemistry of EI Fragmentation of Steroids

Though the elemental composition of all fragments can be determined using high resolution mass spectrometry, it is still useful to understand the general patterns of carbonium ion decomposition which are reflected in the distribution of fragment ions in steroid EI mass spectra. On ionisation, the electron with the lowest ionisation

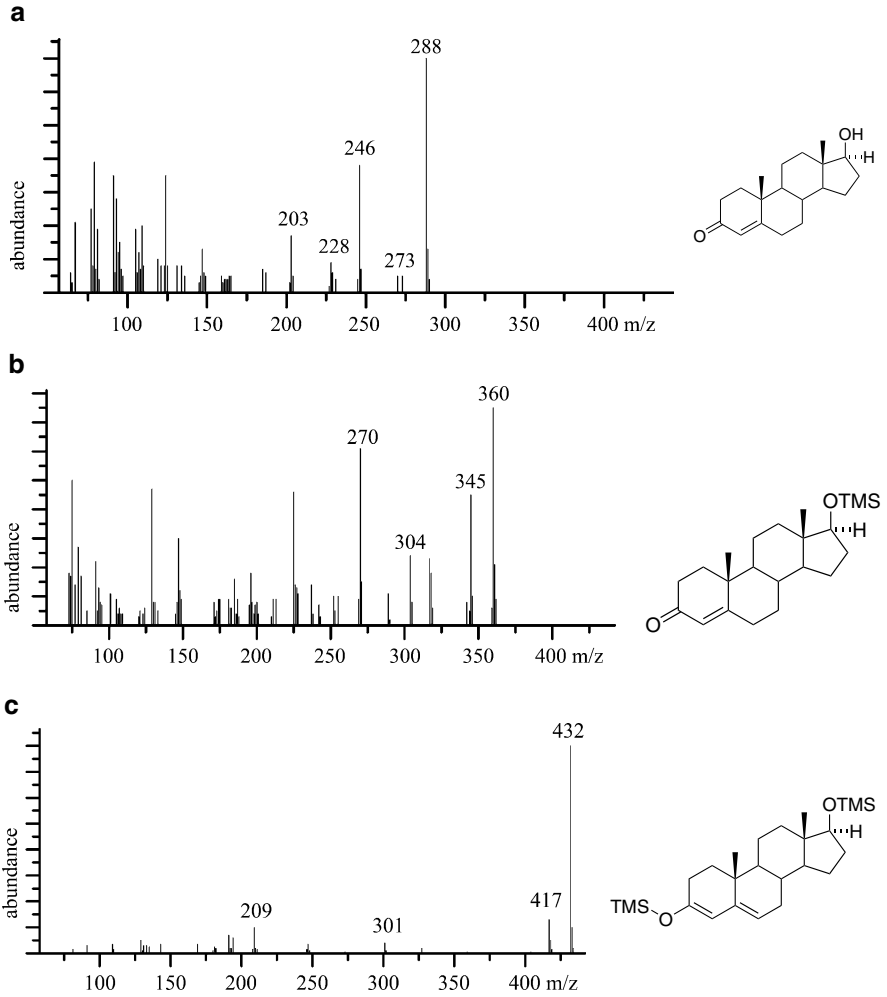


Fig. 2.61 Mass spectra of testosterone before and after derivatisation. (**a**) Testosterone; (**b**) 17β-trimethylsilyloxyandrost-4-en-3-one; (**c**) 3,17β-bistrimethylsilyloxyandrosta-3,5-diene

potential (IP) is lost and the so called "molecular ion" is formed, this is usually a radical-cation $[M]^{+\cdot}$. Radical reactions of individual ions are seldom one-step reactions; the ions often rearrange, dissociate and rearrange again and further dissociate. General rules apply to the stability of the molecular ions formed, i.e. a π-system usually has a lower IP (i.e., is more easily ionised) than a σ-system; a conjugated π-system has a lower IP than an isolated π-system; non-bonded electrons of a heteroatom have a lower IP than a π-double bond. In summary, we can say that the ease of ionisation falls in the following sequence: free electron pair in a heteroatom (halogen, O, S) > C=C–C=C > C=C > C–C > C–H. The resultant molecular ions tend to undergo

three different modes of reactions: *homolytic* cleavage, *heterolytic* cleavage and *rearrangement*. In a *homolytic* cleavage, the β-bond to the charged centre dissociates, one electron remains with the β-carbon (thus creating a new, smaller radical), the other electron shifts towards the charge centre, thus forming a more stable ion with an even number of electrons (no longer a radical, see Eq. 2.15) (Brooks, 1979).

$$-C-C-X^{+\cdot} \rightarrow -C^{\cdot} + C{=}X^{+} \tag{2.15}$$

In a *heterolytic* fragmentation, both electrons from the C–X bond shift to the charge-site (e.g., the heteroatom), in the spectrum we then see the signal of the resultant ion, while the heteroatom, being a radical, is not detected (Eq. 2.16):

$$-C-C-X^{+\cdot} \rightarrow -C-C^{+} + X^{\cdot} \tag{2.16}$$

Rearrangements are numerous (see also Section 2.5.7), often a C–H bond dissociates homolytically, rearranges, and gives products more stable than the molecular ion. The charge may either remain at the original heteroatom (Fig. 2.62, mode a) or may move to a γ-position (Fig. 2.62, mode b) (see also Fig. 2.63).

Because of the complexity of the various types of fragmentation, there is a great difference between interpretation of mass spectra and other types of spectra; in infrared spectroscopy for example, a functional group at one end of the steroid molecule has always the same signals (the same frequency and intensity) regardless of the substitution at the other end, this is not true for mass spectra. For instance the presence or absence of a double bond between C-5 and C-6 decides whether the side-chain will be lost from the position C-17 or not (see Fig. 2.64) (Brooks, 1979).

Thus the EI spectrum of cholesterol shows a prominent signal of the steroid nucleus after the loss of the side-chain as a radical (Fig. 2.65).

Some Important Types of Fragmentation In reality, steroid samples studied are seldom simple hydrocarbons, nevertheless, we may start our survey of steroid fragmentation with the mass spectrum of 5α-androstane. Having no better sites to capture an electron, it is the C–C bonds in the A- and D-rings which are homolytically cleaved (Fig. 2.66). Two pathways of fragmentation prevail, in both a positive

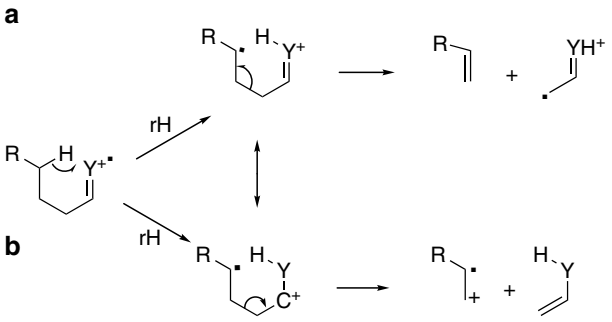


Fig. 2.62 Fragment-ion formation by rearrangement. rH means migration of a hydrogen atom

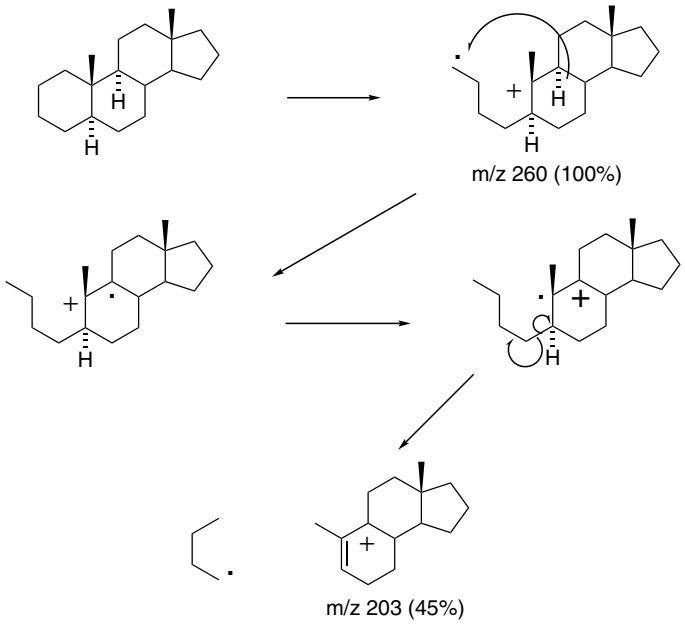
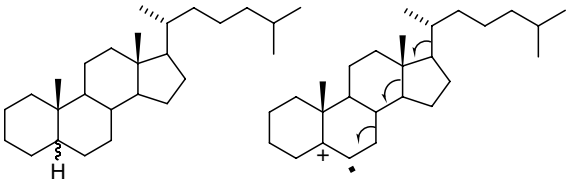


Fig. 2.63 The major fragment formation in a spectrum of 5α-androstane



	$[M]^{+\bullet}$	[M-side chain]$^+$
5α -cholestane	28	1
5β -cholestane	33	1
cholest-5-ene	100	59

Fig. 2.64 Effect (%) of unsaturation on cleavage of the side-chain

charge at a tertiary carbon (C-10 or C-13) is formed initially. The two initially formed radical cations (*m/z* 260) lose neutral fragments and yield cations of *m/z* 217 and 203 in comparable yields.

When a *hydroxyl* group is placed in position C-17, the D ring cleavage prevails and two major fragment ions are found in the spectrum besides the molecular ion (*m/z* 276) (Fig. 2.67).

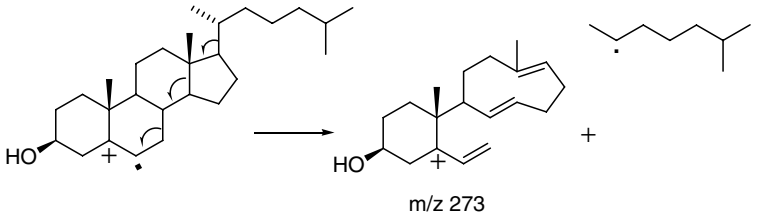


Fig. 2.65 Major fragment ion in a mass spectrum of cholesterol

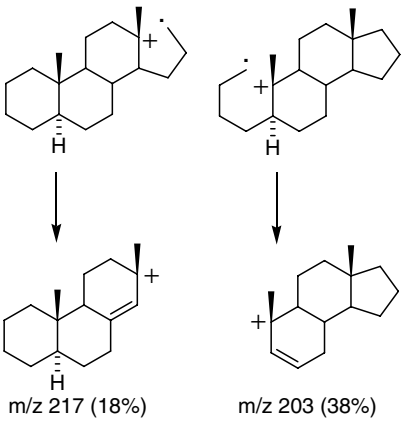


Fig. 2.66 Initial fragmentation of 5α-androstane

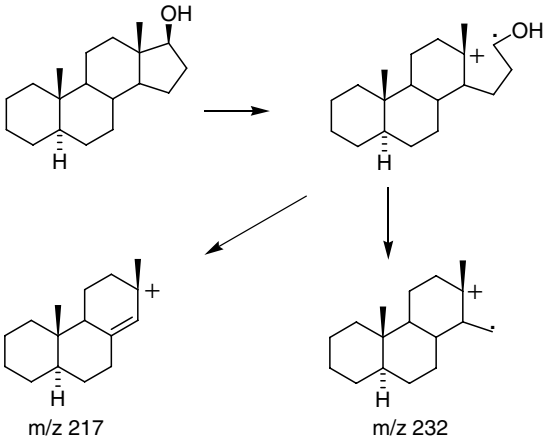


Fig. 2.67 Fragmentation of 5α-androstan-17β-ol

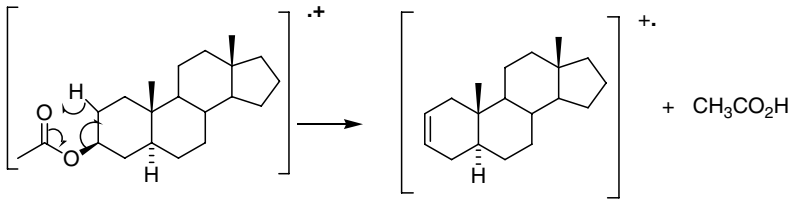


Fig. 2.68 Initial fragmentation of 5α-androstan-3β-ol, 3-acetate

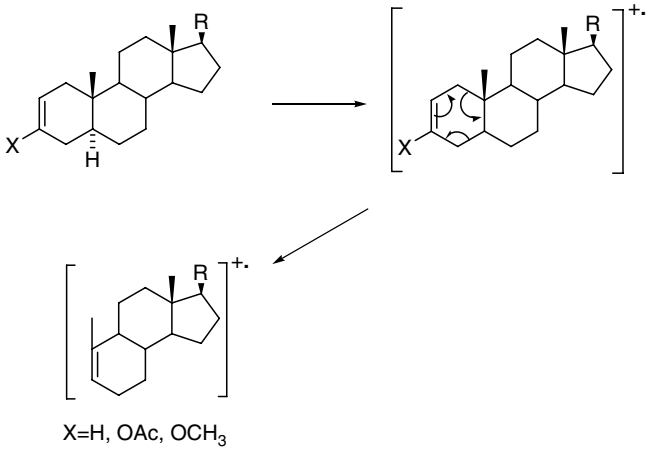
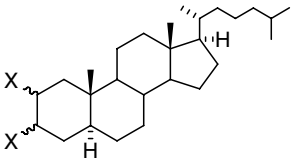


Fig. 2.69 Retro Diels Alder reaction in some Δ^2-steroids

Steroid alcohols are often found as *acetates*. The acetoxy group is often cleaved from the molecular-ion, as the lone pair of the ester group has a low IP and thus is preferentially ionised and can initiate fragmentation (Fig. 2.68).

A double bond is a very frequent grouping in steroid samples. EI of an *olefin* may initiate various rearrangements: Δ^1 and Δ^3-olefins lose the D ring as the saturated analogue above (Fig. 2.67), with no apparent effect of the double bond. On the other hand, the Δ^2-isomer undergoes a retro Diels Alder reaction producing neutral butadiene and a $[M-C_4H_6]^{+\cdot}$fragment-ion (Fig. 2.69). 3α-Enol esters and 3α-enol ethers also lose the equivalent modified butadiene.

A *halogen* atom in a steroid molecule is easily ionised by EI. Usually it is released from the molecule as a neutral hydrogen halide, which is formed by 1,3-elimination and involves the hydrogen atom at a more substituted carbon atom. An axial halogen cleaves from the molecular ion more easily than an equatorial one. Vicinal 2,3-dihalides eliminate a neutral molecule of X_2 leaving behind a charged steroidal Δ^2-olefin, which can then undergo the retro Diels Alder reaction, and additionally fragment in the D ring (Fig. 2.70).



Compound	$(M)^+$	$(M - X_2)^+$	$(M - D\ ring)^+$	$(M - C_4H_6)^+$
X = axial Cl	53	20	100	8
X = axial Br	37	82	30	37
X = equatorial Br	33	45	47	24

Fig. 2.70 Abundance (%) of fragments in spectra of 2ξ,3ξ-dihalo-5α-cholestanes

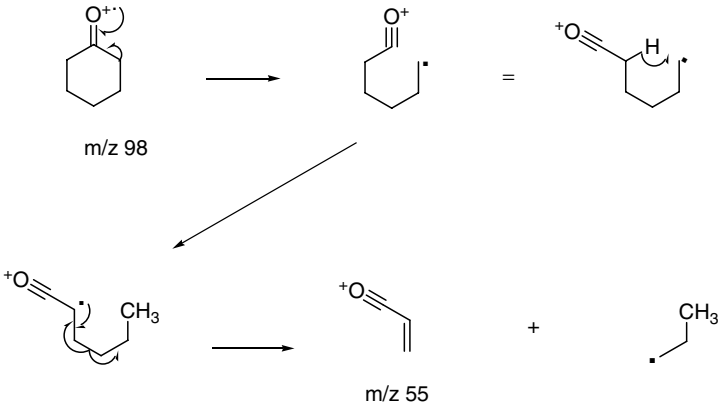


Fig. 2.71 Cyclohexanone rearrangement

Oxo groups are frequently present in steroids. Most of them undergo a "cyclohexanone-type rearrangement". This starts with the α-cleavage and ends with the formation of ions of *m/z* 55 and 98 (Fig. 2.71).

The fragmentation reactions of many steroidal cyclohexanones have been studied using labelled substrates, a few examples are given below. In simple mono ketones the following patterns of fragmentations are found.

The oxo group in 5α-androstan-1-one is ionised and rupture of the C-1–C-10 and C-4–C-5 bonds occurs giving an ion of *m/z* 203 (Fig. 2.72). Alternatively, the molecular ion (*m/z* 274) may lose water (*m/z* 256), or the neighbouring methyl group (C-19) alone (*m/z* 259), or with carbon monoxide (*m/z* 231).

In a parallel process, 1-oxo compounds fragment with rupture of the C-9–C-10 bond which leads to fragments consisting of the former A ring (*m/z* 111 and 124) (Fig. 2.73).

2-Oxo-5α-cholestane undergoes the α-cleavage leading eventually to formal expulsion of acetone. The $[M–58]^+$ fragment is the base peak in the spectrum. Experiments with labelled samples proved a more complicated pathway in which a C-1 cation attracts a proton from position 9α. 3-Oxo-5α-androstane gives a fragment-

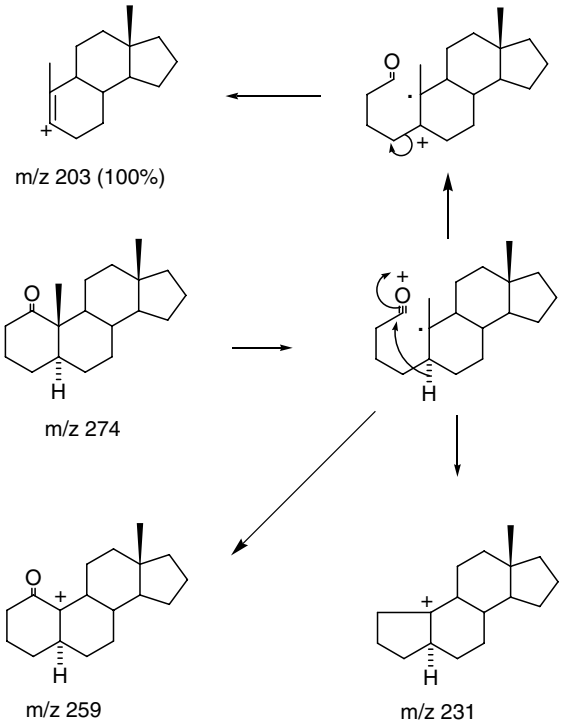


Fig. 2.72 Fragmentation of 5α-androstan-1-one

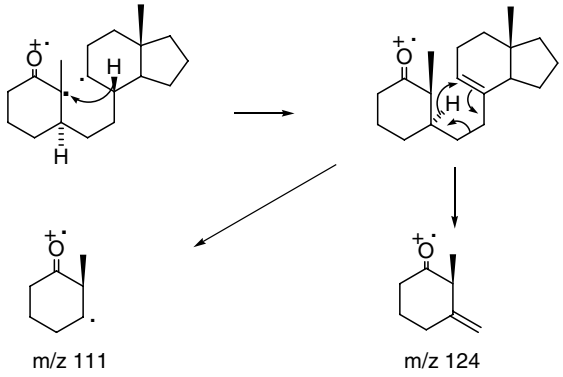


Fig. 2.73 Low molecular weight fragments of 5α-androstan-1-one

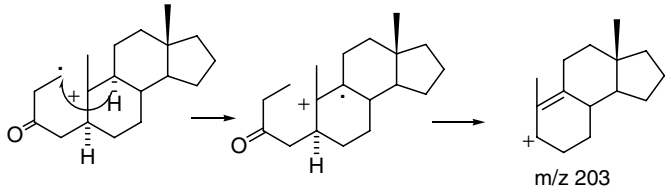


Fig. 2.74 Fragmentation of 5α-androstan-3-one

ion $[M–C_4H_8O]^+$ as the most prominent peak in its spectrum. Apparently, rupture of the C-1–C-10 bond occurs initially, which initiates cleavage of the A ring. The base peak is accompanied by a strong signal due to the $[M–C_4H_7O]^+$ fragment-ion (Fig. 2.74).

It is interesting to compare the fragmentation patterns of 5α-androstan-3-one with its ketal. The ethylendioxy group is a useful protection group in organic synthesis and a useful derivative for mass spectrometric characterisation where it can prevent random hydrogen transfer (Budzikiewicz et al., 1967). Figure 2.75 shows that 3,3-ethylenedioxy-5α-androstane undergoes two α-cleavage pathways producing 2,3-*seco* and 3,4-*seco* intermediates (Audier, 1973). Subsequent hydride shifts initiate rearrangement and cleavage which produces characteristic fragments at *m/z* 99 and 125.

7-Oxo-5α-androstane has two prominent fragments in its spectrum at *m/z* 135 and *m/z* 178 (100%). α-Cleavage opens the C-7–C-8 bond which then produces a cation formed of the C and D rings (*m/z* 135). Alternatively, the C-9–C-10 bond ruptures and initiates expulsion of the neutral A ring with a cation formed consisting of the rest of the molecule (*m/z* 178) (Fig. 2.76).

11-Oxo-5α-androstane is cleaved in the B-ring, yielding a 9,10-*seco* radical. As in the above examples, hydrogen migration and rupture of the C-6–C-7 or C-5–C-6 bonds produced two prominent fragments which are remnants of the former C and D rings (Fig. 2.77).

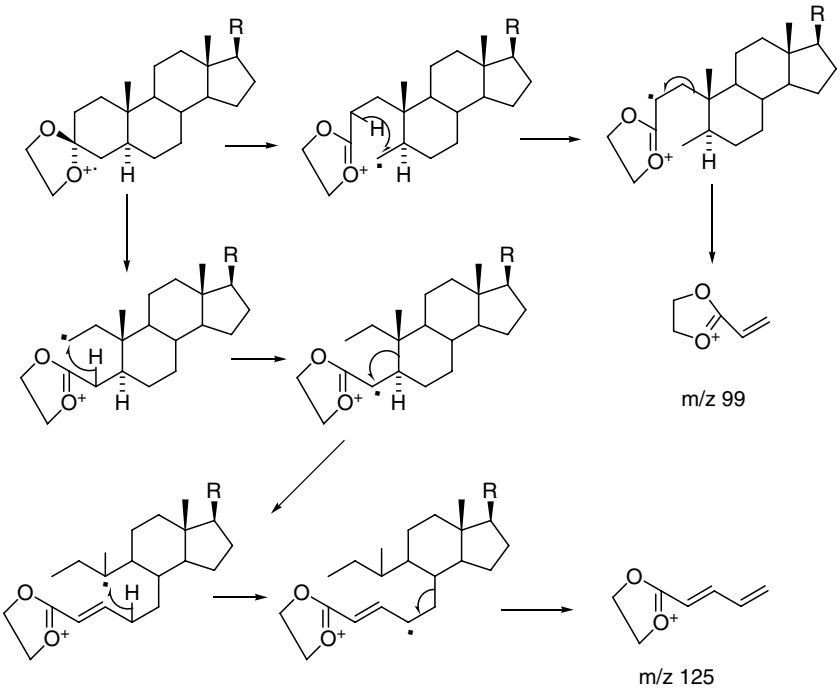


Fig. 2.75 Characteristic fragments of EI spectra of 3,3-ethylenedioxy derivativives

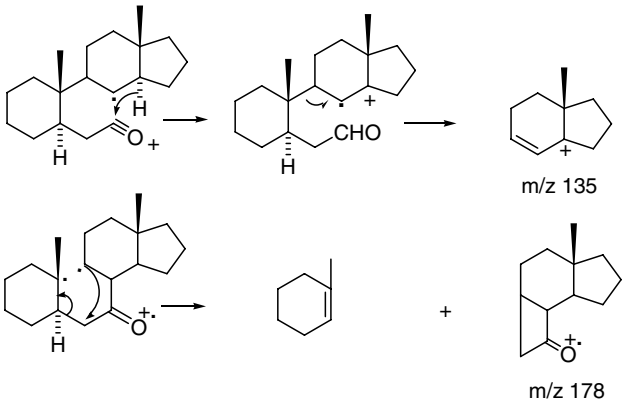


Fig. 2.76 Fragmentation of 5α-androstan-7-one

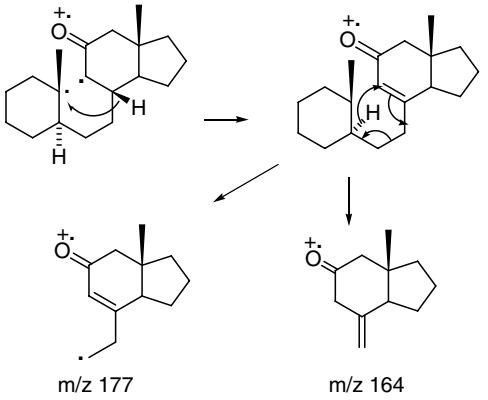


Fig. 2.77 Fragmentation of 5α-androstan-11-one

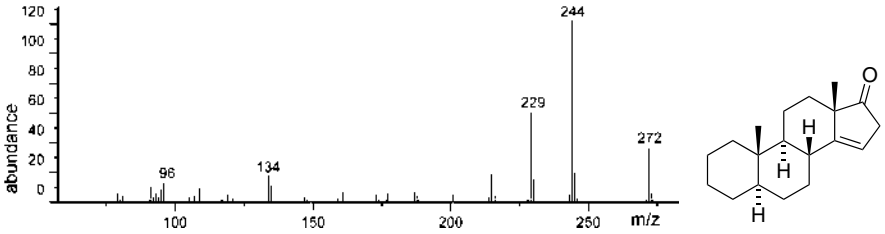


Fig. 2.78 Mass spectrum of 5α-androst-14-en-17-one

17-Oxosteroids are the most frequently quoted representatives of steroidal cyclopentanones. Their fragmentation also starts with the α-cleavage. For instance, in 5α-androst-14-en-17-one ($[M]^{+\cdot}$ *m/z* 272), the oxo group gets ionised and the α-bonds (C_{13}–$C_{17}$and C_{16}–C_{17}) are broken: a neutral fragment (C=O) is split leaving

the positive ion at the steroidal rest (M^+-28 *m/z*). Splitting off of the C18-methyl group leads to the 229 *m/z* fragment. The 96 *m/z* fragment corresponds to methylcyclohexene which was formed from the A ring (Fig. 2.78).

16-Oxosteroids start their fragmentation with the release of the C18-methyl group leading to a relatively stable 13(17)-en-16-one system (the H-17 migrated to the carbonyl group which holds a positive ion). The other part of the molecule – the unsaturated A ring – releases butadiene and the product of the retro-Diels Alder reaction (203 *m/z*) is the base peak in the spectrum. The direct retro-Diels Alder reaction of the molecular ion affords the second most prominent peak at *m/z* 218 (Fig. 2.79).

The above spectra and their description should be taken as examples illustrating the diversity of fragmentation patterns and rearrangements that occur in steroids. As a guide to spectra interpretation it is wise to consider the point where ionisation is most likely to initially occur and which rearrangements are most likely to follow.

Carboxylic acids in the steroid series are mostly represented by bile acids. Their hydroxyl groups are often acetylated, and in their mass spectra fragments corresponding to their elimination products (olefins) form major fragment ions. An intense ion is usually formed by elimination of the whole side-chain (Fig. 2.80).

The above discussion of the EI fragmentation of steroids, serves to illustrate the diversity of possible reactions. For more detailed information concerning EI spectra of steroids the interested reader should consult reviews and books by Budzikiewicz et al. (1964), Budzikiewicz (1972), Elliott (1972), Engel and Orr (1972), Zaretskii

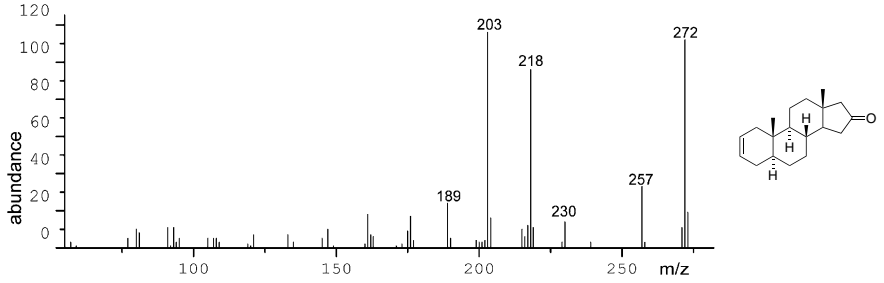


Fig. 2.79 Mass spectrum of 5α-androst-2-en-16-one

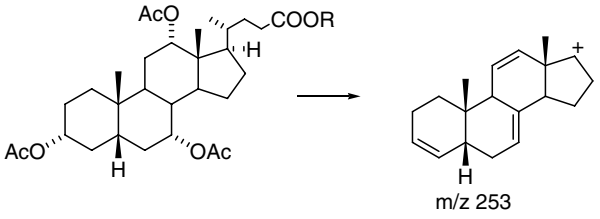


Fig. 2.80 Fragmentation of cholic acid triacetate

(1976), Djerassi (1978), Budzikiewicz (1980), Elliott (1980), Brooks and Gaskell (1980), Gerst et al. (1997), Griffiths et al. (2005).

2.5.9 *Interpretation of EI Spectra*

Even simple EI spectra can afford considerable information, the most important of which is the compound molecular weight, provided by observation of the $M^{+\cdot}$ ion. Exact mass measurement on this ion can provide its elemental composition. However, the molecular ion may not be present in the EI spectrum, but can often be inferred from the fragmentation pattern. For example the observation of $[M-90]^+$ and $[M-180]^+$ in the EI spectra of TMS ethers of dihydroxysteroids indicates the mass of the molecular ion. Alternatively, this information can be provided by the acquisition of CI, ES, or FAB spectra.

Simple EI spectra recorded at low resolution can be used to calculate the number of carbon atoms in an unknown sample, this is a consequence of the natural abundance of the ^{13}C isotope in nature. The number of carbon atoms (X) can be calculated from the relative abundance of a molecular peak (I_A) and its first isotopic signal (I_{A+1}) as follows:

$$X = (I_{A+1} / I_A) \times (89.9) \tag{2.17}$$

Similarly, low resolution spectra suffice to answer the question of how many double bonds or rings (i.e., a double bond equivalent, DBE) exist in the molecule analysed.(2.18)

$$\mathbf{DBE} = \boldsymbol{X} - (\mathbf{0.5} \times \boldsymbol{Y}) + (\mathbf{0.5} \times \boldsymbol{Z}) + \mathbf{1} \tag{2.18}$$

where X = number of carbon atoms, Y = number of hydrogen atoms, or halogens, and Z = number of nitrogen atoms. For example 5α-cholestan-6-one gives a molecular ion (*m/z* 386) with relative abundance (100%) and 30% for the first isotopic peak. According to Eqs. 2.17 and 2.18),

$$\mathrm{X} = (30 / 100) \times (89.9) = 26.97(\text{i.e. } 27)$$

and

$$\mathrm{DBE} = 26.97 - (0.5 \times 46) + 1 = 5$$

In reality, the sample consists of four rings and one oxo group.

Steroids, particularly when studied by GC–MS, are analysed in derivatised forms and general patterns of EI fragmentation of derivatised steroids are discussed in Section 2.5.7. Given in Table 2.22 are the common fragment ions and losses seen in mass spectra of trimethylsilyl (TMS) and methyloxime (MO)-TMS derivatives of C_{19} and C_{21} steroids. Fragment-ion information in combination with molecular weight information can enable structural identification. Today, however, the

Table 2.22 Common fragment ions and losses seen in mass spectra of trimethylsilyl (TMS) and methyloxime (MO)-TMS derivatives of C_{19} and C_{21} steroids (modified from Griffiths et al., 2005)

Fragment ion or loss	Fragment composition or origin	Structures giving designated ions
–29	C_2H_5	3,6-TMS; 3-TMS-6-one
–30	CH_2O (C-18 or -19)	19-TMS-3-one
		18-TMS-17-one
–43	SC	20-one
–44	C-11,12	15-or 20-TMS-11-one
–46	D-ring	11- or 16-TMS-17-MO
–47	A-ring	6-TMS-4-ene-3-MO ($C_{19}C_{21}$)
–56	C_3H_4O (C-1,2,3)	3-TMS-5-ene-17-, 11- or 20-one (C_{21} ,C_{19})
		6-TMS-4-ene-3-one (C_{21})
–59	SC D-ring	7- or 8-ene-20-MO
–71		4-ene-3-one-16-TMS
–85, 86	SC D-ring	20-one
–86		15-TMS-17-MO
87		17-MO (C_{19})
–99 –86	SC D-ring C-16,-17	20-MO
100,87,70	SC D-ring C-16,-17	20-MO
103, –103	CH_2OTMS	18-, 19-, or 21-TMS (C_{19}, C_{21})
116, 117		17-ol-20,21-TMS
		4-ene-3-one,16-TMS
116 or 117		15- or 16-TMS-17-one
117	SC	20-TMS
–117		17,20-TMS
–116	C_2H_3OTMS	1- or 2-TMS-4-ene-3-one
124	$C_8H_{12}O$	(4-ene)-3-one
125		3,11-TMS-17-MO
125, 137, 153	A-ring	4-ene-3-MO
126	SC D-ring	7-or 8-ene-20-MO
129, –129	C_3H_4OTMS	5-ene-3-TMS
		17-TMS(C_{19})
		2,3-TMS
		some 3,6-TMS; 3-TMS-11,17-one
–131		7,17-TMS or 18-TMS-17-one(C_{19})
		15,16-TMS-17-one
		12,17-TMS(C_{19})
	SC	21-TMS-20-one
133		1-TMS-4-ene-3-MO
		15- or 16-TMS-17-MO(C_{19})
		17-TMS-16-MO(C_{19})
138	A-ring, C-19	5β-3,6-MO
142, 143	$C_4H_{5(6)}$ OTMS	4-ene-3-TMS; 3-enol-TMS;2,3-TMS; (1,3-TMS)
–142		4-ene-3-TMS
143		3,11-TMS; 7-or 8-ene-3-TMS;15- or 16-TMS-17-one
–143		4-ene-3,6-TMS; 6-TMS-3-one
–144		3-TMS-11-one (C_{21})
		15 or 16-TMS-17-one

(continued)

Table 2.22 (continued)

Fragment ion or loss	Fragment composition or origin	Structures giving designated ions
		17-TMS-16-one (C_{19})
−145		3,6(β)-TMS
147	$(CH_3)_2SiOTMS$	Di- and poly-TMS (vicinal)
−147		15-TMS-17-MO
−152		18,21-TMS-20-MO
156, 184, 199		3,11-TMS-17-one
156, 188	SC D-ring	16-TMS-20-MO
156, 158, 188	SC D-ring	17-TMS-20-MO (C_{21})
157, 159, 172, 186	SC D-ring	16-TMS-20-one
158	D-ring	18-or19-TMS-17-one(C_{19})
		17-TMS-16-MO(C_{19})
158, 174	D-ring	16-TMS-17-MO (C_{19})
−159	SC C16,17	15,16-TMS-20-one
161		3,6-TMS(C_{21})
		11,21-TMS
169		18-TMS-17-one
169, 182		11,17-TMS(C_{19})
170, 201	SC D-ring	15-TMS-20-MO;20-TMS-16-MO
−171	D-ring	15-TMS-20-one
172	D-ring	15- or 16-TMS-20 one
174, −174	D-ring	16-TMS-17-MO
		17-TMS-16-MO
		16,18-TMS-17- MO(C_{19})
		11,21-TMS-20-one; 15,21-TMS-20-one
175, 188	SC D-ring	21-TMS-20-MO
−187	SC D-ring	21-TMS-20-MO
−188	SC D-ring	15,21-TMS-20-MO
191	$CH(OTMS)_2$	(11,15,16,17,18)-di(tri)-TMS(C_{21})
		C_{21}-poly-TMS
−193	D-ring	16,17-TMS (C_{19})
196		16-TMS-17-one
196, 271		1,3-TMS-11-one(C_{21})
205		16,17-TMS(C_{19})
−205	SC	17,20,21-TMS
217 (218, 219)	$C_3H_3(OTMS)_2$	1,3-TMS, 15,(18),17-TMS(C_{19})
223	C,D rings	6,17-TMS(C_{19})
234[a]	$C_9H_{22}O_3Si_2$	17,20-TMS-21-COOMe
243	$C_5H_3(OTMS)_2$	17,20,21-TMS, 3,7-TMS; 3,5-TMS
−247	SC C,D rings	15,17,20-TMS
258,289	SC D-ring	15,17-TMS-20-MO; 15,21-TMS-20-MO
260	SC D-ring	15,21-TMS-20-one;16,21-TMS-20-one
276	SC D-ring	16,21-TMS-20-MO
276, 246, 244	SC D-ring	17,21-TMS-20-MO
−307		15,17,20-TMS
333	SC C,D rings	15,17,20-TMS

[a]Base peak in methylesters of cortoic acids. fragment formed by Mclafferty rearrangement.
Abbreviations: MO, methoximated carbonyl; TMS, trimethylsilylated hydroxyl; "-One," underivatised carbonyl; SC, side-chain common fragmentations -90,-15 and -31 not included; unless specified otherwise, it is generally assumed that all steroids have a 3-TMS.

identification of compounds from their EI spectra is greatly aided by the use of EI spectral libraries. For steroid chemists the most valuable collection is that of Makin, Trafford and Nolan containing 2,500 spectra.

In summary, mass spectrometry is an indispensable tool in steroid research and medicinal chemistry. GC–MS and LC–MS are used for the routine identification of minute amounts of steroids (pg) in biological samples, while new MS/MS and MS^n technologies are allowing the identification of steroids with novel structures. See Chapter 3 for a full discussion of LC–MS.

2.6 Less Frequently Used Methods of Analysis of Steroids

2.6.1 Raman Spectroscopy

Raman spectroscopy is beyond the scope of this book as the corresponding spectroscope is hardly as widespread in steroid laboratories as other techniques discussed (Gremlich, 2001). It has different selection rules than IR. In principle, an IR spectrum is an *absorption* spectrum: on passing through the sample, the ray loses intensity of some frequencies. The losses correspond to vibration of functional groups present in the sample. Thus, the ensuing ray is modified by the quality of a sample: some colours are more filtered off in intensity than others. On the other hand, a Raman spectrum is a record of light *produced* by the sample when all excited groups are ridding of the added energy. Not always, however, do they emit exactly the same amount of energy, which they gained on irradiation; mostly, they lose some energy beforehand. Thus, the frequency, at which an individual "transmitter" (i.e. a functional group) broadcasts, is usually lower (i.e., less energetic) than the frequency of the given functional group in the standard IR absorption spectroscopy. Many different sample arrangements suitable for Raman spectrometry run under many different conditions (e.g., measurement in a solution, of a crystalline or amorphous sample, of a living tissue). A very simple scheme is shown in Fig. 2.81.

Evidence obtained from IR and Raman spectra is complementary to each other (Parker, 1975). Table 2.23 shows that groups, showing strong signals in one of the methods, often produce weak signals in the other. In general, IR spectra signals are most prominent in cases where a strong dipole is involved (e.g. the O–H stretching vibration), while Raman spectra are more suitable for detection of symmetric bonds (e.g. a CH=CH bond). Thus one of their major advantages is that they are able to detect those symmetrical features of structure, which fail to give IR spectra (Pouskouleli et al., 1983).

Raman spectroscopy is even able to differentiate between diamond graphite and amorphous carbon; it is also good for studying the backbone vibrations of the organic chain C bonds. Crystalline organic compounds yield narrow lines with characteristic patterns caused by the structure of a crystal.

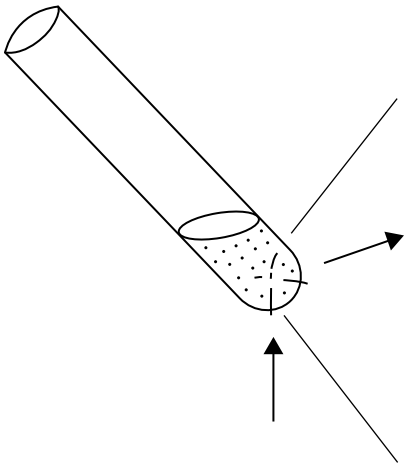


Fig. 2.81 Sample arrangement for Raman spectrometry. A solution is placed in an NMR tube, he axis of which has an angle of 90° relative to the entrance optics. The laser beam comes from the bottom, the scattered radiation is observed within the given conus. So called "Back scattering" can also be used, the angle between the laser and the axis of observed radiation would then be 180° (modified from Schrader, 1995, p. 136)

Table 2.23 Stretching bond frequencies (cm^{-1})

		Frequency range spectra	
Functional group		Raman	IR
ν(O–H)	3650–3000	w	s
ν(N–H)	3500–3300	m	m
ν(=C–H)	3300	w	s
N(=C–H)	3100–3000	s	m
N(–C–H)	3000–2800	s	s
N(–S–H)	2600–2550	s	w
N(C≡C)	2250–2100	vs	w-0
N(C=C)	1820–1680	s-w	vs
N_A(C–O–C)	1150–1060	w	s
ν_S(C–O–C)	970–800	m	W
ν(O–O)	900–845	s	0-w
ν(S–S)	550–430	s	0-w

Another advantage is, that even aqueous solutions of samples and living tissues can be studied (in IR absorption spectra the strong absorption of water would prohibit such measurement even if IR cuvettes survived such experiments). Thus, the technique was used for differentiation between hormone-responsive and hormone-unresponsive tumour cell lines (Beljebbar, 2000; Shafer-Peltier, 2002).

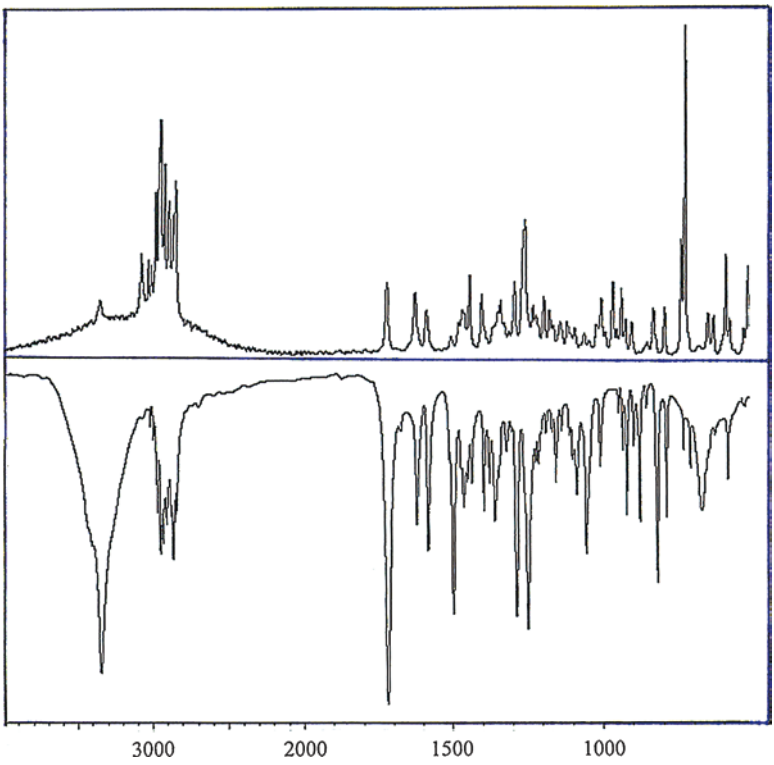


Fig. 2.82 Raman (the *upper half*) and infrared absorption (the *lower half*) spectra of estrone

The drawback of Raman spectroscopy lies in the fact that the presence of impurities producing fluorescence, originally of much higher intensity than that of the emitted radiation, could prohibit a spectrum being taken.

Figure 2.82 shows similarity and differences of both types of spectra. The upper part is the Raman spectrum of estrone, the lower part is its IR spectrum. The most striking difference between the spectra is the intensity of the corresponding peaks: signals hardly observable in one type of the spectra becomes prominent in the other type.

The comparison of the both types of estrone spectra should not give the impression that Raman spectroscopy is limited to the infrared region only. Raman effects are observed in the UV and visible light regions; for analysis of steroids, however, the IR region is optimal.

The classical Raman effect produces only very weak signals. Raman spectroscopy has gone a long way since its discovery by Indian physicist C.V. Raman in 1928. Before the invention of lasers in 1960, radiation emitted by the mercury arc was used for exciting Raman spectra. Today, most types of lasers (‘continuous wave’ (cw) and pulsed, gas, solid state, semiconductor, etc.), with emission lines from the UV to the near-infrared region (NIR), are used as radiation sources for the excitation

of Raman spectra. Especially, NIR Raman spectra are excited mainly with a neodymium doped yttrium-aluminium garnet laser (Nd:YAG), emitting at 1,064 nm (at this wavelength, the spectra are practically free of fluorescence which otherwise represent a practical obstacle). Raman spectra can now be recorded with minimal sample preparation. Raman spectroscopy is capable of non-destructive analysis of any sample, cells and tissues included (Mazurek and Szostak, 2006; Cuffini et al., 2007; DeBeer et al., 2007).

In the past, Raman spectroscopy was not as much used in molecular structure determination as IR absorption spectroscopy (Schrader, 1995) (Steigner and Schrader, 1970), however, the use of lasers and fiber-optic probes have made Raman spectroscopy of steroids more available to everyday needs (Greek et al., 1998; Salmain et al., 2005). Several techniques have lately been discovered, which very successfully enhance the otherwise weak Raman effect. The *resonance Raman spectroscopy* (RRS) where resonance occurs when the photon energy of the exciting laser beam is approximately equal to the energy of an electric dipole allowed transition of a particular chromophore. *The surface-enhanced Raman spectroscopy* (SERS) employs the influence of small metal particles on the elementary process of Raman scattering. These two techniques may even be combined into *surface-enhanced resonance Raman effect* (SERRS). Such spectra are recorded with the same spectrometers as classical Raman spectra, although different conditions of the excitation and special sample techniques are used.

2.6.2 X-Ray Diffraction

In most cases, where the classical steroid skeleton is involved, structural problems were usually solved by more common methods (e.g. combination of MS and NMR spectroscopy), the reason being the inadequate level of technique: in certain cases (e.g. in solving structures of skeletal rearrangement), X-ray diffraction was the last resort. It required a perfect monocrystal of the product and a powerful instrument, which produced raw data on a photographic film that had to be very tediously solved by an experienced crystallographer.

The determination of crystal structures by X-ray crystallography has come a long way since its discovery in 1912. Dorothy Crowfoot Hodgkin became a sole winner of the 1964 Nobel Prize in Chemistry “for her determination by x-ray techniques of the structures of biologically important molecules.” Her first steroid studied was cholesteryl iodide. No longer is “a heavy atom” required for evaluation of the many diffractions. Commercial diffractometers with CCD detectors (“Charge-Coupled Device”) and professional analytical programmes take away a lot of tedious analysis by skilled experts.

Anyway, the method still cannot be used by amateurs and for practical use, a reader should at least understand the basic principle (the concept of reflections, whose geometry and intensities correspond to the position of atoms in the crystal) and then invite a professional for cooperation. Then, he may be asked for a perfect monocrystal

of his sample; often a polarisation microscope would reveal that the "beautiful" – in his eyes – crystal is a mere cluster of crystals only. If the growth of a crystal leads to twins of identical crystals, the problem of interfering diffractions can mostly be solved by adequate software. When several crystals grow independently into a complex product, a better crystallisation procedure will be recommended (Ladd and Palmer, 2003).

The strict demand for a monocrystal seems to clash with recent experience: the X-ray examination of polycrystalline materials has solved complete structure of medium-sized molecules, with up to 60 atoms in the asymmetric unit. The modern developments in the powder method have added a new and powerful tool for the determination of the structure of the many substances that could be obtained *only* in microcrystalline form (particle size ca 10^{-3} m). Nevertheless, in powder diffraction work, fewer reflections are available than with single crystal X-ray crystallography. In addition, the problem of determining the unit cell, indices, space group, and intensities of reflections still make the work much more difficult for a crystallographer.

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Chapter 3
General Methods for the Extraction, Purification, and Measurement of Steroids by Chromatography and Mass Spectrometry

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3.1 Introduction

Steroids consist of an essentially lipophilic (or hydrophobic, non-polar) cyclopentanoperhydrophenanthrene nucleus modified on the periphery of the nucleus or on the side chain by the addition of hydrophilic (or lipophobic, polar) groups. Although steroids are widely distributed in nature and many thousands have been synthesised in the laboratories of pharmaceutical and chemical organisations, this chapter concentrates primarily on the methodology for the analysis of steroids of biological importance to human subjects and in particular on the methods for the analysis of the very low concentrations of steroids found in human biological tissues or formed during *in vitro* or *in vivo* studies. This does not, however, imply that the techniques discussed here may not find applicability in other areas of steroid analysis. This chapter neither discusses specifically the saturation analysis techniques including immunoassay-radioimmunoassay (RIA), enzymeimmunoassay (EIA), which are explained in Chapter 4, nor the analysis of cardenolides, sapogenins, alkaloids, brassinosteroids or ecdysteroids, which present their own analytical challenges but

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are of less interest in a clinical context. Further details on basic principles of mass spectrometry (MS) are discussed in Chapter 2.

During metabolism, steroids generally become more hydrophilic by reduction, further hydroxylation and esterification (conjugation) with glucuronic or sulphuric acid. Bile acids (containing a C24 carboxylic acid group) may be linked through a peptide bond to glycine or taurine. Despite the addition of these polar groups, the essential non-polarity (hydrophobicity) of the steroids means that they are all, to varying degrees, soluble in organic solvents and can thus be extracted from aqueous media by a solvent or solvent mixture of suitable polarity.

In vivo secreted steroid hormones are carried in the bloodstream bound to plasma proteins – specifically to binding globulins, which have high affinity but low capacity (e.g. cortisol binding globulin, sex hormone binding globulin, vitamin D binding globulin, etc.) or non-specifically to albumin, which has low affinity but high capacity. Some steroids (e.g. cholesterol) may be incorporated as an integral part of the structure of the plasma lipoproteins, and these lipoproteins may form part of the structure of cells and tissues from which steroids may need to be extracted.

Steroid hormones exert their *in vivo* effects by binding to specific receptor proteins, which although found in the cytoplasm of responsive cells, may be derived from nuclear receptors that bind avidly to the nuclear chromatin when the active steroid is bound but not when the active steroid is absent. It is well established that sex steroid hormones bind to nuclear receptors, which then act as transcription factors to control biochemical processes. Mounting evidence now indicates that steroids may also influence physiological events more rapidly via non-genomic mechanisms (e.g. Balthazart et al., 2006 and reviewed by Losel et al., 2003; Wehling and Losel, 2006). Direct and indirect action of steroids in mitochondria are new areas of research with relevance to oxidative phosphorylation (Gavrilova-Jordan and Price, 2007; Haider, 2007).

3.1.1 Analysis of Steroids

The presence in tissues and biological fluids of binding proteins and their interaction with the steroid of interest are important considerations when deciding how best to develop a method for the analysis of a particular steroid. The initial question to consider is whether it is possible to assay the steroid directly in the medium without prior treatment; and today, this would appear to be a major objective in the development of tests for routine use in clinical laboratories for diagnostic purposes since such procedures are simple and avoid the need for extraction. Immunoassay tests are often conducted with serum or plasma processed without any extraction or purification of the steroids. Tandem mass spectrometry looks attractive for the analysis of steroids, often with little pretreatment of the sample, but the cost of such apparatus at the present time and the expertise needed may mitigate against its routine use, although leasing arrangements and simplification of operation may

overcome these difficulties. However, when reaching a decision whether to use a direct assay, the specificity of the final quantitation and the requirements of the assay must be taken into consideration. A simple non-extraction assay may provide in many circumstances a semi-quantitative estimate of the concentration of the analyte, which may be of considerable value to clinical colleagues. Although this result may not be accurate, so long as it is precise and reproducible, it may still have value. There have been many reviews of methods of steroid analysis, too many to list here, but readers may find two (Grant and Beastall, 1983; Shackleton et al., 1990b) useful as an introduction to early literature in this area. There are a number of reviews of steroid extraction and separation published since 1999, which may be of use (Wolthers and Kraan, 1999; Shimada et al., 2001; Marwah et al., 2001; Nozaki, 2001; Volin, 2001; Appelblad and Irgum, 2002; Sjövall, 2004; Pujos et al., 2005; Setchell and Heubi, 2006); while some of these reviews restrict their coverage to specific steroids or groups of steroids, they are still useful as the methodology described is almost certainly applicable with modification to other steroids. The relevance of steroid hormone assay in the clinical laboratory has also been reviewed (Holst et al., 2004).

Any analytical procedure has three (or four) major steps:

1. Extraction of the analyte from the matrix.
2. Pre-purification.
3. Quantitation. In some cases, qualitative analysis is sufficient to meet the objectives of the analysis. The detection of synthetic anabolic steroid metabolites in urine of a sports person is thus often sufficient to define the abuse of anabolic steroids (Kicman and Gower, 2003). The list of prohibited steroids continues to grow (van Eenoo and Delbeke, 2006; Borges et al., 2007), and new 'designer' steroids are being found, creating new analytical challenges to doping control laboratories (Nielen et al. (2006)). This topic is discussed in greater detail in Chapter 9.
4. Quality assurance (QA) – both internal and, where possible, external–particularly in laboratories where large numbers of steroid assays are carried out. Excellent external quality assurance for steroid assays is provided to hospital clinical biochemistry laboratories in the UK, and this scheme is discussed in detail in Chapter 13 (see also discussion of a similar scheme for vitamins D metabolites in Chapter 11). It cannot be emphasised too often that good quality assurance is essential and that the maintenance of high standards of output from an analytical laboratory requires scrupulous attention to all quality control safeguards, which should include full information about the patient from whom the sample was taken so that unexpected or unusual results can be more easily detected (Jones and Honour, 2006).

Although blood, urine, and saliva have been the biological fluids most often examined for the presence of steroids, there is increasing interest in steroids in brain (so-called neurosteroids). In addition to the analysis of steroids in tissues and biological fluids, there is an interest in the presence of steroids in unexpected matrices, which are not specifically discussed here. These include

- Nutritional supplements (liquid and solid) that are widely used in sport but have given rise to positive tests in doping control (De Cock et al., 2001; Geyer et al., 2004; Parr et al., 2004; Tseng et al., 2005; Maughan, 2005; Martello et al., 2007; Parr et al., 2007). Methods for the measurement of anabolic steroids in the sports area are explained in more detail in Chapter 9.
- Human hair in which the concentrations of steroids along the hair fibres can be related to the time of exposure (Cirimile et al., 2000; Dumestre-Toulet et al., 2002; Wheeler, 2006; Gambelunghe et al., 2007; Rambaud et al., 2007).
- Water and sewage effluents for the presence of potential endocrine disruptors and as means of assessing contamination (e.g. Sumpter and Johnson, 2006; Szucs et al., 2006) or marine sediment analysis (Hajkova et al., 2007; Li et al., 2007).
- Animal tissues (e.g. Daeseleire et al., 1992) and feeds (e.g. Gonzalo-Lumbreras et al., 2007) for *inter alia* the presence of banned growth promoting steroids.

Clearly, the formulation of medications containing steroids is of considerable importance as is the need to demonstrate purity therein (Kotiyan and Vavia, 2000; Gorog, 2004, 2005), and this application of steroid analysis in the pharmaceutical area is discussed in Chapter 12. Noppe et al. (2008) have reviewed the measurement of steroid hormones in edible matrices.

All the analytical steps are interconnected, and it is obvious that the greater the specificity of the quantitation procedure, the less extraction or pre-purification necessary. High specificity quantitation procedures can thus be used with minimum pre-purification, and there are many examples of methods, which have been developed, that rely on this specificity. The specificity of liquid chromatography (LC) coupled to tandem mass spectrometry has allowed the assay of 12 steroid hormones in 200 μL of human serum, following a simple protein precipitation. On-line extraction/purification occurs prior to LC separation (Guo et al., 2006), and further specificity relies on multiple reaction monitoring (MRM). Such procedures may well provide valuable data, but it is very important to be fully aware of any short cuts, which may have been taken, and ensure that the method is only applied in situations for which it was developed. It is, however, not an uncommon practice that methods developed for one situation are applied uncritically to other situations in which they may not be valid.

3.1.2 Internal Standards

Purification prior to quantitation inevitably leads to loss of analyte, and the more extensive is the purification, the greater is the loss sustained. There must, therefore, be included in any quantitative method some means of assessing losses through the extraction and purification procedures. This is usually done by using an internal standard or radioactive (or stable isotope-labelled) recovery marker, which is added to the matrix at the start of the analysis. There are several requirements which must be considered in the selection of internal standards, which is of course constrained by the choice of the quantitation procedure to be used. The internal standard must,

after addition to the matrix, be distributed (e.g. bound to any protein, etc.) in the same way as the analyte. This is usually achieved by incubating for a period at 37°C, perhaps while gently shaking the fluid under analysis. A note of caution should be expressed here. Steroids are hydrophobic compounds and do not dissolve significantly in aqueous media and are thus added to the matrix dissolved in ethanol, methanol, or other polar solvent which is miscible with water. The volume of such a solvent should be as small as possible in comparison with the volume of fluid used for analysis to prevent denaturation of any protein. Sometimes, the steroid is added to a glass container, the solvent evaporated off and the fluid for analysis added. This practice is not to be recommended since steroids, when added in this way, can be adsorbed to the surface of the glass and may not subsequently be dissolved in the matrix, even when the glass has been inactivated by prior treatment with dimethyldichlorosilane. In addition, some steroids can be destroyed when evaporated to dryness on glass surfaces, particularly when the glass has previously been cleaned by treatment with chromic acid, a practice which used to be common but is now no longer so.

The internal standard chosen ideally must be indistinguishable from the steroid analyte during the process of extraction and purification but must be recognisable at the final quantitation stage. Internal standards can also fulfil a further function apart from their use as a means of assessing recovery. Steroids that are present in very low concentrations may become susceptible to irreversible loss by adsorption to glass surfaces, destruction by metal surfaces, and oxidation. The presence of a larger quantity of internal standard can prevent or minimise such losses. In such cases, the internal standard is also acting as a carrier. For gas chromatography–mass spectrometry (GC-MS) methods, the ideal internal standard is a deuterated or carbon-13 form of the analyte, and such standards should contain at least two but preferably three extra stable isotopes – this process leads to the so-called isotope dilution mass spectrometry (IDMS) procedure, and examples are given below. A number of publications describe methods for the synthesis of labelled steroids and bile acids with different isotopes (carbon and hydrogen) at positions around the steroids with up to eight hydrogens replaced with deuterium (Wudy, 1990; Zomer and Stavenuiter, 1990; Shoda et al., 1993; Furuta et al., 1999, 2003; Sulima et al., 2005; Kiuru and Wahala, 2006; Numazawa and Handa, 2006); however, addition of too many atoms of isotope can lead to chromatographic separation from the natural steroid. Insertion of deuterium into the steroid molecules is usually effected by acid-catalysed deuterium exchange and thus care must be taken during any extraction or pre-purification to avoid acid conditions, which can lead to loss of deuterium. Microwave-assisted synthesis of deuterium-labelled oestrogen fatty acid esters–a technique which may have other similar applications – has been reported by Kiuru and Wahala (2006). Isomers are also acceptable internal standards (e.g. 3β, 5α-tetrahydroaldosterone in the determination of 3α, 5β-tetrahydroaldosterone - Honour and Shackleton, 1977), but only if it is clear that the isomer is not found in the fluid under analysis. Chromatographic separation of analyte and internal standard may cause loss of the carrier effect, and the minimal separation of isotope-labelled standards has a positive advantage in this respect.

A chemical analogue of the analyte can also be used; for example, Δ^1-testosterone can be used in the measurement of testosterone by gas-liquid chromatography (GLC). Close chemical analogues used in this way must have physicochemical characteristics similar to the analyte, but must be distinguished from it before or during quantitation. Steroids labelled with deuterium are less readily available, but good sources to consider are Cambridge Isotopes (Cambridge Isotope Laboratories, 50 Frontage Road, Andover, MA 01810-5413), and recently the National Measurement Institute (Canberra City, ACT2601, Australia). Labelled free and conjugated metabolites are also available (Gartner et al., 2003; Gaertner et al., 2007). Other sources are given in a later section of this chapter (Section 3.9.3).

The use of tritium-labelled standards for use in steroid assays, not involving saturation analysis, has been described, and in such circumstances, a second internal standard has been necessary during the final separation and quantitation step. An example of such use of two internal standards is described (Seamark et al., 1980) in an early method for the measurement of vitamin D metabolites in human plasma by GC-MS. An alternative to actual addition of the standard to the fluid under analysis is to add the analyte to a second sample and process each side by side, the recovery being assessed by the difference between the two results. Such procedures are not recommended. As an added precaution, in some situations, two different internal standards can be used and the analyte quantified by relation to each separately. In such a case, the analyte concentration should be the same irrespective of which standard is used – where this is not so, it suggests that the standard that gives the lower result is incompletely resolved from a contaminating peak.

3.2 Extraction

Early methods of steroid analysis usually involved extraction as the first step and such extractions were normally carried out using solvents. The main drawback of liquid–liquid extraction is emulsion formation, though centrifugation will often overcome this situation but is time-consuming. Standing extraction tubes in dry ice to freeze the aqueous phase is a useful way of decanting an upper organic layer. While the modern trend has been to move away from such extractions because of the need to restrict use of toxic and/or flammable solvents and the problems of disposal, such extractions should not be completely discarded and there may well be situations where such procedures can still be useful. Indeed, a recent publication (Hill et al., 2007), describing the measurement of pregnanolones in third trimester pregnancy plasma, used ether for extraction, freezing the aqueous phase in solid CO_2/ethanol, and pouring off the ether layer. After evaporation to dryness, the residue was further purified by partitioning between *n*-pentane: 80% methanol in water (1:1) – (Ghulam et al. (1999) and Shu et al. (2003)) who describe similar extraction procedures in two assays for serum/plasma corticosterone. 1-Chlorobutane has been used for extraction of serum DHEA metabolites (Labrie et al., 2007). It is not the intention of this chapter to provide recipes for the extraction of specific steroids,

which can be found in the succeeding chapters in this book where more detailed reviews of the analysis of specific steroid groups are given, but merely to give an outline of the general principles of extraction and to draw attention to some of the problems which may be encountered during steroid analysis.

3.2.1 Solvent Extraction

When deciding upon the best solvent for such extractions, consideration must be given to the polarity of the steroid of interest and the interaction of the steroid to binding proteins. The extraction solvent must ideally do two things; it must totally disrupt the binding of the steroid to protein and must extract the steroid of interest quantitatively and leave behind in the aqueous medium other steroids and non-specific interfering substances. In practice, of course, this is never possible and by its very nature such solvent extraction will also extract a number of other steroids of similar polarity and thus similar structure to the analyte which may well interfere in the final quantitation. Depending upon the relative concentration of the steroid analyte in comparison to that of the potentially interfering steroids, it may be necessary to interpolate further purification steps prior to quantitation. For steroids which are incorporated into the lipoproteins, such as cholesterol and vitamin D, it may be necessary to add chemicals to disrupt the lipoprotein structure prior to extraction since without this disruption the recovery of steroid analyte may be very low (e.g. Axelson, 1985). Bile acids and their conjugates bind to protein and while it may seem sensible to extract these acids from acidic aqueous media, better recoveries are often obtained from alkaline media, perhaps because of the disruption of protein binding at pH11 and above. Solvent extraction of bile acids from tissues may be particularly difficult (reviewed by Street et al., 1983).

The more polar steroids such as the glucuronide and sulphate conjugates and sometimes even the polyhydroxylated C21 steroids are not always effectively extracted even by very polar solvents. A very early extraction procedure (Edwards et al., 1953) overcame this difficulty by the addition of ammonium sulphate at saturation concentrations to urine and acidification to pH2 with 10%(v/v) HCl prior to extraction with ether:isopropanol mixture (3:1, v/v). This procedure was quite effective, and majority of steroid glucuronides and sulphates were extracted to a large degree by this procedure. Although these steroid ester conjugates are most commonly found in urine, they are also present in plasma (especially, C19 glucuronides and sulphates and C21 sulphates) and may be extracted with varying efficiency into polar organic solvents. Addition of ammonium sulphate has also been efficacious in improving the extraction of vitamin D from plasma/serum (Hollis and Frank, 1985). Because there is a spread of polarity amongst the steroids, it is possible, by careful choice of solvents for extraction, to provide a considerable degree of selectivity for the steroid of interest, although such selectivity is seldom exclusive and there are many examples of the use of this type of selection. Figure 3.1 shows the range of solvent polarities in comparison to the polarity of various steroid groups.

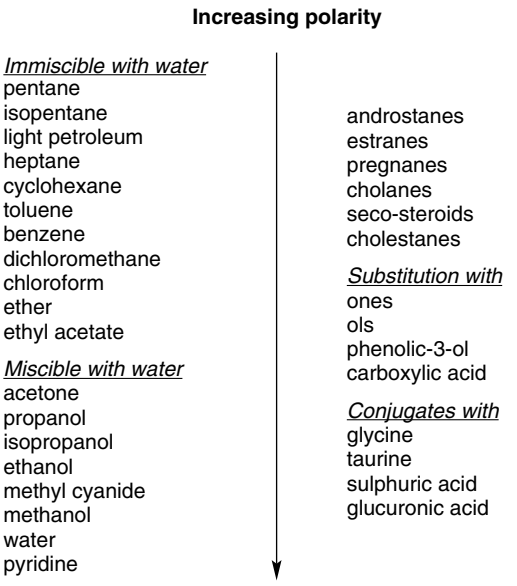

Fig. 3.1 Solvents available for steroid extraction from biological fluids.

When discussing the extraction of steroids, it must be remembered that many steroids often bind very tightly to glass and it is therefore advisable to silanise all glassware prior to use by treatment with dimethyldichlorosilane (1% v/v in toluene) or similar reagent washing afterwards with methanol. It is probably obvious, but still needs emphasising that solvent extraction generally precludes the use of plastic, silicone grease, etc. Considerable care must be taken to exclude all plastic since the occurrence of plasticisers (phthalates) in extracts may interfere in the final analysis. Steroids bound to glass are often difficult to dislodge and this may give rise to problems. It is often the practice, when assessing extractions or assay efficiency, to add radiolabelled or unlabelled steroid in solvent, evaporate the solvent to dryness, and add the medium of interest (plasma, urine, etc.), incubate and hope that the added steroid is distributed in the matrix in the same way as the endogenous analyte. In many cases, however, the steroid that has been added in this way is absorbed largely to the glass of the container and does not dissolve in the aqueous medium. Even subsequent solvent extraction may not totally dislodge the steroid which is bound to the glass and thus any corrections which are made to the final analytical result as a result of the recovery of added standard will give rise to a falsely high value. It is preferable if such procedures are used to ensure that the steroid binding is minimised by prior silanisation of all glassware and that a small amount of polar solvent (ethanol, methanol etc.), which is totally miscible with water is added, to dissolve the steroid prior to the addition of the aqueous medium or alternatively that the standard is added directly to the aqueous medium in a suitable solvent in sufficiently small volumes not to disrupt any binding or to denature any enzyme. Glassware may also present other hazards and it is advisable not to evaporate solvents and leave small quantities of steroids in the dry state for long periods. Some steroids (e.g. the secosteroids)

are particularly susceptible in the dry state to oxidation which can be substantially reduced by storage in suitable solvent.

Because of the occurrence of steroids of different types ranging from oestrogens through corticosteroids to bile acids and bile alcohols, there is no single procedure which efficiently and selectively removes all the steroids from the medium in which they are found. A single secreted steroid may be metabolised to innumerable metabolites of differing degrees of polarity which may then be conjugated with polar acids to assist in excretion. The reverse may happen, and steroid polarity may be decreased by the esterification of hydroxyl groups with fatty acids; for example, cholesterol esters are important constituents of the lipoproteins. Esters of oestrogens have also been described (Hochberg et al., 1991; Larner et al., 1992, 1993; Tikkanen et al., 2002), and the process of de-esterification, saponification, by incubation with alkali, usually KOH, is widely used as a first step in the extraction of a number of steroids from food and other material, although liquid chromatography–mass spectrometry (LC-MS) analysis of oestrogen esters (Miilunpohja et al., 2006) and cholesteryl esters (Liebisch et al., 2006) is now available without prior saponificiation. The wide variety of steroids and the metabolism, conjugation and/or esterification which they can undergo is not the only problem facing the analyst. Choice of the method must also take into account the medium selected for analysis, and, in this context, steroid binding and the problems this presents have already been mentioned. The physical characteristics of the matrix is clearly of importance and methods adopted for the analysis of granular or powdery food material will obviously not be appropriate for the analysis of a steroid in sunflower oil or metabolites of corticosteroids in human urine or plasma. Procedures for the extraction and measurement of cholesterol (Fenton, 1992), vitamin D (Rizzolo and Polesello, 1992; Jones and Makin, 2000) and anabolic steroids in meat (Marchand et al., 2000; Feduniuk et al., 2006; Xu et al., 2006; Nielen et al., 2007) and nutritional supplements (van Thuyne and Delbeke, 2004, 2005; Martello et al., 2007) have been reviewed.

Steroids share their essential non-polar nature with a large number of other lipids and methods, which effectively extract a wide range of steroids, and are likely also to extract a large amount of non-steroidal lipid material. Such lipid material must not be ignored since it can well interfere in subsequent separation or quantitation procedures. Lipid can be removed from the extracts by partitioning with organic solvents. This was an important step when isolating steroids from brain tissue (Ebner et al., 2006).

Steroids exist in hair in an intercellular space between the cuticle and cortical cells. Attention has been given to hair steroids as indicators of steroid abuse. The hair lipids include squalene, wax esters, triglycerides, free fatty acids, ceramides and cholesterol sulphate. Steroids can be extracted from hair using solvent mixtures such as chloroform with methanol (Choi and Chung, 1999). Analysis of steroids in hair as a means of detecting ingestion of anabolic steroids has been reviewed by Kintz (2004 – Kintz et al., 2006; Gambelunghe et al., 2007). Fatty acids can of course be easily removed by an alkaline wash but this may also remove acidic steroids such as oestrogens or bile acids. Saponification, apart from liberating steroids from their esters, also has the added advantage of hydrolysing many neutral lipids

which can then be removed by alkali or acid washes. It may not, however, be possible to remove potentially interfering lipid material by simple washings of solvent extracts and further separatory procedures, such as the use of magnesium oxide mini-columns as described by O'Shannessy and Renwick (1983), may be necessary. Despite these precautions, significant amounts of non-specific non-steroidal neutral lipid material may be present in supposedly clean steroid extracts and this may not always be appreciated since this interfering material usually does not have any recognisable characteristic which immediately betrays its presence. While in most instances, such material can be ignored, there are situations where it can assume importance, for example, in immunoassays where steroids extracted from biological matrices may not always behave in the same way as standard steroids used for the standard curve (e.g. Jawad et al., 1981). While many of these techniques are more than 20 years old, they should not be ignored and may still have applications to particular problems of today, especially when combined with more modern technology (e.g. Makin et al., 2002; Huang et al., 2007).

For the quantitative extraction of neutral unconjugated steroids from aqueous media such as urine, bile, plasma/serum, saliva, *in vitro* incubation media, etc., the use of an equal volume or excess of polar solvent such as ethanol, methanol, acetone or methyl cyanide is very effective. Tissue extraction is usually carried out either after or during homogenisation (e.g. Andersson and Sjövall, 1985). Such extraction systems can also be used to extract steroid conjugates with varying degrees of efficiency when used in combination with acid or alkaline pH and added salts. These solvents have the added advantage that they also disrupt steroid protein binding by denaturing the protein. In the case of cholesterol or vitamin D, this denaturation may have to be taken further by the addition of ammonium sulphate (Hollis and Frank, 1985) or pentylamine (Axelson, 1985) or other material to disrupt the lipoprotein. The protein can then be removed by centrifugation, leaving a 50:50 solvent:water mixture which can be further extracted by the addition of suitable solvent such as chloroform which is immiscible with water. This causes the formation of two layers, the bottom of which contains the unconjugated steroids which can be removed. Use of chloroform:methanol mixtures for such extractions produces exactly the same end result and such extractions have been widely used over the last 50 years (Bligh and Dyer, 1957). Many modifications of this procedure have been introduced which may well have advantages for particular purposes but do not greatly improve the general applicability of the original procedure for steroid extraction. Acetonitrile is widely used as an extraction solvent for vitamin D metabolites in a similar fashion and the aqueous extract after removal of the protein by centrifugation can be used for solid-phase purification using Sep-Pak silica cartridges (see Chapter 11). On-line deproteinisation during high-performance liquid chromatography (HPLC) can be achieved using a polymer-coated mixed functional silica column (Okumura et al., 1995).

Careful choice of solvents can provide a considerable degree of selectivity. It is clearly a relatively simple task to use solvent extraction to discriminate between non-polar neutral steroids and their conjugates with glucuronides and sulphates. It is not so simple to rely on solvent extraction to discriminate between steroids of similar polarity but it is possible to achieve quite simple separations by judicious choice of

solvent. There are numerous examples of such solvent selectivity in the literature, the use of isopentane to separate aetiocholanolone (96% extracted) and 11β-hydroxy-aetiocholanolone (2.5% extracted) (Few, 1968) and the separation of the oestrogens, oestradiol and oestrone (>95% extracted) from oestriol (4% extracted) using benzene:light petroleum (1:1, v/v)) (Brown, 1955). It might be argued that such examples of solvent selectivity, being 35–50 years old, are not appropriate for a book on modern methods of analysis but if such procedures, which are simple and quick, provide a solution to today's problems their age should not debar them from use.

There are other examples of such solvent selectivity. It has become fashionable in the age of the rapid and simple immunoassay method, which when applied directly to plasma without any tedious extraction or separation procedure, gives a very rapid result to rely too heavily on the advertising message on the side of the reagent pack without much critical appraisal. A good example of such reliance and the value of a simple extraction prior to assay is reported in the excellent paper by Wong et al. (1992) who carefully investigated the nature of the steroids in neonatal plasma that interfered in a "non-extraction" immunoassay for 17-hydroxyprogesterone, an important assay used in the early diagnosis of congenital adrenal hyperplasia (CAH), where a falsely high result could have severe consequences. The interference was demonstrated to be due to 5-en-3β-ol steroids conjugated with sulphuric acid, particularly 17-hydroxypregnenolone sulphate, which are formed in the fetal adrenal cortex (Shackleton, 1984) and are present in neonatal plasma. A simple extraction of the plasma with a relatively non-polar solvent, isopropanol:hexane (3:97,v/v), removed 17-hydroxyprogesterone leaving behind the interfering steroid sulphates. Two immunoassays for plasma 17-hydroxyprogesterone have been evaluated (Nahoul, 1994), comparing results with an in-house method which used ether extraction followed by column chromatography on LH-20 – both assays were found to give high values and it was concluded that neither was suitable for use with infants or women in the follicular phase of their menstrual cycle. Analysis of 17-hydroxyprogesterone by LC-MS/MS has a wider range for accuracy than RIA (Etter et al., 2006). Direct assays for testosterone are entirely inappropriate in the first 6 months of life (Fuqua et al., 1995). Urine free cortisol measurements with direct RIA measurements can have poor specificity and recovery (Gray et al., 2003; Horie et al., 2007). These examples illustrate very well the need to remember the value of a simple extraction or chromatographic step, which can provide a much needed increase in the selectivity of a method (Davison et al., 2005; Schirpenbach et al., 2006) and also demonstrate the need to remember that assays developed and validated using one medium (adult male plasma) may not be valid when applied to other media (neonatal or female plasma).

Urine is a complex medium which contains a wide variety of metabolic products of the secreted steroid hormones (androgens, oestrogens, corticosteroids, preganediols, cholesterol, etc.) which may be unconjugated or conjugated with glucuronic or sulphuric acids. A comprehensive analysis of urine presents, therefore, a considerable analytical challenge. While the analysis of urine for clinical diagnostic purposes is not as popular as it once was, the use of steroid profiles by capillary GLC can still provide very valuable information. Of interest also is the analysis of urine for the identification

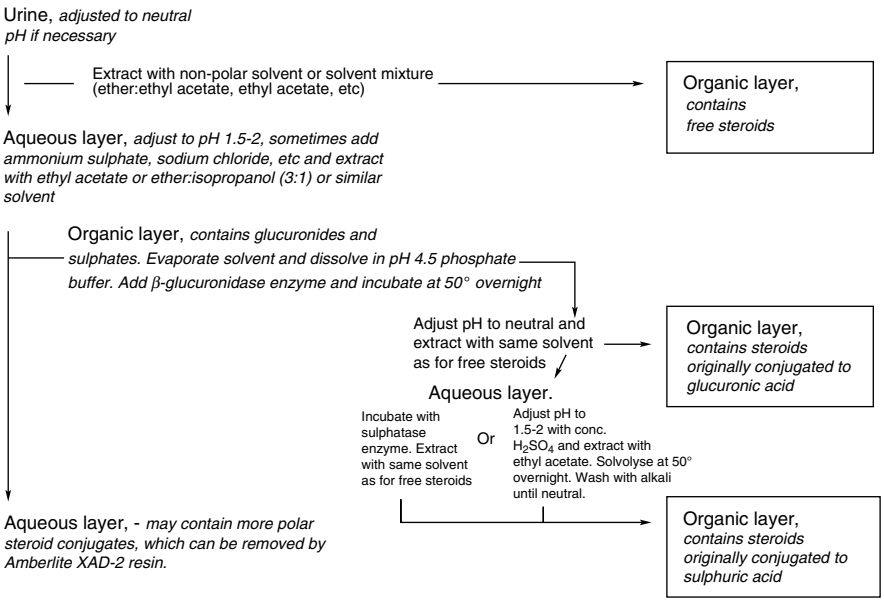

Fig. 3.2 Scheme for the extraction and separation of steroid groups from urine without chromatography.

of the metabolites of administered steroids, such as 4-hydroxyandrostenedione, an aromatase inhibitor, which may be of value in monitoring the treatment of oestrogen-dependent breast cancer (Foster et al., 1986; Poon et al., 1992). Figure 3.2 illustrates a non-chromatographic approach to the extraction of steroids from urine using solvent extraction and subsequent hydrolysis of the conjugates. A method of solvent extraction has been described for the extraction of testosterone and epitestosterone that uses an aqueous two-phase system composed of 1-butyl-3-methylimidazolium chloride and dipotassium hydrogen phosphate (He et al., 2005). In a similar manner, the efficiency of extraction of steroid sulphates using ion-pairing with a number of reagents has been evaluated (Cawley et al., 2005).

3.2.2 Solid-Phase Extraction

Although many of today's publications concerned with steroid analysis still usefully employ liquid-liquid extraction as an initial step in the purification of steroids of interest, it is in general true to say that over the last 20–25 years the most important step forward in steroid analysis has been the advent of solid-phase extraction (SPE), particularly the availability of microparticulate silica coated with octadecasilane packed into syringes or cartridges. The use of Sep-Pak C18, for the extraction of steroids was initially described by Shackleton and Whitney in 1980. A survey of methods

for steroid analysis published in 1993 indicated how popular such solid-phase procedures had become in that more than 70% of publications used such extractions. Although subsequent surveys have confirmed this trend, solvent extractions are still used today. Extraction of steroids from aqueous media has been carried out using neutral (Amberlite XAD-2 – Bradlow, 1968, 1977) and ion-exchange resins (DEAE-Sephadex – Derks and Drayer, 1978a), and such procedures are still used successfully even today, particularly for the extraction of polar steroids (e.g. Deboer et al., 1992; Poon et al., 1992); Dowex AG resins have been used in conjunction with adsorption cartridges for the purification of catechol and guaiacol oestrogens (Saegusa et al., 1993). An interesting but limited comparison of the uses of solid-phase extraction (SPE) and their value in contrast to solvent extraction in the pre-HPLC stage of the analysis of plasma cortisol and corticosterone has been published (Hariharan et al., 1992).

There are a wide variety of solid-phase materials available for use in the extraction of steroids and examination of the chapters in this book and the catalogues of suppliers of these materials will confirm this. The field is bedevilled by the use of trade names which sound nice but are not helpful when considering their method of action. Extrelut, Chem-Elut, VacElut, Bond-Elut, Tox Elut might appear to be different trade names for the same thing but they are not. SPE material appears to fall into two distinct groups. Firstly, those systems based on Keiselguhr (Celite – a diatomaceous earth) treated in various and sometimes unspecified ways (e.g. Chem-Elut, Analytchem & Extrelut, E.G. Merck) to inactivate the material and then sieved into different size ranges (e.g. Perona and Pavan, 1993; Saegusa et al., 1993; Suzuki et al., 1993). The material is then packed into syringes, cartridges, etc. of various sizes and shapes made from a variety of different plastics. The aqueous medium is poured onto the material, which takes up the water, and the steroids are then eluted with organic solvents. This process would appear to be a simple liquid–liquid partition chromatography process similar to the celite partition column chromatography of the past. Since these columns have a finite capacity to absorb water, it is possible inadvertently to overload and if aqueous material passes through the column, steroids of interest will also pass through still dissolved in the aqueous matrix. Tox Elut (Varian), a similar type of system but using a more granular material designed for the analysis of drugs of abuse has a dye incorporated into it, which indicates how far down the column the added aqueous medium has reached. Such systems are still occasionally used for preliminary purification before quantitation (e.g. Ibrahim et al., 2003; Fiet et al., 2004; Davison et al., 2005).

The second type of SPE material is based upon microparticulate silica either used directly or modified in an ever increasing variety of different ways. Some of the non-polar, polar and ion-exchange sorbents, which are in use today and can be obtained from a variety of sources (e.g. Biotage IST, Biotage GB Ltd., Duffryn Industrial Estate, Ystrad Mynach, Hengoed CF82 8RJ, UK and Analytchem International, Harbour City, CA, USA) are illustrated in Fig. 3.3, which is taken from the catalogue of Biotage IST, as an example of what is available. These sorbents can be packed into syringe-like reservoirs or pre-packed cartridges (e.g. Sep-Pak, Millipore-Waters) of different sizes, which can cope with differing loads. These SPE systems are based on a variety of absorption and partition. Most steroid

BIOTAGE RESIN AND SILICA BASED SORBENTS
Table adapted and updated from 2004 Catalogue of Sample Preparation Products and Services

Sorbent	Description	Structure	
Resin based sorbents: primary interactions are strongly NON-POLAR			
EVOLUTE ABN	Surface modified polystyrene divinylbenzene		
ENV+	Hydroxylated polystyrene-divinylbenzene		
101	Polystyrene-divinylbenzene		
Silica based non-polar sorbents: primary interactions are NON-POLAR			
Non-endcapped sorbents exert stronger polar and ionic secondary interactions than endcapped sorbents			
C18*	Octadecyl	-Si-$C_{18}H_{37}$	
MF C18	Octadecyl monofunctional	-Si-$C_{18}H_{37}$	
C8*	Octyl	-Si-C_8H_{17}	
C6	Hexyl	-Si-C_6H_{13}	
C4	Butyl	-Si-C_4H_9	
C2*	Ethyl	-Si-C_2H_5	
CH†	Cyclohexyl	-Si- 	
PH*	Phenyl	-Si- 	
CN*	Cyanopropyl	-Si-$(CH_2)_3CN$	
Silica based mixed-mode sorbents: primary interactions are NON-POLAR and IONIC			
HAX	C8 and strong anion exchange		
HCX	C8 and strong cation exchange		
HCX-3	C18 and strong cation exchange		
HCX-5	C4 and strong cation exchange		
HCX-Q	C8 and weak cation exchange		
Multimode	C18, strong anion and cation exchange		
Silica based ion exchange sorbents: primary interactions are IONIC			
Silica based anion exchange sorbents			
			Secondary interaction (aqueous matrix)
NH2	Aminopropyl	-Si-$(CH_2)_3NH_2$	Weak non-polar
PSA	Primary secondary amine	-Si-$(CH_2)_3NH(CH_2)_2NH_2$	Weak non-polar
SAX	Quaternary amine	-Si-$(CH_2)_3N^+(CH_3)_3$ Cl^-	Weak non-polar
PE-AX	Quaternary amine	-Si-$(CH_2)_3N^+(CH_3)_3$ OAc^-	Weak non-polar
Silica based cation exchange sorbents			
			Secondary interaction (aqueous matrix)
CBA	Propylcarboxylic acid	-Si-$(CH_2)_3COOH$	Weak non-polar
SCX	Benzenesulfonic acid	-Si-  -SO_3^- H^+	Medium non-polar
SCX-2 (PRS)	Propylsulfonic acid	-Si-$(CH_2)_3SO_3$-H^+	Weak non-polar
SCX-3	Ethylbenzene sulfonic acid	-Si-$(CH_2)_3$-  -SO_3^- H^+	Strong non-polar
Silica based polar sorbents: primary interactions are POLAR			
			Secondary interaction (non-aqueous matrix)
CN	Cyanopropyl	-Si-$(CH_2)_3CN$	
SI	Silica	-Si-OH	Weak ion exchange
DIOL	2,3-dihydroxypropoxypropyl	-Si-$(CH_2)_3$-OCH_2-CH(OH)-CH_2(OH)	
NH2	Aminopropyl	-Si-$(CH_2)_3NH_2$	Weak ion exchange
PSA	Primary secondary amine	-Si-$(CH_2)_3NH(CH_2)_2NH_2$	Weak ion exchange
FL	Florisil	$MgO•3.6\ SiO_2•0.1$ OH	
AL-A	Alumina – acidic		
AL-N	Alumina – neutral		
AL-B	Alumina – basic		

* Endcapped sorbent available † Cyclohexyl is not endcapped

Fig. 3.3 Biotage resin and silica based sorbents. Table adapted and updated from 2004 Catalogue of Sample Preparation Products and Services

extractions are carried out using reverse-phase methodology where the silica has been modified by linking the silanol groups to hydrocarbons of varying chain length, the most popular being octadecane forming octadecasilyl silica (ODS or C18) (Shackleton and Whitney, 1980), although other chain lengths have been used (e.g. C_2) for extraction of steroids, such as oestrone (e.g. Ciotti et al., 1989), from serum. Depending upon the treatment used to form the ODS material, a proportion of silanol groups on the silica may still be unchanged. Further treatment, with a silylating material to produce trimethylsilyl ethers (TMS) of these polar groups, known as 'end-capping' can be carried out. The presence of untreated silanol groups leads to a significant adsorption as well as a reverse-phase partition and deliberately produced non-fully end-capped material (Bond-Elut C18-OH) has been put to good use by combining the extraction and subsequent separation of vitamin D metabolites on the same cartridge by changing solvents, so-called 'phase-switching' (Hollis, 1986), and this procedure is now incorporated into a commercial method for 1α, 25-dihydroxyvitamin D assay.

Comment has been made before about the value of washing organic extracts with alkali and similar purification can be achieved with amino (NH_2) columns in series with C18 extraction columns (Schmidt et al., 1985). These silica-based sorbent columns too have finite capacity but are now available, as mentioned above, in different sizes or alternatively the packing material can be supplied as such and appropriate amounts made up in columns of suitable sizes for the particular application. When setting up a method using these SPE columns, attention must be paid to the material in which the silica is packed. Not all the plastics in which the C18 material was packed (including cartridge, syringe and frits, etc.) react to solvents in the same way and material may be eluted from the plastic which is innocuous when the eluent is used in one method but devastating in another. Small SPE columns can be inserted into HPLC systems (Lopez de Alda and Barcello, 2001; Barrett et al., 2005; Kataoka, et al., 2007). Miniaturised fibre-packed injection needles have recently been used as a novel extraction device prior to GLC, although it has not yet been applied to steroid analysis (Ogawa et al., 2007; Saito et al., 2007).

Before being applied to the column, any steroid–protein binding must be disrupted and in the case of the silica C18 extraction procedures this is usually today carried out by the use of acetonitrile or methanol which is added in equal amounts, vortex mixed and the protein plug removed by centrifugation. The acetonitrile:water mix is then applied to the cartridge and the steroids can be eluted with methanol, after washing with various concentrations of water in methanol to elute polar material. Sometimes, the protein plug is re-extracted and combined with the original extract applied to the Silica C18 SPE system. This procedure is very effective usually leading to near-quantitative recovery. For some steroids, such as vitamin D itself and cholesterol, more rigorous procedures may be required in order to disrupt protein binding (reviewed in Porteous et al., 1987). There are numerous papers, which can be referred to, that use these SPE methods for the extraction of steroids and all are used in essentially the same way. The use of BondElut C18 cartridges for the extraction of bile acids from serum required dilution of serum with 0.1 M NaOH or 0.5 M triethylamine sulphate and heating for 30 min at 64°C prior to application (Rodrigues and Setchell,

1996). It will be noted from Fig. 3.3 that ion-exchange SPE material is now available, and these are now being increasingly used for steroid extraction (e.g. Strahm et al., 2008). For the separation of free and conjugated steroids, mixed mode anion-exchange SPE cartridges were used (Ebner et al., 2006). Further applications of ion-exchange SPE are to be anticipated. Solid-phase extraction was also used to remove lipids from brain extracts (Liere et al., 2004; Ebner et al., 2006).

Reverse-phase extraction using C18 (ODS) coated silica is by far the most popular material for SPE, although subsequent separatory procedures using SIL (untreated silica) or –CN (cyano, particularly useful for separating steroids with oxo groups) have proved valuable. Indeed, Sep Pak C18 cartridges have been used successfully to extract bile acids from serum, urine, liver biopsy extracts, bile, gastric juice and faeces (Guldutuna et al., 1993). ODS-coated silica has also been used for steroid extraction prior to HPLC by the use of a guard column, packed with ODS-silica, in place of the injection loop. Diluted samples were injected into the column, washed and then eluted onto the analytical HPLC column with methanol (Wade and Haegele, 1991a).

The Waters company are producing a range of OASIS cartridges. There are five available Oasis® sorbent chemistries, which are designed to meet just about all sample preparation needs. They are all built upon unique water-wettable Oasis® HLB copolymer and provide exceptional results (AbuRuz et al., 2003). The sulphonic acid (MCX) and quaternary amine (MAX) derivatives of Oasis® HLB provide dual modes of retention enabling greater cleanup selectivity and sensitivity for both acidic and/or basic compounds – even if the sorbent in the wells runs dry. Oasis® WCX (weak cation exchanger) and WAX (weak anion exchanger) are also derivatives of Oasis® HLB. These sorbents are specifically designed to offer the same benefits and features as HLB with the ability to retain and release strong acids (e.g. sulphonates) and bases (e.g. quaternary amines). All of the five patented Oasis® chemistries are available in several device formats (e.g. cartridges, 96-well plate, and µElution plate) to fit specific needs. These cartridges were used more recently in the processing of steroids from prostate (Higashi et al., 2005c), brain extracts (Ebner et al., 2006), bovine milk (McDonald et al., 2007) and rat biofluids and foetal tissue (Samtani and Jusko, 2007).

Other solid-phase extraction procedures for steroid extraction from biological media have been described such as the use of graphitised carbon ("Carbopak", Supelco, Poole, Dorset, BH17 7NH) for the extraction of oestrogens and their conjugates from urine, serum and amniotic fluid, but can also be used for separation of steroids (illustrated in Fig. 3.4, Andreolini et al., 1987). Cyclodextrin, with which steroids form inclusion compounds, has also proved valuable as a means of extracting steroids prior to GC-MS (De Brabandere et al., 1993) or HPLC (Wade and Haegele, 1991b). Oestradiol-17β, oestriol and 17α-ethinyloestradiol have been analysed in environmental waters by LC with fluorescent detection following on-line solid-phase microextraction using a polymer monolith inside a polyether ether ketone (PEEK) tube (Wen et al., 2006).

Automated liquid sampling handling systems are now being used to improve analytical efficiency. SPE can be incorporated in to such procedures and has been

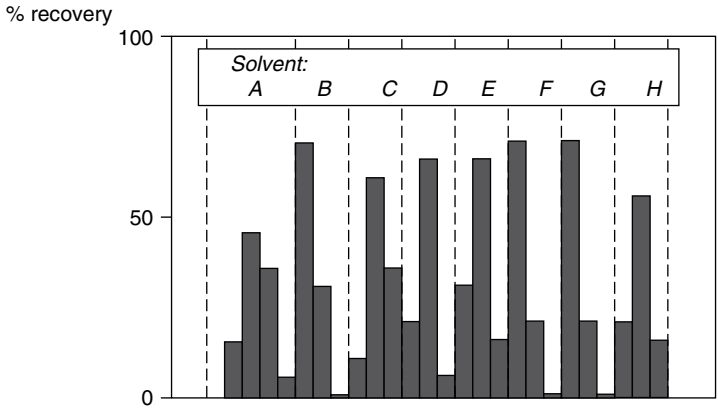

Fig. 3.4 Group separation of some steroids and their conjugates using graphitised carbon black cartridges (Carbopak, Supelco). These cartridges consist of a 6 cm x 1 cm i.d. cylindrical polypropylene tube which is one-sixth filled with 250 mg of carbon black with a particle size ranging between 20 and 125 μm. The absorbent bed is held in place by polyethylene frits. After removal of phospholipids by passing a methanol extract of serum or amniotic fluid through a C 18 Sep-Pak, the eluent in methanol:water (80:20) was percolated through the Carbopak cartridge and steroid groups were recovered by sequential elution with a variety of solvents figure illustrates the separation in this system of a number of different standard steroids identified as follows: solvent A, (methanol) elutes androsterone; solvent B, (100 mmol/1 of formic acid in methanol) elutes estradiol; solvent C, (chloroform: methanol, 60:40) elutes androsterone glucuronide; solvent D, (chloroform: methanol, 27:73, with 250 mmol/1 formic acid) elutes estradiol 3-glucuronide; solvent E, (chloroform: methanol, 60:40, containing 250 mmol/1 acid) elutes estradiol 17-glucuronide; solvent F, (chloroform: methanol, 10:90, containing 5 mmol/1 of tetramethylammonium hydroxide) elutes androsterone sulphate; solvent G, (chloroform: methanol, 80:20, containing 0.5 mmol/1 of tetramethylammonium hydroxide) elutes estradiol 3-sulphate; solvent H, (chloroform: methanol, 80:20, containing 5 mmol/1 methylammonium hydroxide) elutes estradiol 3-sulphate 17-glucuronide. (Reprinted with permission from Andreolini *et al*., 1987. Copyright 1987 American Chemical Society.)

tested for example in pharmaceutical applications (Tamvakopoulos et al., 2002) in urine steroid analysis (e.g. equilenin and progesterone – Rule and Henion, 1999; 6β-hydroxycortisol and cortisol – Barrett et al., 2005) and anabolic steroids – Haber et al., 2001) and for the measurement of low concentrations of oestrogens in natural and treated water, coupled to LC-MS (Rodriguez-Mozaz et al., 2004). In a similar fashion, solid-phase extraction can be incorporated into the LC system and thus are susceptible to automation. A recent example of this is the extraction of cortisol from human saliva using a Supel Q PLOT column for extraction in series with the analytical LC column (Kataoka et al., 2007).

A novel approach, coating absorptive material onto stir bars (Kawaguchi et al., 2006a), has been used for the extraction of estrone and oestradiol-17β (Stopforth et al., 2007a), testosterone and epitestosterone (Stopforth et al., 2007b) from human urine and oestradiol-17β from river water (Kawaguchi et al., 2004, 2006b) – the stir bars were removed and washed. The steroids were derivatised in the gas phase and then introduced into a GC-MS system by thermal desorption. Steroid conjugates, absorbed onto these bars could be treated in a similar way, de-conjugation taking place once the

bars had been removed. The use of these stir bars is not confined to GC-MS, and a similar procedure has been described for the extraction of urine spiked with oestriol, methyltestosterone and progesterone, and subsequent elution of the steroids for analysis by LC-MS (Huang and Yuan, 2007). A similar method using sol-gel coated polydimethylsiloxane/β-cyclodextrin stir bars has been described for extraction of oestradiol-17β from river water for subsequent LC-UV analysis (Hu et al., 2007).

3.2.3 Hydrolysis of Steroid Conjugates

Hydrolysis of steroid glucuronides is usually carried out using a β-glucuronidase preparation, although effective hydrolysis can also be achieved by oxidation with periodate that removes the glucuronide residue, leaving behind a mixture of free steroid and steroid formate. Hydrolysis of steroid sulphates is on the other hand usually carried out by acid solvolysis in ethyl acetate which is very effective for most steroids but does not always cleave the ester link of some bile acids. Parmentier and Eyssen (1977) described an alternative acid hydrolysis procedure using methanol:acetone (1:9,v/v) containing approx. 1% (v/v) HCl (18 h at 37°C) which they used to hydrolyse bile acid sulphates on C3, C7 and C12. A similar method was published in 1989 (Tang and Crone, 1989) which used anhydrous methanolic HCl for the hydrolysis of glucuronides, sulphates and phosphates. Enzyme hydrolysis of steroid sulphates can also be carried out using sulphatase preparations from a variety of sources (*Patella vulgata*, *Helix Pomatia* etc.) most of which also contain β-glucuronidase activity (Shibasaki et al., 2001), although such preparations do not always work efficiently (Epstein et al., 1983), and before use, they should be tested using a model steroid sulphate. Use of these enzyme preparations can also occasionally lead to further problems and it has been, for example, reported (Schmidt et al., 1985) that the recovery of DHEA from urine treated with *Helix pomatia* preparations drops dramatically as the concentration of enzyme increases. While enzyme hydrolyses such as those illustrated in Fig. 3.2 can be carried out without extraction, it has been noted by Schmidt et al. (1985), and many times in the past, that improved hydrolysis with less enzyme can be achieved after extraction, presumably because of the presence of interfering compounds in unextracted urine. In preparations containing both glucuronidase and sulphatase activity, sulphatase activity can be inhibited by incubating in pH 4.5 phosphate buffer. A study of the optimum conditions for steroid glucuronide hydrolysis was carried out by Ferchaud et al. (2000) who recommended the use of abalone entrails at pH 5.2 at 42°C. Hydrolysis of steroid conjugates, while rendering analysis of the steroid composition of these fractions simpler, does obscure information and it would clearly be better to analyse these conjugates without prior hydrolysis (e.g. Gaskell, 1990; Shackleton et al., 1990a). The advent of LC-MS has provided an ideal approach towards the analysis of intact glucuronides and sulphates, and this has been used for the analysis of intact urinary 17-oxosteroid sulphates and glucuronides using LC-MS (Jia et al., 2001) and serum androsterone glucuronide and androstenediol glucuronide using LC-MS/MS (Labrie et al., 2006). A more comprehensive approach

to this problem has been published by Antignac et al. (2005) who separated 14 steroid glucuronides and sulphates in a single run in their LC-MS-MS system. Figure 3.5 illustrates this separation. A similar extraction procedure using Bakerbond C18 cartridge followed by a quaternary ammonium cartridge (Bond Elut SAX) has been

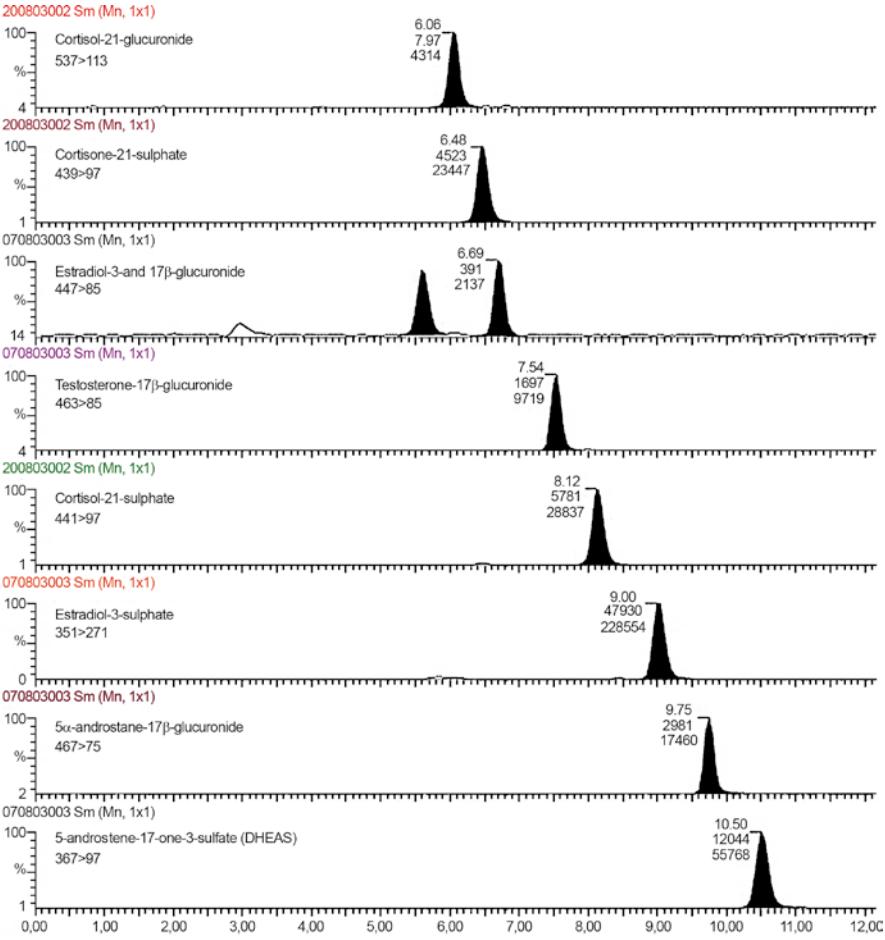

Fig. 3.5 Figure 3 from Antignac et al. (2005). Typical ion chromatograms obtained for a standard solution of steroid and corticosteroids phase II matabolites (10ng injected). Data were acquired using a Quattro LC® triple quadrupole analyzer (Micromass®, Manchester, UK) operating in electrospray ionization mode (ESI). Nitrogen was used as nebulization and desolvation gas, with flow rates of 90 and 600 L/h, respectively. Source and desolvation temperatures were 120 and 350 °C, respectively. The electric potential applied on the capillary was 3.5 kV, and the sampling cone voltage was optimized for each molecule. The LC–MS/MS experiments were performed using argon as the collision gas at a pressure of 4.0×10^{-14} mbar and a collision energy adapted for each compound. The ions selected in MSI and those monitored in MS2 after CID are shown in upper left of each panel. [Further data is given in Antignac et al. (2005) from which this Figure is taken with permission.]

used to extract 19-norandrosterone sulphate from human urine prior to LC-MS-MS (Strahm et al., 2007). Sodium cholate micelle capillary electrophoresis has been used to determine 16 oestrogens, DHEA and their glucuronide and sulphate conjugates in 100 μL of serum (Katayama et al., 2003).

Bile acids are excreted in more complex forms than most other steroids, being not only conjugated to glucuronic and sulphuric acids but also joined, via a peptide bond at C24 to either glycine or taurine. Hydrolysis of bile acid glycosides is influenced by the source of the enzyme, position of sugar moiety, enzyme activity and incubation conditions (Momose et al., 1997a), but is usually attempted using cholylglycine hydrolase (e.g. Paauw et al., 1996; Gatti et al., 1997). Other derivatives of bile acids have been described (Niwa et al., 1993). Hydrolysis to free acids therefore obscures even more information than normal. An increasing number of GC and LC-MS systems have been developed which allow bile acid conjugates to be analysed directly without hydrolysis (e.g. Street et al., 1986; Iida et al., 1992; Ikegawa et al., 1992; Yang et al., 1997; Tessier et al., 2003; Ando et al., 2006; Caron et al., 2006). In addition, a 2D HPTLC method with cyclodextrin/aqueous methanol solvent has been developed (Momose et al., 1998) as well as an ion-pair HPLC method using di-*n*-butylamine acetate as a mobile-phase additive (Sasaki et al., 2000). Analysis of bile acids is dealt with in greater detail in Chapter 10.

The common lack of reference metabolites in conjugated forms precludes validation of all urinary methods. A number of papers describe synthesis of conjugates using tissue and recombinant enzymes and chemical techniques for steroids (diMarco et al., 1998; Sasaki et al., Iida*[1] and Nambara, 2000; Kuuranne et al., 2002, 2003) and bile acids (Momose et al., 1997b; Gall et al., 1999; Lida et al., 2002; Kakiyama et al., 2005; Caron et al., 2006; Jantti et al., 2007).

3.2.4 Immunoaffinity Extraction

In an ideal world, the initial extraction procedure would not be necessary as the quantitation method would be so specific that neither the biological matrix nor steroids with similar structures to that of the analyte would interfere. Unfortunately, although some direct immunoassays are described, there are very few of these analytical methods for steroids which cannot be improved by some form of extraction and/or pre-purification before quantitation, although there is a report of an immunoassay for 17-hydroxyprogesterone which was not improved by interpolation of a column chromatographic step (Lim et al., 1995). Extraction procedures, which removed only the steroid of interest from the matrix, would be of considerable

[1]Note that Lida, T and Iida, T appear to be the same person but are spelled differently in PubMed. In the text and reference list the spelling adopted by PubMed is used. Please check both spellings in the reference list.

value in some circumstances and one extraction method has shown considerable promise in this area. Use of columns packed with immobilised antibodies (immunoaffinity columns - IAC) for extraction can provide a means of selective extraction and purification (Glencross et al., 1981). The selectivity depends of course upon the specificity of the antibody for the analyte but has, for example, been successfully applied in the GC-MS analysis of oestradiol in human plasma (Gaskell and Brownsey, 1983), prior to immunoassay (Webb et al., 1985) or prior to HPLC by incorporating an antibody bound to Si60 in the injection loop of the LC system (Nilsson, 1983). In these methods, the antibody is chemically attached to a support (e.g. Sepharose or Sephacryl) and kits are available to carry out this procedure (e.g. Stanley et al., 1993). The immobilised antibody is then packed into a column and the matrix percolated through it. After washing, the analyte(s) can be released from the antibody and thus eluted from the column by altering the salt concentration. Highly specific antibodies obviously provide a selective extraction but there may be situations when a less selective, broad spectrum extraction is required. This can be achieved by using an antibody with broader specificity. Tsikas (2001) has reviewed the application of IAC prior to GC-MS and it has been used subsequently for clean up prior to HPLC-ToF (Time of flight) analysis of oestrogens in sewage-impacted urban estuary water (Reddy and Brownawell, 2005). The development of a multi-target IAC for oestrone, oestradiol and oestriol in urine prior to separation by micellar electrokinetic chromatography (MEKC) has also been described (Su et al., 2005). Testosterone in male urine has been successfully determined by partial filling micellar electrokinetic chromatography (PF-MEKC) after immunoaffinity SPE (Amundsen et al., 2007). Microemulsion EKC (MEEKC) has been used for the measurement of natural and synthetic oestrogens in pharmaceutical preparations (Tripodi et al., 2006) and scanning MEKC has been applied to the measurement of corticosterone and 17-hydroxycorticosterone in plasma and urine (Chen et al., 2004).

3.2.5 Extraction Using Molecularly Imprinted Polymers and Restricted Access Material

Since the first edition of this book, new forms of selective extraction have been introduced but not yet widely applied to steroid analysis. Firstly, molecularly imprinted polymers (MIPs Haginaka, 2001), which could be regarded as synthetic 'antibodies'. For example, oestradiol was extracted into an MIP packed into a microcolumn and subsequently eluted using microwave-assisted extraction (Bravo et al., 2005). Other examples again using oestradiol have been outlined (Dong et al., 2003; Szumski and Buszewski, 2004; Watabe et al., 2006). Specific extraction procedures for cholesterol, using molecularly imprinted cyclodextrin microspheres (Egawa et al., 2005) and sorbents (Pichon, 2007), have also been described. There is a comprehensive review of the literature (up to 2003) on MIP and its appli-

cations (Alexander et al., 2006). Use of MIP and RAM materials in SPE-LC-MS for monitoring oestrogen contamination of water has been reviewed by Rodriguez-Mozaz et al., (2007).

The second development uses the so-called restricted access material (RAM) coupled on-line to LC-MS (van der Hoeven et al., 1997; Petrovic and Barcelo, 2002; Petrovic et al., 2002; Christiaens et al., 2003, 2004; Souverain et al., 2004) or by the use of column switching – after absorption onto a C4-alkyl-diol silica RAM. The absorbed steroid was backflushed onto a conventional C18 column followed by ES-MS (Chang et al., 2003).

After extraction, it may still be necessary to carry out some form of purification before quantitation and this may be necessary for a number of reasons. Firstly, such purification removes potentially interfering compounds of similar structure which may on occasions be present in higher concentrations than the analyte itself. It may also be necessary to carry out a preliminary fractionation to separate steroids in particular group. There are a multiplicity of methods of purifying steroids after extraction from the biological matrix, all using some form of chromatography. Today, only two chromatographic techniques are however widely used, high-performance liquid chromatography (HPLC) and gas–liquid chromatography (GLC), both of which provide high resolution and thus considerable specificity which is further improved when these systems are coupled to a mass spectrometer (MS). Use of HPLC and the hyphenated techniques of HPLC-MS and GC-MS are discussed in separate sections below. Only a brief outline is given here of the other chromatographic techniques, which although less popular than they once were, may still have a role to play in certain situations and in countries where expensive HPLC and/or GLC apparatus is not available.

Steroid hormones circulate bound to albumin and/or specific binding globulin and it is accepted that the physiologically active steroid is the unbound portion which comprises around 10% or less of the total. Solvent extraction, with or without the use of protein denaturing agents and/or binding displacers disrupts the binding and thus extracts the total steroid, although there is one description of the use of 1 μL *n*-octane suspended from the tip of a microsyringe needle to extract free progesterone (Jeannot and Cantwell, 1997). In the clinical context, 'free' or unbound plasma/serum concentrations of steroids may be of value. Measurement of steroids in saliva (Simard, 2004), which is an ultrafiltrate may be a useful, albeit indirect, method of assessing free steroid in the circulation although a number of problems have been identified in salivary testosterone measurements (Granger et al., 2004). For direct separation of the free fraction, some sort of dialysis (e.g. Torma et al., 1995) or ultracentrifugation is still the method of choice today, usually combined with GC-MS as the concentrations involved are at least an order of magnitude lower than total concentrations (van Uytfanghe et al., 2005). A recent survey of androgen assays recommends the use of dialysis, ammonium sulphate precipitation or calculation (Vermeulen, 2005). A recent review on the measurement of androgens deals with the assessment of 'free' steroids as well as hair analysis (Wheeler, 2006).

3.3 Column Chromatography

This technique, as its name implies, relates to the separation of steroids on material packed into columns (usually glass, but can be inert plastic), which can be of any diameter and/or length. The basis of the separation can be:

- Partition chromatography, straight-phase (ordinary) or reverse-phase chromatography
- Absorption chromatography

In partition chromatography the steroid of interest is separated by its relative solubilities in mobile phase (usually, an organic solvent) and a stationary phase (usually, a hydrophilic water based solvent). The solvents can be reversed and the hydrophilic phase can be used as the mobile phase in which case the system becomes reversed-phase (RP) chromatography. In this technique, the stationary phase is mixed with an inert support, usually Celite (a diatomaceous earth) which has been washed and sieved, and the mixture is carefully packed into the column and the mobile phase allowed to pass through by gravity usually from a reservoir attached to the top of the column. Note that the method of packing such columns can exert considerable influence on the final resolving power (Edwards, 1969a). The mobile phase can remain the same throughout the separation (isocratic elution) or it can be varied (gradient elution). The variation of the solvent is achieved by pre-column mixing from two or more separate reservoirs and can be achieved in a number of different ways. These columns may have considerable resolving power which to some degree is determined by their diameter and length and as a rule of thumb the narrower the diameter and the longer the length, the greater the resolution. They do not however approach the resolution which is achieved using HPLC. They are, however, cheap, relatively simple to set up, and capable of dealing with large quantities of material. Many excellent reviews of the use of column chromatography have been published in the past (e.g. Neher, 1964), and interested readers can learn more about this technique from these reviews, which, even though written more than 40 years ago, are still valuable today, particularly as very little recent research has been carried out on this technique. Both straight-phase and reverse-phase partition columns are still in use today. In an investigation of C19 steroid glucuronides in premenopausal women with non-classical congenital adrenal hyperplasia, androsterone and dihydrotestosterone (DHT) were separated by reverse-phase chromatography on Celite columns eluting with increasing concentrations of ethyl acetate in isooctane using ethylene glycol as the stationary phase (Whorwood et al., 1992) or, in the separation of aldosterone prior to RIA, eluting with increasing concentrations of ethyl acetate in *n*-hexane using 30% formamide in water as the stationary phase (Schirpenbach et al., 2006). A very similar technique using ethylene glycol as the stationary phase (Fig. 3.6) was used for the purification of 5α-androstane-3,17-dione from peripheral plasma by Milewich et al., (1992). The same reverse-phase Celite chromatography, in mini-columns as illustrated in this figure, continues to be used (Bixo et al., 1997; Morineau et al., 1997; Silvestre et al., 1998; Ibrahim et al., 2003; Sieber-Ruckstuhl et al., 2006) – even one paper describing the separation of estradiol, testosterone and

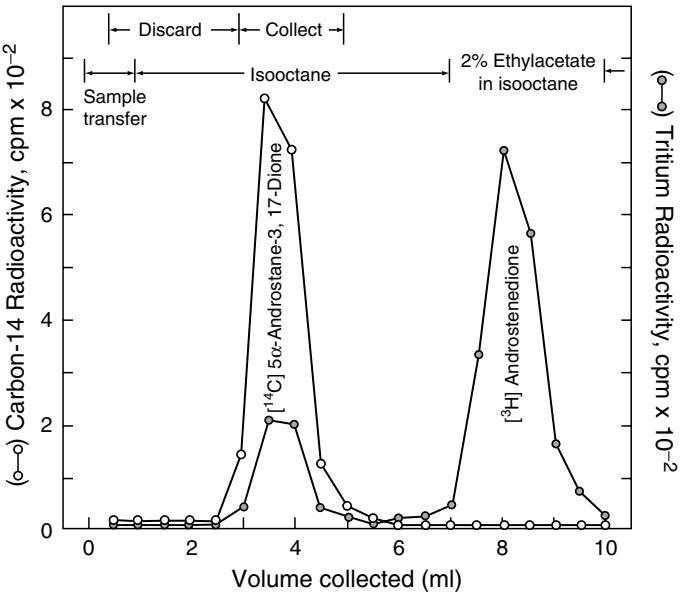

Fig. 3.6 [4-^{14}C]5α-Androstanedione (~2000c.p.m.) was separated from [1,2,6,7-^{3}H]androstenedione (~2000c.p.m.) on a Celite–ethylene glycol column by elution with iso–octane, A mixture of Celite (2.0g) and ethylene glycol was packed into 5ml disposable serological pipettes and conditioned with 5.5ml iso-octane. Samples were added to the column dissolved in iso-octane. After discarding the transfer volume (2 × 0.5ml) and the initial 1.5ml of iso-octane eluent, the next 2ml contained ~98% of [4-^{14}C]5α-androstanedione applied to the column. The bleeding of ^{14}C into the tritium channel was 30%. This chromatographic system was used to separate plasma 5α-androstanedione from more polar steroids. (Reprinted from Milewich *et al.,*1992, with kind permission from Elsevier Science Ltd, The Boulevard, Langford Lane, Kidlington OX5 IGB, UK)

androstenedione in the egg yolks from wild Louisiana alligators (Conley et al., 1997)! For quantitative analysis of allopregnanolone in serum or plasma by radioimmunoassay silica micro-column chromatography was necessary when the steroid levels were low (Bicikova et al., 1995). The method included permanganate oxidation of steroids to remove progesterone which was the main cross-reacting steroid. The separation and characterisation of pregnenolone-3-stearate in rat brain by HPLC required the ethyl acetate extracted steroids of brain to be purified further by silica gel chromatography (Shimada et al., 1997).

The column as an alternative can be packed with adsorbent material and steroids can be separated by selective adsorption to this material being eluted from the column by solvents of increasing polarity. Because these adsorptive materials contain varying degrees of water, the separatory process still involves partition to a small degree. Adsorbents which have been used are Florisil (magnesium silicate), aluminium oxide and silica. An example of the use of aluminium oxide columns is given in Fig. 3.7. Florisil has recently been used for purification of sterols in marine sediments after derivitisation prior to GC-MS (Li et al., 2007). The availability of microparticulate silica which can be pre-packed in cartridges or syringes has been

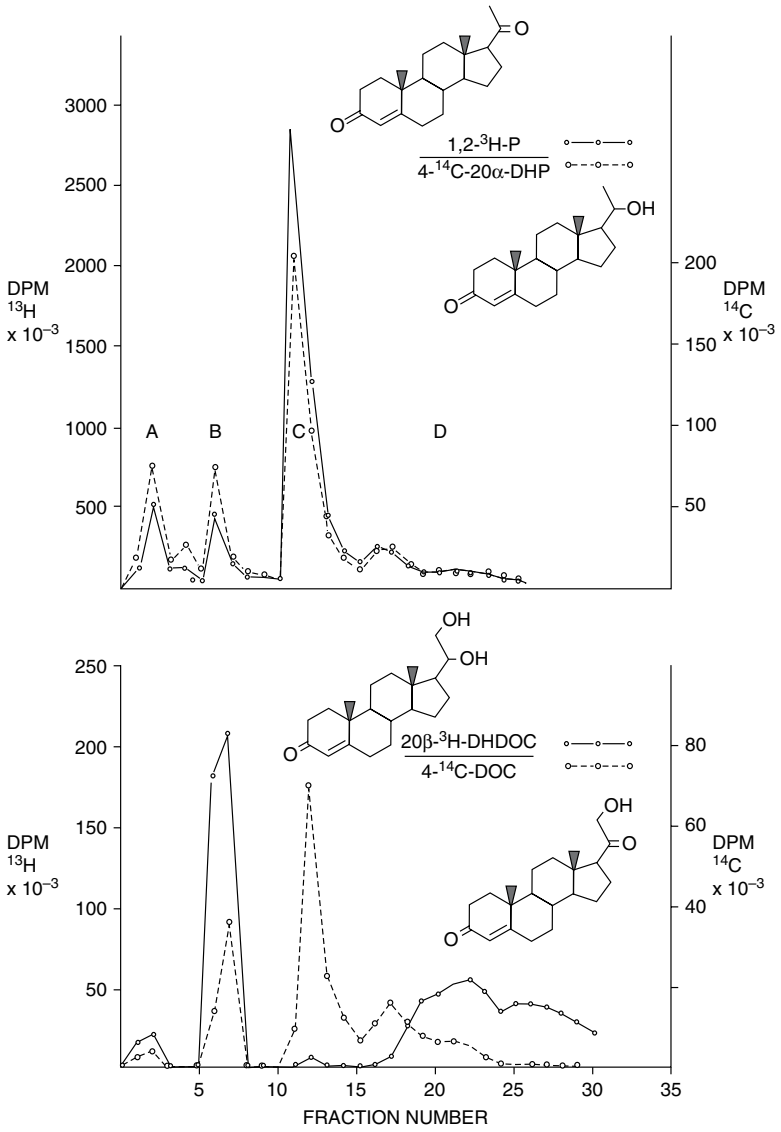

Fig. 3.7 Aluminium oxide separation of rabbit urinary metabolites of progesterone and 20α reduced progesterone (upper trace) and 11-deoxycorticosterone and 20β-reduced 11-deoxycorticosterone. Urine extracts were treated with a β-glucuronidase preparation in 0.2 M acetate buffer, pH 4.5, for 48 h. The aqueous solution was then adjusted to pH 2 with HCI and extracted with ether:ethanol (3:1). After careful drying, the extract was added to an alumina column: 20 g of alumina, deactivated by the addition of water (5%, v/w), added as a slurry in benzene to a glass column (2 cm i.d.). The column was then eluted with ether:ethanol (3:1), 50 ml, ethanol, 20 ml, 50% aqueous (v/v) ethanol, 20 ml, 0.1 M sodium acetate buffer, pH 5, 100ml, and 1 mM sodium acetate buffer, pH 5, 100ml. Eluate fractions (10 ml) were collected and the radioactive content was determined by liquid scintillation counting of a small aliquot of each fraction. Fractions A-D represent: A: 21-deoxysteroids; B: 21-hydroxysteroids; C and D: steroid C-21 acids. (Reprinted from Senciall *et al*., 1992, with kind permission from Elsevier Science Ltd, The Boulevard, Langford Lane, kidlington OX5 1GB, UK.)

discussed in the previous section where ODS-treated silica has been used for the solid-phase extraction of steroids from biological matrices. Microparticulate silica can give excellent separations and has replaced previous column separations using ordinary silica (e.g. use of silica-DIOL column giving comparable separation of cortisol and 11-deoxycortisol to that achieved in mini-celite columns – Morineau et al., 1997). Once again, details of separations of steroids on columns of silica, Florisil and aluminium oxide are given in Neher (1964). Use of microparticulate silica can provide rapid and simple purification and if cartridges are used they may, after washing, be re-used many times and can thus be very cheap. However efficient such cartridges or columns are for small numbers of samples, it must be remembered that where large numbers of samples have to be processed use of any kind of column chromatography can be cumbersome and time-consuming since columns are by their very nature sequential.

The use of SPE cartridges has become very popular for the preliminary fractionation of vitamin D metabolites, but one report (van Hoof et al., 1993) has attempted to resuscitate the use of paper chromatography, pointing out quite correctly that large number of samples can be processed in a single paper chromatographic run. This methodology was subsequently used prior to immunoassay of calcitriol (van Hoof et al., 1999). Similar considerations apply to the use of thin-layer chromatography (TLC) and both these techniques will be briefly discussed below. It is probably not correct to extrapolate from this simple demonstration of the use of paper chromatography for the separation of vitamin D metabolites to suggest that it has much of a role in modern steroid analyses. Separations of steroids using microparticulate silica can provide very satisfactory solutions to many of the problems facing the steroid analyst, and if batch separations are required TLC is most likely to be the preferable option to move to. Microparticulate silica cartridge separation of 11-deoxycortisol and cortisol after solvent extraction (see Fig. 3.8a), prior to immunoassay, has provided a useful method of measuring plasma cortisol levels in patients taking metyrapone (an 11-hydroxylase inhibitor), in whom the concentrations of 11-deoxycortisol rise and interact with the antiserum used in the cortisol immunoassay, achieving a comparable purification to that obtained using reverse-phase HPLC (Wiebe et al., 1991) and mini-celite and sil-DIOL colums (Morineau et al., 1997). Reverse-phase ODS silica has also been used for the separation of steroids and has been used to improve the specificity of an immunoassay for 3α-hydroxy-4-pregnen-20-one by separating the steroid using methanol:water (3:1) prior to immunoassay (Fig. 3.8b)). Microparticulate silica coated with ODS (e.g. Street et al., 1985; Payne et al., 1989) and attached to quarternary amine (Bond-Elut SAX, Scalia, 1990) have been used for the fractionation of steroids into conjugate groups prior to further analysis.

One form of column chromatography which is still widely used is ‘size exclusion’ or ‘gel filtration’ chromatography based on Sephadex, a cross-linked dextran, which has found considerable application (Murphy, 1971) in the separation of steroid groups, such as sulphates, glucuronides etc. Sephadex was designed for use with hydrophilic compounds in polar aqueous solvents but modified Sephadex (e.g. hydroxyalkoxy-Sephadex) lipophilic materials, such as LH-20 (a beaded, cross-linked dextran which has been polyhydroxypropylated) and Lipidex (hydroxyalkoxypropyl-Sephadex), are now available for gel filtration of hydrophobic compounds, such as steroids.

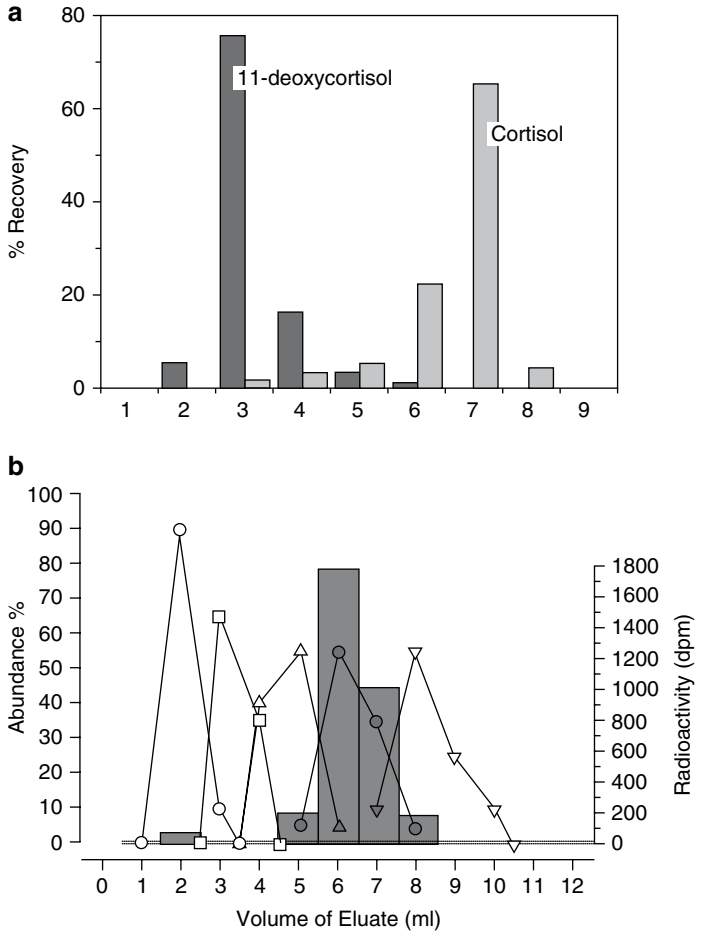

Fig. 3.8 Use of small SPE-C-18 columns or cartridges for the fractionation of steroids. (a) separation of cortisol and 11-deoxycortisol on Sep-Pak SIL cartridges. A mixture of radiolabelled steroids was applied to the cartridge, prepared as recommended by the manufacturers, and the column was sequentially eluted with 5 x 2 ml 1.5% methanol in chloroform (fractions 1-5) followed by 3 x 1 ml of methanol (fractions 6-8). Fractions were collected and the radioactivity was assessed by liquid scintillation counting (courtesy of W. H. Bradbury). (b) Separation of steroids by C-18 minicolumns (6 mm x 42 mm). [^{3}H]-3α-Hydroxy-4-pregnen-20-one (shaded bars), 4-pregnene-3, 11,20-trione (○), 3α-hydroxy-4-androsten-17-one (□), progesterone (Δ), 3α-hydroxy-4-pregnen-20-one (•), and 4-pregnene-3α,20α/β-diols (∇) were added to rat serum. After extracting with ether and washing with NaOH, the dried extract was taken up in 100 µl of methanol:water (3:1) and loaded on to the mini column. The columns were then eluted with methanol:water (3:1) at a flow rate of 0.3 ml/min, using a low vacuum. Fractions (each 1 ml) were collected and monitored by UV absorbance and scintillation counting for radioactivity. (Reprinted from Wiebe *et al.,* 1991, with kind permission from Elsevier Science Ltd, The Boulevard, Langford Lane, Kidlington OX5 1GB, UK.)

Sephadex LH20 columns for the fractionation of steroids was described by Wong et al. (1992) who extracted plasma with acetone:ethanol (1:1, v/v) and applied the dried extract, dissolved in methanol:chloroform (1:1, v/v) to an LH20 column, which was

then eluted with the same solvent, separating the steroids into three fractions; free steroids and steroid glucuronides, monosulphates and, after further elution with methanol, disulphates. Sephadex LH-20 has been used over many years for multi-component steroid analysis and has been incorporated into an automated pre-immunoassay chromatographic system for plasma pregnenolone and 17-hydroxypregnenolone (Riepe et al., 2001). A recent application was the separate measurements of 18-hydroxy desoxycorticosterone (DOC) and 18-hydroxy B from a single plasma extract (Fig. 3.9 – Riepe et al., 2003). Testosterone and many other steroid assays by RIA are notoriously unreliable in the newborn period without prior chromatography. Results for testosterone were up to 3.8 times higher with RIA alone compared with an analysis after Sephadex LH-20 eluted with methanol in benzene (Fuqua et al., 1995) although a safer solvent system would be recommended today. Lipidex is also widely used as a means of purifying steroid trimethylsilyl ethers formed by incubation with trimethylsilylimidazole (TSIM), the reagent being retained in the Lipidex, while the steroid derivative is eluted with hexane (Shackleton and Honour, 1976). Sephadex can also be modified to produce, for example, diethylaminoethyl(DEAE)-substituted Sephadex which acts as an ion-exchange column but still with size exclusion properties. A combina-

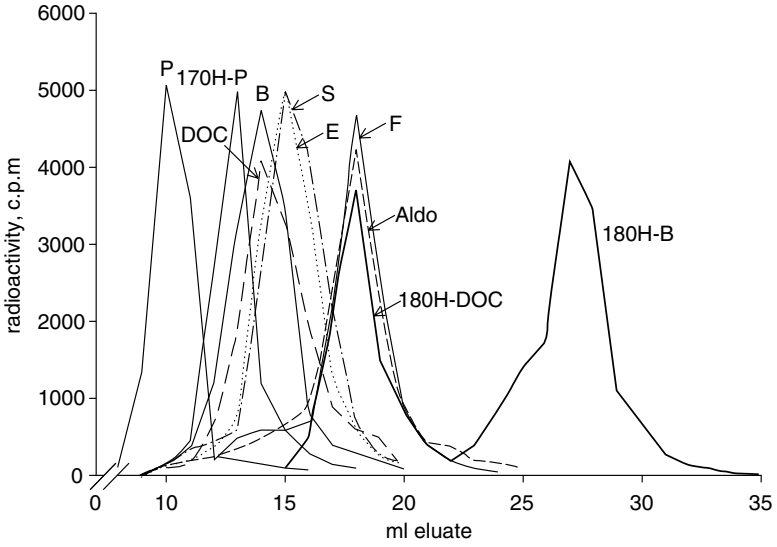

Fig. 3.9 Figure 1 from Riepe et al. (2003) [Chromatogram of tritiated steroids eluted from 45-cm Sephadex LH-20 columns using methylene chloride-acetone (1:2, v/v) as solvent. Peaks eluted: P, progesterone; 17-OH-P, 17-hydroxyprogesterone; B, corticosterone; DOC, 11-deoxycorticosterone; E, cortisone; S, 11-deoxycortisol; F, cortisol; Aldo, aldosterone; 18OH-DOC, 18OH-deoxycorticosterone; 18OH-B, 18OH-corticosterone. A Sephadex LH-20 chromatography system with 450 × 10 mm columns was used for steroid separation. Plasma extracts were redissolved in 1,000 µl of the solvent system and injected into Sephadex LH-20 packed chromatography columns. Elution was performed following gravity. From Riepe et al., 2003 with permission.]

tion of SPE, Lipidex 5000, Amberlyst A-26 and LH-20 chromatography has been used for the extraction of a variety of steroids from blood, liver and faeces prior to GC-MS after derivatisation (Al-Alousi and Anderson, 2002). QAE Sephadex and DEAE Sephadex have been used in a complex pre-GC-MS separation system for oestrogens in urine (Knust et al., 2007).

Urinary steroids can be fractionated into free glucuronide and sulphate fractions after extraction using Sep Pak C18 cartridges using columns of DEAE-Sephadex A-25 (see Fig. 3.10, Hämäläinen et al., 1991) as a preferable and more efficient alternative to the solvent separation procedures previously illustrated in Fig. 3.2. Adlercreutz et al. (2004) have developed a more complex separation system using a variety of ion-exchange resins in sequence to separate oestrogens and phytoestrogens in urine. The use of triethylaminohydroxypropyl (TEAP)-LH-20 as means of extensive and efficient fractionation of steroid groups prior to GC-MS has been described by Axelson and Sahlberg (1983), and this paper also references some of the early works in this field from Sjövall's laboratory. Bile acids can be separated into various conjugated fractions by the use of piperidinohydroxypropyl Sephadex LH20 (PHP-LH20) (e.g. Ikegawa et al., 1992) and the use of ion-exchange columns for steroid separation has been reviewed by Heikkinen et al.,(1983). Many other examples of the use of modified Sephadex for the separation of steroids have been

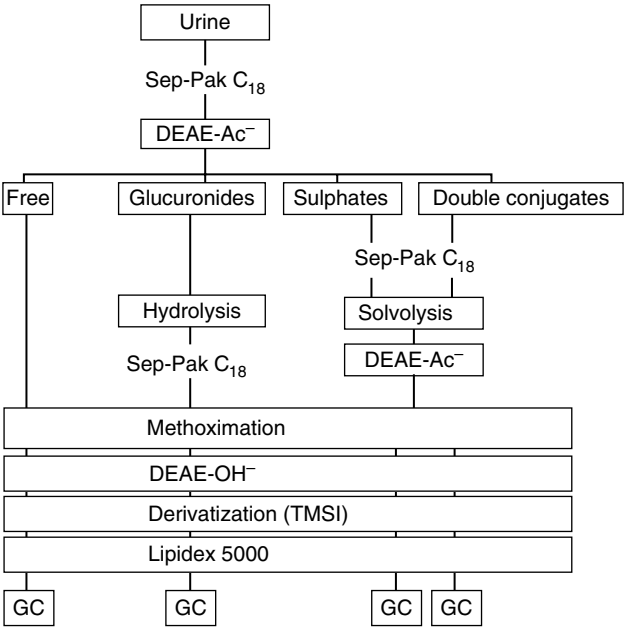

Fig. 3.10 An example of the use of modified Sephadex for the fractionation of urinary neutral steroids prior to GLC analysis (from Hamalainen *et al.*, 1991., with permission)

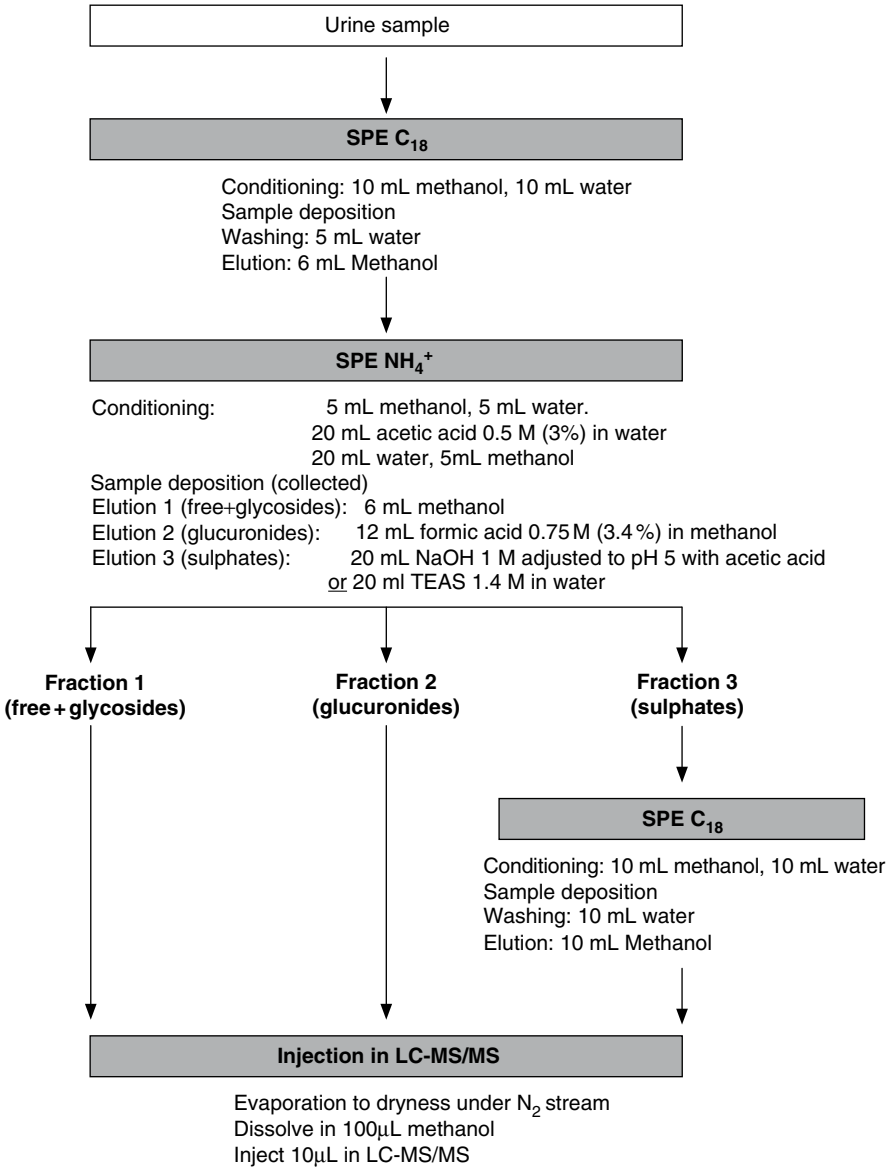

Fig. 3.11 Analytical protocol proposed for a complete separation of the free, glucuronide and sulphate steroids and corticosteroids. (from Antignac et al., 2005, with permission - Figure 4 [*Copyright 2005 Elsevier*])

described (e.g. Setchell and Shackleton, 1973; Archambault et al., 1984; Lisboa et al., 1991; Geisler et al., 2000; Wudy et al., 2002; Riepe et al., 2003; and see review by Sjövall, 2004). A similar separation to that illustrated in Fig. 3.10 can now be achieved using only C18 and NH_4^+ cartridges (see Fig. 3.11).

3.4 Thin-Layer Chromatography

The adsorptive material is coated onto a plate for thin-layer chromatography (TLC). Thin-layer plates can be either prepared in the laboratory or alternatively bought from a number of suppliers. While original TLC was carried out using glass plates, today the support is usually coated onto aluminium foil, which has the advantage that it is light, unaffected by solvents used for elution and areas of interest can be removed by cutting with scissors rather than the alternative procedure of scrapping off the adsorbent material. TLC of steroids is still widely carried out and the adsorptive materials used is usually silica gel although aluminium oxide has been used for the separation of C19 androgens. TLC has the advantage that a large number of samples can be processed in a single chromatographic run. The difficulty with TLC is the need to identify the areas on the plate which correspond to the steroid of interest. Steroids which are ultraviolet (UV) absorbing (e.g., delta-4-3-ones absorb at 240 nm) can be visualised with the use of UV light and adsorptive material is available which contains a fluorescent compound which enhances the UV absorbance of the steroid of interest. Steroids which do not absorb in the UV may have to be visualised by spraying part of the plate to identify the position of standards which have been run together with the samples of interest. There are a wide variety of methods of visualising steroids on TLC plates usually involving spraying and/or heating with a variety of reagents which may or may not be specific for particular types of steroids, and these have been admirably summarised by Edwards (1969b). If standards have not been run, a narrow side strip of the TLC plate can be removed and the steroids located. Fenske (2008) described a TLC method for plasma cortisol which involved dipping the plate into isonicotinic acid hydrazide and quantifying the cortisol by fluorimetry using a scanner.

A further advantage of TLC is that radioactive steroids can be identified by placing the plate in contact with x-ray film, producing an autoradiogram. Dalla Valle et al., 2004; Jang et al., 2007), although metabolites of [^{3}H]progesterone, for example, have been separated by TLC on silica gel, and located by cutting the plates into 1-cm bands and subsequent liquid scintillation counting (Carey et al., 1994). The availability of microparticulate silica has enabled the introduction of the so-called 'high-performance' TLC, which is claimed to have increased resolving power. Reverse-phase systems for TLC have been described but are no longer widely used. Although there are fewer examples of the use of TLC for steroid separation, it is still not uncommon (Fiorelli et al., 2002; Matsunaga et al., 2002, 2004; Robinzon et al., 2004; Waxman and Chang, 2006) The separation of hydroxylated metabolites of progesterone produced by the fungus *Aspergillus fumigatus* has been described (Smith et al., 1994) using Kieselgel 60 F254 (E.G. Merck) eluting with a solvent of ethyl acetate:petroleum (65:35 v/v) and catechol oestrogens, again on silica gel, eluting with benzene:heptane:ethyl acetate (5:2:3:, v/v) or chloroform:ethyl acetate (3:1, v/v) (Jellinck et al., 1991, 2006). The separation of five androstane isomers by TLC has been carefully

optimised for solvent system (Cimpoiu et al., 2006). Placental metabolites of synthetic corticosteroids were examined using TLC to address particularly the activity of HSD11B2 (Murphy et al., 2007). Jellinck et al. (2005) have separated 7α- and 7β-hydroxy DHEA by TLC on silica gel using xylene:ethyl actetate:chloroform (40:20:40) eluting solvent.

Vitamin D metabolites have also been separated by high-performance TLC (Thierry-Palmer and Gray, 1983) and the use of TLC in the separation of the vitamin D metabolites has been advocated as a useful method in countries where HPLC is not available (Justova and Starka, 1981).

Although TLC is still used for steroid separations, very little development has taken place in the last 30 years and it remains very much as it was in the 1980s. Some excellent reviews have been published but a useful introduction to methods available for a variety of steroids was given by Heftmann (1983) from which an introduction to the past literature can be obtained. TLC is still widely used to demonstrate purity of pharmaceuticals (Kotiyan and Vavia, 2000) and to demonstrate the reactivity of tissues and transfected genes (Lee et al.,1998; Godin et al., 1999; Gupta et al., 2001). Pharmaceutical impurities of less than 0.5% are possible without detection (false negative). Data from TLC experiments may thus need to be supplemented by more sensitive tests such as GC, GC-MS, HPLC, LC-MS (Engelbrecht and Swart, 2000; Gorog, 2004; Jellinck et al., 2005, 2006). Table 3.1 gives some examples of the use of TLC in the period 1995-2006, illustrating the continuing popularity of this simple and cheap technology and its wide application in the steroid field, from oestrogens to bile acids.

3.5 Paper Chromatography

Paper chromatographic systems, both straight- and reverse-phase Bush (1961), are seldom used today although careful scanning of the modern literature will still occasionally unearth the use of a paper chromatographic separation. Such separations have been used for the purification of radiolabelled steroids prior to use (Whorwood et al., 1992) and indeed may sometimes be used for the purification of a steroid prior to quantitation – in one such case, testosterone was purified by descending paper chromatography using a Bush A system (Bush, 1961) prior to immunoassay (Swinkels et al., 1992). It is interesting to note here that these authors are the same group that recommended the use of paper chromatography for the separation of vitamin D metabolites (van Hoof et al., 1993), although it is not clear precisely what advantage the use of paper chromatography has in this context, although these same authors have used it subsequently (Van Hoof et al., 1999). Androgens in seminal plasma were significantly lower by RIA after paper chromatography (Zalata et al., 1995). Measurement of cortisol in patients with chronic renal failure, who were given dexamethasone to suppress the cortisol

Table 3.1 Some examples of methods using of thin-layer chromatography for steroid separation since 1995

Application	TLC type	Eluting solvent	Ref
Testosterone metabolites	Silica gel (Whatman LK5DF)	$CHCl_3$:acetone(9:1) followed by CH_2Cl_2:EtAc: 95%EtOH (7:2:0.5)	Agrawal et al. (1995)
7-Hydroxylated metabolites of pregnenolone in mouse brain microsomes	Silica gel (Merck F254)	Ethyl acetate	Doostzadeh and Morfin (1997)
Steroidal alkaloid glycosides	Silica gel	$CHCl_3$:MeOH: ammonia soln (7:2.5:1)	Tanaka et al. (1997)
Analysis of androst-5-enediol in human prostate cancer cells	Not specified	Toluene:95% ethanol (9:1) x2	Miyamoto et al. (1998)
Bile acids and conjugates	? + Methyl-β-cyclo-dextrin	RP-HPTLC 2D	Momose et al. (1998)
C19 steroids formed in mammalian cells	Silica gel (Whatman) impregnated with $AgNO_3$	Toluene:acetone: $CHCl_3$ (8:2:5)	Godin et al. (1999)
Microsomal metabolites of DHA	Silica gel (F254)	2-D (1) Tol:MeOH (9:1) (2)$CHCl_3$:ether (1:1)	Schmidt et al. (2000)
Estradiol in pharmaceutical preparations	Silica gel 60F254	$CHCl_3$:acetone: IPA:gla. Acetic acid (9:1:0.4:0.1)	Kotiyan and Vavia (2000)
Isolation of aldosterone ester from heart	Florisil	Ethanol:$CHCl_3$ (2:98)	Gomez-Sanchez et al. (2001)
Estrogen metabolism in colorectal cancer cells	Silica gel Merck F254)	CH_2Cl_2:EtAc (4:1)	Fiorelli et al. (2002)
7-Hydroxylation of DHEA in Alzheimer's	Silica gel (F254)	$CHCl_3$:EtAc (4:1)	Weill-Engerer et al. (2003)
7-hydroxylation of DHEA in pig liver microsomes	Silica gel GF200 m	EtAc:Hx: gla Acetic acid (18:8:3)	Robinzon et al. (2004)

(continued)

Table 3.1 (continued)

Application	TLC type	Eluting solvent	Ref
DHEA metabolism in brain	Silica gel (F254)	Xylene:EtAc: $CHCl_3$ (40:15:45)	Jellinck et al. (2005, 2006)
HPTLC of androstenes	Silica gel (F254)	Bz:MeCN, Bz:EtAc Bz:dioxane	Perisic-Janjic et al. (2005)
Androgen metabolism in synovial cells	Silica gel (Merck F254)	Tol:MeOH (9:1) then at right angles $CHCl_3$:ether (1:1)	Schmidt et al. (2005)
Estradiol as aromatase inhibitor in breast cancer cells	Silica gel 60 F254	$CHCl_3$:EtAc (4:1) or CycloHx:EtAc (1:1)	Pasqualini and Chetrite (2006)
6β-Hydroxylation of testo. by CYP3A	Silica gel	CH_2Cl_2:acetone and $CHCl_3$:EtAc:EtOH	Waxman and Chang (2006)
Interconversion of 7α- and 7β-OH DHEA	Silica 60	$CHCl_3$:EtOH: H_2O (87:13:1)	Muller et al. (2006)
Optimisation of separation of 5 androgens	Silica 60	7 Different systems	Cimpoiu et al. (2006)
P450c17 assay in fish tissues	Silica Gel (Merck 5729)	CH_2Cl_2:EtAc:MeOH (85:15:3)	Zhou et al. (2005)
Purification of pyridine-carboxylate oxosteroid derivatives	Silica gel 60 F254 (Merck)	Not given	Yamashita et al. (2007a)
Metabolism of synthetic steroids by human placenta	Silica gel 60 F-254	$CHCl_3$:MeOH (95:5 or 97:3)	Murphy et al. (2007)
Microglia conversion of DHEA to androst-5-enediol	Silica gel	Xylene:EtAc:$CHCl_3$ (10:25:75)	Jellinck et al. (2007)
5α-Reductase activity	HPTLC Keiselgel 60 F254	$CHCl_3$:acetone (9:1) × 3 air-drying between each run	Bratoeff et al. (2007)
5α-Reductase activity	Silica gel	Toluene:acetone (8:2)	Jang et al. (2007)
Application	TLC type	Eluting solvent	Ref

(continued)

Table 3.1 (continued)

Application	TLC type	Eluting solvent	Ref
Cholesteryl ester hydroperoxide isomer analysis	Silica gel 60 F254	Hx:ether:acetic acid (70:30:1) blotting onto PVDF membrane prior to GC-EI(+)-MS	Minami et al. (2007) and Kawai et al. (2007)
DHEA conversion to 5-androstenediol	Silica gel	Xylene:EtAc:$CHCl_3$ (10:25:75)	Jellinck et al. (2007)
Steroid sulfatase activity - separation of E1 & E2	Silica gel G/UV-254 Alugram	13% EtOH in toluene	Stute et al. (2008)
DHEA metabolism in songbird brain	Silica gel	$CHCl_3$:EtAc (4:1)	Pradhan et al. (2008)
Estradiol and progesterone inhibit sulfatase activity	Silica gel 60 F254	$CHCl_3$:EtAc (4:1)	Chetrite et al. (2007), Pasqualini & Chetrite (2008)
Novel P450c17 in fish	Silica gel Merck 5729	CH_2Cl_2:EtAc:MeOH (85:15:3)	Zhou et al. (2007)
Brain microglia express steroid converting enzymes in mice	Silica gel aluminium sheets	$CHCl_3$:EtAc:xylene (62:21:17) and (68:23:9)	Gottfried-Blackmore et al. (2008)
5α-Reductase in normal and neoplastic prostate biopsies	Kieselgel 60 F254	EtAc:Bz (2:1)	Oliveira et al. (2008)
Separation of testosterone & esters	Variety of plates: silica, ODS and aluminium oxide	Variety of solvents in chamber for horizontal HPTLC at 20°C	Zarzycki and Zarzycka (2008) Zarzycki (2008)

production, were superior after paper chromatography than by direct RIA (Van Herle et al., 1998). It is our experience that paper chromatography using Bush systems is time-consuming and significant amounts of potentially interfering non-specific material is usually eluted from the paper together with the steroid of interest, giving rise to high blank values. While careful washing of the paper prior to chromatography can often reduce the blank values, this is not always the case and in our view there is very little to recommend the use of paper chromatographic systems for steroid separation today.

3.6 Gas–Liquid Chromatography

Gas–liquid chromatography (GLC or GC) is a partition system where the steroid solute is in the vapour phase. Because of the relatively high molecular weight of steroids and their derivatives, GLC has to be carried out at high temperatures, usually in excess of 200°C. The vaporised steroid, once introduced into the GLC column, is carried through the system by a gas, usually helium, because it is less dense than nitrogen, gives improved separation but is of course much more expensive to buy. Better separation can be achieved with hydrogen (Impens et al., 2001) but there are safety issues to be considered. For many years, the separation procedure with the greatest resolving power was gas chromatography (the early years of GLC were reviewed by Horning, 1968), originally carried out using packed columns but today capillary columns of glass or fused silica are more popular. The analysis of bile acids by GLC was reviewed by Batta and Salen in 1999, and since then they have extended the analysis of faeces to achieve a lipid profile (Batta et al., 2002). The measurement of glucocorticoids in biological fluids using, inter alia, GLC has been reviewed by Holder (2006).

3.6.1 Column Technology

Steroids of interest are separated by their relative solubility in the stationary phase which is a thermostable compound, liquid at the column temperature used, which is coated onto an inert support, usually Celite in packed columns or onto the walls of the tube in capillary columns. Wall-coated columns give better separation as would be expected from the increased number of theoretical plates produced by this methodology. There are a wide variety of stationary phases which have been used for the separation of steroids and the catalogue of any chromatography supplier will give lists of such material. Stationary phases are, however, usually substituted siloxane polymers which can be modified chemically. The commonest non-selective (i.e. separation is achieved purely by molecular weight which is related to vapour pressure) stationary phase was SE30 which has now been superseded by OV1 or OV101. Chemical substitution of other groups onto the siloxane polymer gives rise to further stationary phases which may have selective characteristics. For example, the use of cyano-substituted siloxane polymers is useful for the separation of ketones. It is necessary that these stationary phases be thermostable and that they are not eluted from the column. The elution of the stationary phase from the column, so-called column ‘bleed’, is less with capillary columns than packed columns and has been largely overcome by the use of chemically bonded stationary phases. There are a wide variety of stationary phases and pre-coated columns available and these are usually listed, together with their characteristics, in chromatography catalogues from a large number of commercial firms active in this area. Good examples of this are the catalogues

published by Chromatography Products and Supelco (Poole, Dorset, BH17 7NH) or Jones Chromatography (Hengoed, Glamorgan CF8 8AU). Complete resolution of all the steroids of interest may not be achieved using a single column, and it may be necessary to use a combination of columns. The separation of 6-hydroxy bile acids is an example, where a combination of CP-Sil-19 CB and CP-SIL-5 CB columns were needed (Batta et al., 1995).

Originally, the columns used for GLC were coiled glass columns of approximately 0.4 cm in diameter, 1–5 m in length, silanised by treatment with dimethyldichlorosilane, and packed with the stationary phase which was coated onto an 'inert' support which was usually acid-washed and sieved Celite. On many occasions, the 'inert' support was in fact not as inert as it was claimed to be and adsorption problems occurred leading to poor peak shape and sometimes loss of steroid. GC separation of bile acids has been reported using stainless steel capillary columns (Iida et al., 1995). The modern capillary columns, now usually wall-coated open tubular (WCOT) where the stationary phase is coated on the inside of fused silica columns of diameter of approximately 0.2 mm, although megabore columns (0.5–0.75 mm i.d.) with greater capacity, but reduced resolution, are also available. The thickness of the coating (from around 0.1 μm upwards) affects the capacity of the column, the greater the thickness, the greater the capacity. The effect of the thickness of phase coating are interdependent with those of changes in internal diameter and this relationship can be expressed as a 'phase ratio' (β), to which capacity has an inverse relationship. WCOT columns like their predecessors still suffer from the problem of column bleed at high temperatures and attempts have been made to overcome this problem by the use of chemically bonded stationary phase. This process can affect retention times of the steroids. Capillary columns can be of any length up to around 30 m, and at such lengths, they provide one of the most effective means of separating steroids with extremely high resolution. Short and micro bore columns are becoming fashionable for fast chromatography – a urine steroid profile using such columns can be obtained in less than 8 min with a 10-cm fine column compared with 40–60 min on a 30-m column. The GC needs an injector and flow control that withstands very high pressures when narrow columns are used. Short columns can enable faster separation but narrow bore columns can be easily overloaded with sample. Few publications have yet described the benefits for steroids (Rossi et al., 1994).

The increased resolution of capillary columns has reduced the need to use more than one stationary phase and for most applications a polar bonded-phase column is effective. When GC is used alone, polar columns are still necessary to fully separate all steroids but in GC-MS a single column, particularly when SIM is employed, is generally adequate in most situations. In our instruments, we use J & W (J & W Scientific, Folsom, CA 95630 - see www.chromtech.com) 15 m DB-1 polar bonded-phase columns of 25 mm i.d. and 25 μm film thickness, which are solvent rinseable. The upper temperature limit for sustained periods is 325°C, although some columns can be used to 400°C or more without significant column degradation. Some years ago, there was a need to separate methyl ether and trimethylsilyl ether derivatives of cortolic acids by gas chromatography without mass spec-

trometry. This was a case where only a polar column was effective in separating α-cortolic, α-cortolonic, β-cortolic and β-cortolonic acids. A Carbowax 20 M column (polyethylene glycol stationary phase) was used but like most polar columns, it had limited lifetime (Shackleton et al., 1980a). While Carbowax 20 M can still be purchased, superior performance is offered by J & W in the form of DB wax. These newer columns have bonded ethylene glycol stationary phase so are solvent rinseable, more robust and have less bleed than earlier columns.

3.6.2 Sample Injection

Apart from the qualities of the column itself, the length of time a column can be left untouched in an instrument is largely dependent on the injection system. In injection systems where only volatiles reach the capillary (solid injection, splitless injection with cold trapping), the first few centimetres of the column remain relatively clean for many injections over several weeks. In injection systems where solubilised material goes directly into the column (split injection, on-column injection), the first part of the column soon contaminates and must be cut off periodically. This results in a gradual shortening of column length which often does not affect resolution but may require data-system re-programming for automated instruments since the retention times of expected components change markedly.

The most generally applicable injection system for steroid derivatives is *splitless* injection with *cold-trapping*. This system allows a large injection volume (~2 mL) to be used. This vaporises in a removable glass insert in the heated injection port and the heavier (less volatile) components condense in the initial part of the column which is maintained at near ambient temperature (cold-trapping). During temperature programming, the solutes re-vaporise when they get to their volatilization temperature and are duly separated. This system keeps the column relatively clean since most of the involatile material is deposited in the injector glass insert. These inserts must be replaced every few days and for some separations silanisation of the insert is advisable. There are refinements to injection systems with pulsed and pressure features. Injection liners come in many shapes to affect maximum delivery of the sample depending on volatility, sample volume and sensitivity.

The *falling needle* solid injection system and its automated variant which employs a carousel containing a multitude of glass sample capillaries (Shackleton and Honour, 1976) are less commonly used. This system was favoured for many years before the introduction of the splitless system but it does have the disadvantage that in automated mode samples remain dry for many hours before analysis. Unstable derivatives are particularly vulnerable in this system. The solid injection systems have a great advantage in that the total amount of sample can be concentrated on the needle or in the injection vial. In syringe injection systems, particularly automated ones, the amount of sample that can be injected is usually only a small fraction of the solvent volume.

On-column injection from a syringe is particularly useful for minimizing gas chromatographic discrimination against high-mass components, but the heavy column contamination resulting mitigates against using the technique. There also seem to

be few reasons outside industrial analysis for using *split* injection since this both wastes much of the analyte solution and results in heavy column contamination.

3.6.3 Derivative Formation

GLC, as already mentioned, requires the steroid solute to be present in the vapour phase and capable of analysis without destruction at the high temperatures required for this. Androgens and oestrogens can be analysed by GLC without derivatisation since they are stable at the oven temperatures required but have long retention times and occasionally may be dehydrated. In addition, the presence of underivatised hydroxyl groups may impair the chromatographic resolution due to adsorption during chromatography. C21 steroids with the 17-hydroxycorticosteroid side chain undergo thermally-induced side-chain cleavage giving 17-oxo steroids and vitamin D metabolites undergo B-ring cyclisation, giving *pyro*- and *isopyro*-isomers (see Chapter 11 on vitamin D in this book). In order to improve chromatographic resolution and to prevent sidechain cleavage, chemical derivatisation is usually carried out prior to chromatography.

The majority of methods using GC (and GC-MS) for identification and measurements of steroids and metabolites with low detection limits require formation of derivatives prior to GC. A review of some useful derivatives, albeit in the context of GLC-mass spectrometry, is given by Brooks et al. (1983) and the place of GC-MS in the era of molecular biology is commented on by Wudy and Hartmann (2004). Halket has published a number of excellent reviews on this topic (Halket and Zaikin, 2003, 2004, 2005, 2006; Zaikin and Halket, 2003, 2004, 2005, 2006). Trimethylsilyl ether (TMS) and other alkylsilyl ethers (such as tertiary-butyldimethylsilyl ether) are suitable for hydroxyl groups and *O*-methyloxime (MO) for ketones. Enol-TMS ethers are formed on ketones with *N*-methyl-*N*-trimethylsilylfluoroacetamide-trimethylsilyliodosilane. These enol-TMS ethers are used in doping control for the detection of anabolic steroids (Saugy et al., 2000) and for corticosteroids (Choi et al., 2002). There have been some doubts recently about the reliability of this derivative procedure for anabolic steroids (Meunier-Solere et al., 2005) and oestrogens (Shareef et al. 2004, 2006). Pentafluorophenyldimethylsilyl-TMS ether (flophemesyl) derivatives give intense molecular ions in MS that are attractive for GC-MS analysis (Choi and Chung, 1999; Choi et al. 2001). Heptafluorobutyrates have been used for quantitative analysis of several steroids (Scherer et al., 1998; Wudy et al., 2000, 2001, 2002). Bismethylenedioxy-pentafluoropropionate derivatives have been used in the analysis of cortisol metabolites (Furuta et al., 2000a, b). The simultaneous analysis of fatty acids, sterols and bile acids as *n*-butyl ester TMS derivatives has been reported (Batta et al., 2002).

Hydroxyl groups can be derivatised in a number of ways but usually ethers are formed. Use of dimethyldichlorosilane, trimethylchlorosilane and bistrimethylsilylacetamide (BSA) or bistrimethyltrifluoroacetamide (BSTFA) will form trimethylsilyl derivatives on most of the steroid hydroxyls. Use of BSA or BSTFA alone will only form such derivatives on non-sterically hindered groups, whereas trimethylsilylimidazole (TSIM) will react with sterically hindered groups (such as the 25-hydroxyl in 25-hydroxyvita-

min D or the 17α-hydroxyl in the corticosteroids). Other silyl derivatives have also been used for the separation of bile acids (Iida et al., 1992, 2001; Batta et al., 1998). Other ester derivatives such as formates and acetates have also been used. The choice of derivative which is formed will depend upon the method of quantitation as well as the need for good chromatography. The use of tertiary butyldimethylsilyl ethers for quantitative GC-MS is very common since these derivatives give mass spectra without extensive fragmentation (Finlay and Gaskell, 1981; Masse and Wright, 1996). Vicinal hydroxyls can be derivatised to give cyclic alkyl boronate esters and the formation of these derivatives with unknown steroids gives an indication of the structure of the steroid (Brooks and Harvey, 1969). Oxo groups can be derivatised as oximes using methoxyamine hydrochloride in pyridine although enol-trimethylsilyl ethers can also be formed. The carboxyl group of steroid acids must also be derivatised prior to GLC and usually methyl esters are formed and there are simple methods available for this (e.g. Lillington et al., 1981). Mixed esters can also be used, such as the formation of cyclic boronate esters across vicinal hydroxyl groups and subsequent trimethylsilyl ether formation on the remaining hydroxyl groups, which has been used with vitamin D metabolites (Coldwell et al., 1984, 1990). The choice of derivatives for GLC must be a balance between the requirements of the chromatography and those of the detector.

3.6.4 GLC Detectors

Steroids separated by GLC are detected by one of the three main methods, the commonest of which is the flame ionisation detection (FID), which responds to all steroids with varying response factors. In order to use GC-FID for the quantitation of steroids, therefore, it is necessary to set up a standard curve or establish a response factor for the steroid of interest, assuming a straight-line response. Compounds containing nitrogen atoms can be detected with a nitrogen-phosphorus detector (NPD), but as most steroids commonly encountered do not contain nitrogen, using this detection system requires the formation of nitrogen-containing derivatives such as methyloximes (e.g. Vanluchene et al., 1990). This detection system can therefore be quite useful for the selective measurement and detection of steroids containing oxo groups. The third method of detection involves the use of electron capture, a potentially extremely sensitive detector, which requires electron-capturing moieties (usually, halogen atoms) in the analyte. With a few synthetic exceptions, steroids do not contain halogen atoms and thus derivatives (e.g. halogenated trimethylsilyl ethers, Pinnella et al., 2001) have to be made. A good example of the use of halogenated silyl ether derivatives is in a method for the measurement of DHA in plasma by GLC with electron capture detection after the formation of iodomethyldimethylsilyl ethers (Chabraoui et al., 1991). This report is also of interest in that it used reverse-phase Celite columns to purify the analyte after extraction with ether and aluminium oxide columns to purify the silyl ether derivative prior to GLC. Reports of methods using GLC with electron capture are becoming increasingly rare as these detection systems are difficult to use and are susceptible to detector contamination. GLC-EC, although extremely sensitive, is also very difficult to use quantitatively and although it was of

considerable value in early measurements of plasma steroid hormones, it has largely been replaced by interfacing the GLC with a mass spectrometer which when operated correctly can be as sensitive as electron capture and more selective. Pentafluorobenzyl and pentafluoropropionate derivatives have been used as electron capturing derivatives for oestrogens by GC-NI-CI-MS (Brandon et al., 1999; Kim et al., 2000; Evans et al., 2005). Other derivatives have been reviewed by Shimada et al., (2001).

GLC can be linked to mass spectrometers and the availability of simple bench-top spectrometers has reduced the cost of GC-MS. The application of MS to the analysis of steroids is discussed below. The use of capillary gas chromatography for the analysis of urinary steroid profiles is a very good example of the valuable use of GLC-FID using trimethylsilyl ether-*O*-methyloxime derivatives (Shackleton et al., 1980b - Figure 3.12) and when such systems are also linked to an MS, allowing identification of unusual peaks, they can be of immense value (e.g. Palermo et al., 1996; Shackleton, 2008). An example of such a profile for a normal man is given in Fig. 3.12). This is a typical example of such a profile, which provides useful and rapid information in

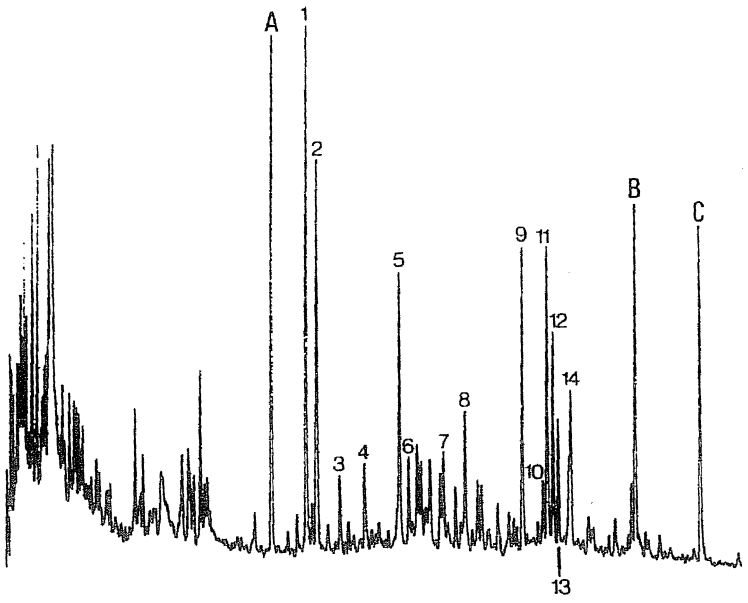

Fig. 3.12 Urinary steroid profile from a normal man 28 years of age. Urine samples were treated with β–glucuronidase and sulphatase enzymes and steroids extracted with Amberlite XAD-2 and Sephadex LH-20. Steroids were then derivatised forming pertrimethylsilyl ethers on all hydroxyl groups and *O*-methyl oximes on oxo groups. GLC was carried out using a 15–25m WCOT column with OV1 or OV101 and using a temperature programme from 160°C to 260°C at approximately 2.5°C/min. Three internal standards were used for the GLC: A, 5α-androstane-3α, 17α-diol; B, stigmasterol; and C, cholesteryl butyrate. Steroids (underivatised) are identified as follows: 1, androsterone; 2, aetiocholanolone; 3, DNA; 4, 11-oxo-aetiocholanolone+11-oxo-androsterone; 5, 11β-hydroxyandrosterone+17α-hydroxypregnanolone; 6, 11β-hydroxyaetiocholanlone; 7, pregnanetriol; 8, androstenetriol; 9, tetrahydrocortisone; 10, allotetrahydrocorticosterone; 11, tetrahydrocortisol; 12, allotetrahydrocortisol; 13, α-cortolone; 14, β-cortolone+β-cortol (from Shackleton et al., 1980b, with permission)

the clinical setting. A recent modification (Pujos et al., 2004) of this technique for urinary steroids involved solid-phase extraction and separation of the extracted steroids using a second SP cartridge into three fractions containing DHEA sulphate, corticosteroids and androgens. Each fraction was analysed separately, the corticosteroids being oxidised to androgens before analysis. It is not clear what advantage is conferred by this somewhat cumbersome methodology.

Some methods not requiring hydrolysis of steroid conjugates have been described. DHEA-S decomposes with loss of sulphuric acid at the elevated temperatures of the GC injector to give reproducibly one of the three isomers with double bonds in the A and B rings. Methyl ester formation before TMS ether will protect glucuronides for GC (Zemaitis and Kroboth, 1998). A method for the separation of the 3-glucoside and 3-glucuronide conjugates of bile acids was described (Iida et al., 1995). Applications of GLC systems, not using mass spectrometry, for the separation of steroids are decreasing, with the exception of the use of urinary steroid profiling, which is still a useful clinical tool, even when GC-MS is not used (Taylor, 2006). There are some useful reviews (Wolthers and Kraan, 1999; Shimada et al., 2001; Volin, 2001; Appelblad and Irgum, 2002).

3.7 High-Performance Liquid Chromatography

HPLC has become an increasingly important chromatographic technique for steroids because:

(i) High temperatures are not required.
(ii) Choice of stationary and mobile phases for optimal separation.
(iii) Material can be recovered from the column eluates for further analytical procedures.
(iv) The resolution achieved by HPLC is superior to TLC and paper chromatography,
(v) HPLC offers the potential and versatility for separation of intact conjugates.
(vi) Although most steroid metabolites are virtually without ultraviolet absorption, which is the most useful of current detectors, some further metabolites of steroids can be detected with a refractive index or an electrochemical detector or by the use of pre- or post-column reaction with compounds which cause enhanced UV absorbance, fluorescence etc.
(vii) Methods of linking HPLC to mass spectrometers have greatly improved, allowing the routine use of LC-MS and LC-MS-MS (see below).

Methods for HPLC separation of steroids up to 1987 have been reviewed by Makin and Heftmann (1988). Reviews by many authors (Volin, 1995, 2001; Marwah et al., 2001; Shimada et al., 2001; Gorog, 2004) consider a number of HPLC separations of steroids in relation to clinical applications and sensitivity, respectively. Diode array detection (DAD) improves the quality of UV data, but is less sensitive.

There have been a few papers since 1995 with new applications of HPLC (e.g. Hu et al., 2005) and Table 3.2 is a selection of LC methods in the period 1990-2008 but does not include LC-MS or LC-MS-MS methods, which are discussed later (see also review by Honour, 2006). LC-MS-MS (i.e. tandem mass spectrometry) seeks to use the flexibility of the detector to obviate the need for extensive clean up of samples. In essence, a short HPLC column is little more than an injection system and "dilute and shoot" analysis is expected. There are a number of dangers here that will be addressed elsewhere in this chapter.

3.7.1 Columns

The separation of steroids with HPLC can be effected by absorption, partition, ion-exchange, reversed-phase (RP) and reversed-phase ion-pair chromatography. High-performance silica and alumina columns give excellent separation of steroids. RP columns eluted with polar binary solvent mixtures, usually methanol or acetonitrile with water, are now used widely. RP columns using microparticulate silica coated with C18, C8, C2 and phenyl materials (listed in Fig. 3.3) have been used. The chromatography depends largely on partition so that selectivity will vary to some extent with the carbon chain length and the nature of the mobile phase. A risk with RP packings is that very non-polar material will accumulate on the columns and decrease separation. This can be prevented to some extent by the use of a guard column (30–70 mm in length) containing the pellicular equivalent of the analytical column. Guard columns are cheap and can be dry-packed. The first few millimetres of packing from the analytical column can also be replaced at intervals.

RP columns with 60,000–80,000 theoretical plates per metre are common. These offer excellent resolution and sharp peaks permit detection by UV absorption of around 1 ng of steroid injected onto the column. Typically, columns are 100–300 mm in length and around 4–5 mm internal diameter. Cyano and amino phases have been used to effect the separation of corticosteroids (Ando et al., 1986). HPLC of polar oestrogens has been achieved on ion-exchange columns (Musey et al., 1978). Micro-bore columns (<2 mm i.d.) may permit increased sensitivity by narrowing the elution peak, but depending on the volume of sample and the total mass of material in the extract, there may be a loss of peak shape and resolution.

The complete separation of naturally occurring mixtures of steroid hormones poses problems due to the wide range of polarities and the tendency for steroids of similar polarity derived from different metabolic pathways to elute in clusters. Careful selection of the stationary phase from the range of commercially available products can enable a system to be devised with high selectivity (O'Hare et al., 1976; Schoneshofer and Dulce, 1979). Silica packings to which are bonded octadecyl or diol groups are most popular for general use. Supports differ in particle size, porosity and levels of residual accessible silanol groups. Synthetic polymers may be more inert than silica. The physical characteristics of many packings have been studied with various solvent gradients. There seems to be no

easy means to identify the most suitable packing for a particular separation. Selective differences cannot be firmly attributed to alkyl chain length or to shape of the packing. Immobilised cyclodextrins, macrocyclic polymers of glucose, which have been used for the extraction of steroids because of their ability to form inclusion complexes (vide supra), have also been introduced as stationary phases (cyclobond) for steroid chromatography (Agnus et al., 1994; Zarzycki et al., 2006; Clifton et al., 2007 and see Table 3.2) and may have advantages to offer but that may depend on carbon load (Zarzycki et al., 2002). Carbon-coated zirconia was compared with porous graphitic carbon stationary phase for separation of equine conjugated estrogens giving separations superior to C18 and alkyl-bonded silica phases (Reepmeyer et al., 2005). In a similar fashion, graphitised carbon, which has been used for crude steroid fractionation (Andreolini et al., 1987, see Fig. 3.4), may also have use as a stationary phase for steroid HPLC since it is micro-crystalline and contains no unreacted silanol groups such as those on silica-based materials and thus may be considered to be a suitable inert material for RP chromatography.

Supports have variable and often incomplete coverage of residual silanol groups ('uncapped') which affects separation, peak shape and recovery. Some packings with about 5% of uncapped silanol groups are chemically reactive with steroids due to intramolecular hydrogen bonding. This leaves the phase acidic and may explain the instability of certain steroids in such systems. Aldosterone and 18-hydroxylated steroids are susceptible to a number of reactions on certain columns which can influence the quality of the HPLC result. Acid, such as may be found on uncapped HPLC supports, can lead to ring closure of such steroids with a bridge of C-18 to C-20 or C-21. In the presence of methanol, this may lead to the formation of methyl ethyl ketals. Other products, dimers and isomers are possible leading to the production of a number of peaks in the HPLC analysis of a single compound. These products can have retention times spread throughout a solvent gradient elution of a RP column. This may be disastrous in the interpretation of a metabolic study unless products are characterised by other means. Some supports are not recommended for aldosterone and related steroids, e.g. 18-hydroxycorticosterone (O'Hare et al., 1980). The extent to which a packing is not covered (end-capped) can be determined by a methyl red absorption test (O'Hare and Nice, 1981). Glycine-conjugated bile acids can be separated by using RP coated silica columns (Nambara and Goto, 1988) and Fig. 3.13 illustrates an example of such a separation carried out on a reverse-phase ODS silica column, using gradient elution with mixtures of acetonitrile:methanol:water using fluorescence detection of the 4-bromoethyl-7-methoxycoumarin derivatives formed pre-column (Guldutuna et al., 1993).

3.7.2 Mobile Phases

Chromatographic systems suitable for HPLC of steroids are based upon or can be tested with TLC (Hara, 1977; Cimpoiu et al., 2006). Useful separations of steroids can be achieved using isocratic chromatography on silica gel with binary solvents (Hara et al., 1978; Capp and Simonian, 1985). The separation of a range of steroids is best achieved with

Table 3.2 Some selected references (1990–2008) for HPLC methods of steroid analysis not involving mass spectrometry

Steroid Analyte(s)	Column	Solvent	Special conditions	Detection	Reference
Digoxin and metabolities (standards)	Spherisorb ODS II	Acetonitrile:water (20:7)		Electrochemical detection of 3,5-dinitrobenzoyl derivatives	Embree and McErlane (1990)
15 Bile acids and pregnanediol, pregnenediols	Finepak SIL C18	50 nM imidazole buffer, pH 6.0:THF (approx. 1:1) or :acetonitrile (25:75)	40°C	3a-OHSDH to give 3-oxoste-roids, forms dansylhydra-zones. Post-column detection by peroxy-oxalate chemiluminescence	Higashidate *et al.* (1990)
Some anabolic steroids (standards)	Bondex C18	Methanol:water (7:3)		UV at 254 and 280 nm	Noggle *et al.* (1990)
Studies on aromisation of 19-oxygenated 16-OH-androgens	Radial Pak C18	0.5% Phosphate buffer, pH 3.0:methanol (1:1)		Electrochemical	Numazawa *et al.* (1990)
Corticosterone in rat urine	Capcell PAK CN	MeCN:water (24.5:75.5)	40°C	Post-column mixing with 65% sulphuric acid at 65°C. Measures fluorescence (exc. 460 nm, em. 510 nm)	Sudo (1990)
Bile acids and conjugates	Develosil ODS-5 YMC-GEL C_8 mBondapak C_{18}	MeCN–0.5%$(NH_4)_2SO_4$ (or KH_2PO_4, pH4)±b-cyclodextrin in various mixtures (i.e. 5:18, etc.)		Pre-column formation of 3-(1-anthroyl) derivatives. Measures fluorescence (exc. 370 nm, em. 470 nm)	Shimada *et al.* (1990)
Urinary corticosteroids	Zorbax ODS	Methanol:water:0.01 M Na acetate (70:30:5)		Mixed post-column with lucigenin/ Triton X-100/0.28 MKOH and induced chemiluminescence monitored	Takeda *et al.* (1990)
Plasma cortisol and 11-deoxycortisol	Supelcosil-LC-18-DB	Gradient of increasing water in acetonitrile		UV at 242 nm	Underwood *et al.* (1990)
Serum bile acids	Reversed-phase	Gradient elution MeCN–MeOH–water mixtures		Pre-column derivatisation with 4-bromomethyl-7-methoxycou-marin and fluorescence detection (exc. 320 and em. 385 nm)	Wang *et al.* (1990)

(continued)

Table 3.2 (continued)

Steroid Analyte(s)	Column	Solvent	Special Conditions	Detection	Reference
Corticosteroids in serum	Shimpack CLC-ODS	MeOH:THF:water (26:18:56)	48°C	UV at 254 nm in series with fluorescence detector for E1, E2, E3 (exc. 285 and em. 310 nm)	Wei *et al.* (1990)
Prednisone, prednisolone and 20-reduced metabolities in perfusion media	2 Brownlee Spheri-5 RP18 columns in series	MeOH:water (57.5:42:5)	40°C	UV at 242 nm	Cannell *et al.* (1991)
Bile acids in human bile	Resin-based reverse-phase PLRP-S	Gradient of MeCN in NaOH	35°C	Pulsed amperometry	Dekker *et al.* (1991)
Free bile acids	Nova-Pak C18	2-propanol:MeCN:water (65:10:25)		Pre-column formation of pentachlorophenyl esters—UV at 230 nm	Ferreira and Elliott (1991)
Prednisone, prednisolone and major urinary metabolities	Zorbaz SIL	Methylene chloride:glacial acetic acid:MeOH (91.3:7.5:1.2)		UV at 254 nm	Garg and Jusko (1991)
3-Oxo bile acids in serum	Nova-Pak Phenyl	3% MeOH in 0.3% phosphate (pH 7.0) buffer:MeCN (8:5)		Pre-column formation of 2-anthroylmethyl oximes. Fluorescence (exc. 260 and em. 405 nm)	Goto *et al.* (1991)
Cortisol and cortisone in saliva	Keystone C8	MeCN:MeOH:water (10:40:50)	50°C	UV at 240 nm	Wade and Haegele (1991b)
	Keystone Hypersil	33% EtAc in hexane, 0.5 satd with water		Pre-column formation of 2-anthroyl esters. Fluorescence (exc. 305–395, em. 430–470 nm)	Haegele and Wade (1991)
Cortoic and cortolonic acids	Pecosphere CR-C18	MeCN:MeOH:water (34:34:32)		1-Pyrenylmethyl esters UV with photodiode detector	Iohan and Vincze (1991)

Anabolic steroids in tissues	Supelco C18	MeCN:0.01 M KH_2PO_4 (pH 3) (48:52 and 46:54). Gradient of MeCN in water. KH_2PO_4 (pH3):MeCN:MeOH: THF (60:21:7:12)		Electrochemical, UV and fluorescence depending upon steroid	Lagana and Marino (1991)
Prednisone, prednisolone and cortisol in plasma	Axxiom ODS	THF:water (25:75)		UV at 240 nm	McBride *et al.* (1991)
Bile acids and conjugates	YMC-GEL C8	MeCN:water (3:1)±5 mM methyl-b-cyclodextrin		Pre-column formation of 1-bromopyrenacyl esters. Measures fluorescence (exe. 370 nm, em. 440 nm)	Shimada *et al.* (1991)
Estetrol, estriol, cortisol and cortisone in amniotic fluid	Medipola-ODS	Phosphate buffer, pH 3.1:MeCN:MeOH (20:2:7)	40°C	UV at 245 nm for E and F, electro-chemical detection for estrogens	Noma *et al.* (1991)
Serum cortisol	TSK	MeCN:THF:19mMK biphthalate (pH 1.85)		Pre-column formation of sulphuric acid fluorophores (exc. 365 nm and em. 520 nm)—*cf.* Nozaki *et al.* (1992)	Nozaki *e t al.* (1991)
Serum steroid profile in Cushing's	NS-Gel C18	Gradient of MeOH in water	50°C	RIA	Ueshiba *et al.* (1991)
Steroids in human ovarian follicular fluid	ChromSpher	Gradient of MeCN–water–methanol		UV detection at 242 nm or 206 nm (estrogens)	Vanluchene *et al.* (1991)
Plasma cortisol and cortisone	Shandon Hypersil-C18	Water:THF:MeCN (80:10:8)+ 5 ml triethylamine/1 and citric acid to adjust pH to 6.5		UV detection at 242 nm	Hariharan *et al.* (1992)
Conjugated bile acids in urine	Bile Pak II	Gradient elution with MeCN–MeOH–10 mM phosphate buffer, pH 7.3		Post-column immobilised 3a-OHSDH/NAD. NADH released by reaction with bile acid measured by chemiluminescence using isoluminol microperoxidase and 1-methoxy-5-methyl-phenazinium methyl sulphate	Ikegawa *et al.* (1992)

(continued)

Table 3.2 (continued)

Steroid Analyte(s)	Column	Solvent	Special Conditions	Detection	Reference
Urinary free cortisol	TSK ODS-80	MeCN: water or phosphate buffer (pH 1.85) mixture		Pre-column formation of ethanol-H_2SO_4 fluorophorescence. Fluorescence (exc. 365 nm and em. 520 nm)-*cf.* Nozaki *et al.* (1991)	Nozaki *et al.* (1992)
Free and sulphated estrogens	Beckman ODS	2% Tetrabutyl-ammonium hydroxide (pH 3): 33% MeCN in water (6.5:93.5)		UV at 210 nm or fluorescence (exc. 280 nm and em. 312 nm)	Su *et al.* (1992)
3α, 5β-Tetrahydroaldo-sterone and cortisol in human urine	L-column ODS	MeOH: MeCN: 0.5 mM ammonium acetate (50:10:40)		Pre-column formation of quinoxalines by reacting with 1,2-diamino-4,5-methylenedioxy-benzene. Fluorescence detection (exc. 350 and em. 390 nm)	Yoshitake *et al.* (1992)
Serum cortisol and cortisone	Ultrasphere ODS	MeOH:THF: water (25.5:9.0:65.5) or MeOH:MeCN: 58 mM NaH_2PO_4 containing 6 mM heptanesulphonic acid		Photodiode array detection—UV absorption	Volin (1992)
Steroids with delta-4-3 oxo group	RP C18	MeCN:water (1:1); 0.01 M Tb nitrate, 0.1 M SDS in 20% MeCN		Post-column mixing with terbium nitrate in 0.1 M SDS or micellar LC. Detection by sensitised terbium fluorescence	Amin *et al.* (1993)
Ethinyl estradiol in rabbit plasma	Novapack C18	50 mM phosphate buffer, pH 3.6–MeCN–MeOH (10:7:3)		Electrochemical detection	Fernandez *et al.* (1993)
Free and conjugated bile acids in a variety of tissues	Ultrasphere ODS	Gradient of MeCN–MeOH–water		Pre-column derivatisation with 4-bromomethyl-7-methoxycou-marin.Fluorescence detection	Guldutuna *et al.* (1993)
Plasma cortisone and corticosterone	Shandon ODS-Hypersil	Water:THF:MeCN (80:10:8)+ 5 ml/l of triethylamine and citric acid, pH 6.5		UV at 242 nm	Hariharan *et al.* (1993)

Plasma corticosteroids	Zorbax ODS	Water:MeOH (25:75) containing 5 mM tetramethyl-ammonium hydrogen sulphate		Pre-column derivatisation with 2-(4-carboxyphenyl)-5,6-dimethyl-benzimidazole. Fluorescence (exc. 334 nm and em. 418 nm)	Katayama *et al.* (1993)
Plasma estrogens	Wakosil-5 C18	Water:methanol (1:9)		Same as above. Fluorescence (exc. 336 nm and em. 440)	Katayama and Taniguchi (1993)
Urinary free cortisol	RP 18	MeOH:water:n-propanol containing 20mM SDS (pH6) (18:80:2) and (38:60:2)		UV at 240 nm	Li *et al.* (1993)
Bile acids and glycine conjugates in human bile	YMC Gel C8	MeCN:MeOH: water (6:7:8)±2.5 mM y-cyclodextrin		Pre-column formation of 7-methoxy-1,4-benzoxazin-2-one-3-methyl ester. Fluorescence detection (exc. 280 and em. 320 nm)	Shimada *et al.* (1993)
Catechol and guaiacol estrogens	Inertsil-ODS-2	0.5% phosphate buffer, pH 3.0: MeCN (59:41)		Electrochemical detection	Suzuki *et al.* (1993)
Pregnanolone (standard)	Cyclobond I (b-cyclodextrin bonded phase)	MeOH:water (65:35)		UV at 241 nm—indirect absorption using testosterone as a probe	Agnus *et al.* (1994)
Hydroxylated metabolites of equilin in rat bile	Cosmosil 5C18-AR	0.5% acetate buffer, pH 5-MeOH-MeCN (60:9:30)		Electrochemical	Ikegawa *et al.* (1994)
Estrogens in human urine	Beckman ODS	MeCN:water (25:75) containing 14 mM cyclodextrin	40°C	UV at 280 nm	Lamparczyk *et al.* (1994)

(continued)

Table 3.2 (continued)

Steroid Analyte(s)	Column	Solvent	Special Conditions	Detection	Reference
Salivary cortisol	CN- or ODS		Post-column mixing with H_2SO_4	Laser-induced fluorescence	Okumura et al. (1995)
Serum cholesterol, cholestanol	-	-	1-Eicosanol as IS post-column Pt-catalysed reduction on-line	Pre-column derivatisation with 2-[2-(iso-cyanate) ethyl] -3-methyl-1,4-naphthoquinone. electrochemical detection	Nakajima et al. (1995)
Bile alcohols	NovaPak Phenyl and TSK-GEL ODS	MeOH- H_2O (83-85%)	2,4-Dinitro-phenyl hydrazones-formed after 3α-OHSDH-ase	UV at 364 nm	Une et al. (1996)
Serum conjugated bile acids	ODS	MeCN: MeOH (60:40) and H_2O Gradient	hydolysis with cholylglycine hydrolase	Fluorescent derivative with 2-bromoacetyl-6-methoxy-naphthalene	Gatti et al. (1997)
Free Glucucorticoids in plasma/urine	Nucleosil 120-C18	H_2O: MeCN (76:24)		UV at 254 nm	Hay and Mormede (1997)
Oestriol-3- and 16-glu. in pregnancy urine	YMC-Pak C4 (A) and Ph(B)	MeOH: 0.5% Tri Ethylamine at 40°C	Column switch A to B	Derivatisation with 6,7-dimethoxy-1-methyl-2(1H)-quinoxaline-3-propionylcarboxylic acid hydrazine. Fluorescence	Iwata et al., (1997)

In vitro hydroxy testosterones	Supelcosil LC-18	MeOH:H_2O Gradient	Cortexolone As IS	UV at 254 nm	Purdon and Lehman-McKeeman (1997)
Prenenolone-3-stearate in brain	-	-	Derivatisation with dansyl hydrazine or 4-(N,N-dimethyl aminosulfonyl)-7-hydrazino-2,1,3-benzoxa-diazole	Fluorescence	Shimada et al. (1997)
Urine free Cortisol	LiChrospher 100 C-18	MeOH: MeCN:H_2O (43:3:54)	Meprednisolone as IS	UV	Turpeinen et al. (1997)
21-oxo-steroids	RP		Pre-column formation of dansyl derivative	Post-column 1,1'-oxalyl-diimidazole-peroxyoxalate induced chemi luminescence	Appelblad et al. (1998)
Progesterone, 17-OH-Prog + 4 x 3-oxo-steroids	Wakosil 5C4	MeCN:H_2O (7:3)	Pre-column derivatisation with 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-propiono-hydrazide (BODIPY FL-hydrazide)	Fluorescence-chemiluminescence	Katayama et al. (1998)

(continued)

Table 3.2 (continued)

Steroid Analyte(s)	Column	Solvent	Special Conditions	Detection	Reference
Urinary cortico steroids	SIL	Propan-2-ol: Hx; (2:98), then (7:93)		Pre-column formation of 9-anthroyl Cyanide Fluorescent cpd	Neufeld et al. (1998)
Glucocorticoids in plasma/urine	Cosmosil 5SL (x2)	Diethylene dioxide: EtAc:CHCl3 Hx:pyridine (500:100: 100:1400:21)	9-Anthroyl nitrile + base catalyst to form 21-fluorescent esters	Fluorescence	Shibata et al. (1998)
Oestrogen in breast tumour tissue by RIA	Hypersil-5μ- ODS	MeCN -phosphate Buffer, pH 3.5		UV at 280 nm	Geisler (2000)
Urine 6β-OH-cortisol	Novapak C18	CH3COOH-MeCN-KH2PO4 50 mM at	25°C and 45°C	UV at 245 nm	Homma et al. (2000)
Progesterone and neuroactive metabolites in serum	Novapak SIL	EtOH in CH_2Cl_2		Fraction collection for RIA	Murphy and Allison (2000)
16a-Hydroxy-DHA and metabolites	5 m C2/C18 Pharmacia	H_2O: MeOH: MeCN	30°C	UV at 238 nm and 210 nm and radio activity	Schmidt et al. (2000)
Urine 2-OH-oestradiol-17-S in Pregnancy	Mightysil RP-18GP	5 g/L $NH_4H_2PO_4$ pH3 - 40oC		Electrochemical detection	Takanashi et al. (2000)

Alphaxalone and pregnanolone in rat plasma	Microsphere C18	25 nM acetate (pH3.9):MeCN (ca 40:60)		Pre-column formation of dansyl derives. Fluorescence detection.	Visser et al. (2000)
Oestradiol in plasma	CapcelPak ODS	MeCN:H_2O (85:15)	Pre-column formation of dansyl derivatives	Post-column chemiluminescence with H_2O_2 and bis(2,4-dinitro-phenyloxalate.	Yamada et al. (2000)
17-Ketosteroid sulphates and glucuronides	Luna C18	0.1 M NH_4Ac: MeCN (35:25)		Sonic spray ionisation Hitachi M8000 ion trap	Jia et al. (2001)
Steroids	CN column	MeOH in ethoxy nonafluoro butane			Kagan (2001)
Serum urso-deoxycholic acid	LiChrospher 100 C18	MeCN:H_2O (6:4)		Derivatisation with 2-bromo-2'-acetonaphthone UV at 245 nm	Nobilis et al. (2001)
Hydroxy testosterones	TSK-gel ODS- 80Ts	MeOH-THF		UV at 254 nm	Tachibana and Tanaka (2001)
Biliary bile acids	Luna C18	MeOH: MeCN: H_2O (53:23:24) +30 mM NH_4Ac pH5.6		Evaporative light scattering detection (ELSD)	Torchia et al. (2001)

(continued)

Table 3.2 (continued)

Steroid Analyte(s)	Column	Solvent	Special Conditions	Detection	Reference
Hydroxy testosterones	Hypersil-BDS C18	THF:MeCN- H_2O		UV	Whalley et al. (2001)
Hepatic estradiol metabolites	Hypersil ODS-5μm	CH_3COOH buffer pH4.5 -MeCN		Electrochemical detection	Cheng et al. (2001)
Microsomal oestradiol-3- and 17-glucuronides	Alltima-phenyl	MeCN: 50 mM NH4PO4 (pH 3) (35:65)		Fluorescence detection	Alkharfy and Frye (2002)
Plasma Oestradiol	CalcellPAK C8 UG-120	0. 0.1%TFA: MeCN (1:1) And 20 mM PO4 buffer(pH9): MeCN (1:1)		Electrochemical detection	Yamada et al. (2002)
Adrenal steroids in CAH	BDS-Hypersil	H_2O-MeOH		Fractions for RIA	Fernandes et al. (2003)
Testo and Epi-T	Hypersil C18	H_2O-MeCN		UV at 245 nm	Ganzalo-Lumbreras et al. (2003)
Cortisol and 6β-OH-cortisol in urine	Symmetry sheild RP8	NH_4 acetate (pH 4)-MeCN		Diode array at 244 nm	Rouits et al. (2003)
Plasma testosterone	C18	KH_2PO_4(pH 4)-MeCN-MeOH		UV at 240 nm	Ng and Yuen (2003)
Corticosteroids anabolics	Hypersil-ODS	Binary solvent Mixtures with SDS - micellar LC	Diode array 190-360 nm		Ganzalo-Limbreras and Izquierdo-Hornillos (2003)

Prednisolone and cortisol in plasma and urine	Hypersil SIL	CH_2Cl_2-H_2O-MeOH-THF-Gla CH_3COOH	UV at 240 nm	AbuRuz et al. (2003)
Androst-5,16-dienes and androst-4,16-dienes	Luna 5 µm, C18	H_2O-MeOH	UV at 210-215 nm	Decreau et al. (2003)
DHA and 7-OH-metabolites in brain	C18 ET250/4 Nucleosil 100-5 MeOH	MeCN-H_2O-NH_4HCO_3-	UV and radio activity	Kazihnitkova et al. (2004)
6β-OH-F and F in urine	Synergi 4 m Polar-RP 80A	Phosphate-acetic acid and Phosphate-acetic acid-MeCN	photodiode array	Furuta et al. (2004)
25- and 24,25-Hydroxy-vitamin D3 in serum	Ultrabase C18	MeOH:water linear gradients from (90:10) to (50:50)	Photodiode array detection	Quesada et al. (2004)
Synthetic corticosteroids	Synergi Max-RP	MeCN:H_2O (35:65)	Post-column chemiluminescence Using luminal + hexacyanoferrate III As catalyst	Vasquez et al. (2005)
16-Androstenes in boar testis	Phenomenex 5 mm C!8	MeCN-H_2O (85:15)	Radioactivity and UV at 200 nm	Sinclair et al. (2005)
Testosterone and epi-T from urine	µBondapak C18	MeCN-MeOH TRIS-HCl(pH6.9)	UV at 245 nm	He et al. (2005)

(continued)

Table 3.2 (continued)

Steroid Analyte(s)	Column	Solvent	Special Conditions	Detection	Reference
Cortisol and cortisone	Hypersil C18	Micellar medium with SDS		UV at 254 nm	Izquierdo-Hornillos et al. (2005)
Cortisol and cortisone in urine	Supelco DSC 18	MeOH: H_2O (63:37) at 30°C		UV at 254 nm	Gatti et al. (2005)
6β-OH- and cortisol Urine and plasma	Inertsil-PH-3	MeCN: H_2O gradient		UV at 245 nm	Hu et al. (2005)
DHEA and DHEA-S A-dione and Testo	C8-SIL	Ion-pairing		Circular diochroism at 295 nm	Gergely et al. (2006)
Oestradiol and Catechol oestrogens in Catfish	Luna C18	MeCN-$NH_4H_2PO_4$ (pH 3)		Electrochemical	Mishra and Joy (2006)
Reviewed in Honour (2006)					
Oestrogen catabolism	LiChroCART 250-4 RP18	MeOH:H_2O (8:2 to 9:1) Gradient		Derivatised with 2-(4-carboxyl-phenyl) 5,6-dimethyl-benzimidazole. Fluorescence detection Ex:336 nm, Emm: 440 nm	Delvoux et al. (2007)
Human fetal cord blood steroids	Supelcosil LC-18	30% MeCN in H_2O + 12 nM β-cyclodextrin at 29°C	Photodiode Array		Clifton et al. (2007)
Metabolomics		35% MeCN			Zarzycki et al. (2006)

Butane acid-DHEA-diester in rat plasma	Diamonsil C18	MeCN:H_2O - gradient from 75-98% MeCN and back to 75%	Pre-column dansyl derivatives - laser-induced fluorescence detection (Ex350 nm, Emm520 nm)	Peng et al. (2007)
Plasma cortisol in children	No details of HPLC assay are provided - merely that the samples were analysed by 'a central laboratory (Quest Diagnostics) using HPLC'			Chrousos et al. (2007)
Free cortisol/ cortisone ratio in human Urine	Varian Pursuit C18 coupled to Waters Atlantis C18	MeCN:H_2O 3:7 at 28°C	UV detection at 240 nm	Al Sharef et al. (2007)
Serum Cholesterol	Develosil C30-UG-3	MeCN:2-propanol (9:1) with 50 mM $LiClO_4$ at 20°C	LC-4C electro-chemical detector	Hojo et al. (2007)
Cholesterol in biological samples	Novopak C18	MeCN:2-propanol (9:1)	Jones oxidation and UV detection at 240 or 250 nm	Dong et al. (2007)
Steroids in animal feeding water	Several RP columns	MeCN:water (35:65)	UV-diode array detection (190-360 nm)	Muniz-Valencia et al. (2008a) see (2008b) in animal feed
Estrone in environmental and drinking water	RP C18 column	MeOH:water (55:45)	UV detection	Wang et al. (2008)
25-Hydroxy- and 24,25-di-hydroxy-vitamin D3 in serum	Using same automated method as previously described (Quesada et al., (2004))			Mata-Granados et al. (2008)

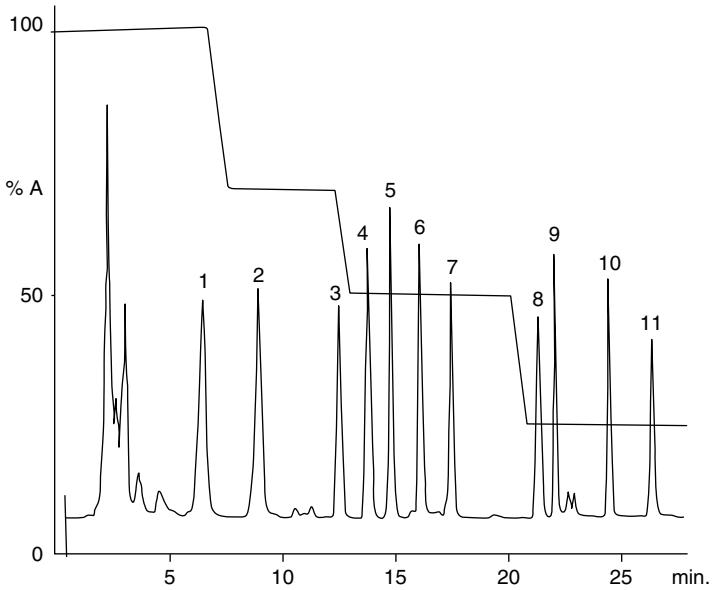

Fig. 3.13 HPLC of standard free and glycine conjugated bile acids: 1, glycoursodeoxycholic acid; 2, glycocholic acid; 3 glycochenodeoxycholic acid; 4, glycodeoxycholic acid; 5, ursodeoxycholic acid; 6, cholic acid; 7, glycolithocholic acid; 8, chenodeoxycholic acid; 9, deoxycholic acid, 10, lauric acid; 1l, lithocholic acid. HPLC was carried out on an Ultrasphere IPC18 column (250 mm x 4.6 mm) at 35°C using a solvent gradient from (A) acetonitrile:methanol:water (100:50:75) to (B) acetonitrile:methanol (100:50). The elution gradient from mobile phase A (100%) to B (drawn line) is marked in the figure. Pre-column derivatisation of bile acids to their 4-bromomethyl-7-methoxy-coumarin derivatives were detected by fluorescence (from Guldutuna *et al.*, 1993, with permission).

gradient elution and there exists methodology to assist optimisation (Nikitas and Pappa-Louisi, 2005). Additional pH, ion-pair and modifier effects can be incorporated. Retention times are reproducible between runs provided that the column is equilibrated to the starting solvent mixture. Methanol:water gradients effect the separation of the major adrenal sterids. Dioxane is a better choice for the separation of polar adrenal steroids and acetonitrile is preferred for resolving testicular steroids. Peak shape, resolution and reproducibility can be improved by maintaining the column at a fixed temperature above ambient, e.g. at 45–60°C that may need optimising for the required separation (Dolan, 2002). At these temperatures, the eluant viscosity is reduced (Burgess, 1978). If working at ambient temperature, it is advisable to have a room with well-controlled temperature to achieve reproducible retention times or use a jacketed column with temperature control. Temperature gradients have been tested with C8 and C18 columns (Dolan et al., 2000a, b). The difficulties in choosing the appropriate column packing for a particular separation have been eased to some extent by using three and four solvents in a mobile phase system. Systematic, statistical procedures for solvent optimisation have been developed (Hara and Hayashi, 1977; D'Agostino et al., 1985; Wei et al., 1990). Column packings may not be consistent and chromatographic conditions may have to be adjusted (Dolan et al., 2002). Derks and Drayer

(1978b) reported the separation of very polar 6α– and 6β-hydroxylated metabolites of cortisol by isocratic elution from a silica column with water:chloroform:methanol.

An LC separation of a complex mixture containing 14 androgenic anabolic steroids (natural and synthetic) for anabolic steroid screening purposes has been carried out. The optimization of the method assessed the use of binary, ternary and quaternary mobile phases containing acetonitrile, methanol or tetrahydrofuran as organic modifiers (Izquierdo-Hornillos and Gonzalo-Lumbreras, 2003). The effects of different reversed-phase packings, and temperature on the separation using acetonitrile as organic modifier were also studied. The optimum separation was achieved by using a water–acetonitrile (55:45, v:v) mobile phase and a Hypersil ODS (250 × 4.6 mm) 5 μm column (30°C) in about 38 min, allowing the separation of all 14 compounds tested (when danazol was excluded, 13 out of 14 were separated in 23 min). Calibration graphs were obtained using bolasterone, methyltestosterone and canrenone as internal standards. Detection limits were in the range 0.012-0.11 μg/mL (Gonzalo-Lumbreras and Izquierdo-Hornillos, 2000).

In some cases, phosphate is incorporated into the mobile phase (Shimada et al., 1979). With these systems, the buffer anion and pH exert significant effects on the separation (Shimada et al., 1986). Salts used in the eluting solvent (see section on analysis of oestrogen conjugates) may in the long term corrode the steel of the columns and tubing. Oestrogens can be effectively separated when silver nitrate is included in the mobile phase to give 2 g of silver nitrate with 60 mL methanol and 40 mL water at 0.55 mL/min (Tscherne and Capitano, 1977). To prevent metallic silver building up on the column, a water:methanol (50:50, v/v) mobile phase is used each evening to flush excess silver nitrate from the system. Even so, a small build-up can occur which requires a rinse with dilute nitric acid or replacement of the tubing when back-pressure rises.

Addition of cyclodextrins to the mobile phase has been claimed to improve the HPLC separation of a variety of steroids, from C21 corticosteroids (Shimada and Nonaka, 1991) to bile acids (Shimada et al., 1990) and oestrogens (Lamparczyk et al., 1994). There is an increasing literature on this subject, particularly from Shimada's laboratory but it is not entirely clear how this improvement is effected. There are a variety of different cyclodextrins and not all confer the same improvement in resolution. It is often necessary to try a number of the polymers before discovering the best for the particular separation. Addition of other compounds to the mobile phase have also provided advantages such as the use of micellar chromatography which can be achieved by the addition of sodium dodecyl sulphate (SDS) (Izquierdo-Hornillos and Gonzalo-Lumbreras, 2003; Izquierdo-Hornillos et al., 2005). Use of micellar chromatography and a two-column system has allowed the measurement of serum cortisol (Nozaki et al., 1991) and urinary free cortisol (Nozaki et al., 1992) by direct injection of urine onto the first column, washing off protein and elution of the concentrated analyte onto the second analytical column. Cetyl trimethyl ammonium bromide has been used to improve the separation of betamethasone and dexamethasone (Pena-Gracia-Brioles et al., 2004).

Ion-pair chromatography is preferred for chromatography of steroid conjugates and bile acid products. Andreolini et al. (1987) have shown excellent separations of

oestrogens on RP-18 packings by eluting with a gradient of acetonitrile/methanol and phosphate buffer containing cetyltrimethylammonium bromide. Gradient elution is usually necessary to elute a series of steroids and bile acids after extraction from biological fluids. Gradient elution reduces analysis times and depending upon the gradient shape can optimise separation and improve peak symmetry. Non-linear, stepped and linear gradients have been used, largely dictated by the available facilities for programming the pumps. Flow and temperature programming can also be used.

The separation of bile acids by HPLC can be achieved using normal-phase or reversed-phase columns. In a normal phase system, an organic acid is usually added to the mobile phase. Bile acids are eluted in the order of decreasing number of hydroxyl groups on the steroid nucleus. The chemically bonded ODS-RP column is the most widely used column for the resolution of bile acids (see Fig. 3.13). The retention of bile acids is markedly influenced by the pH of the mobile phase. The acidity of the eluent and the pK of the bile acid have to be considered (unconjugated pK = 6, glycine-conjugated pK = 4.5, taurine-conjugated pK = 1.5). With an anion-exchange column, bile acid conjugates are readily separated.

For the separation and detection of bile acids in biological specimens with an ODS column, an acidic mobile phase is used to separate glycine- and taurine-conjugated bile acids. The unconjugated bile acids are then eluted with a neutral or weakly alkaline mobile phase. For the analysis of complex mixtures of bile acids, it is preferable to fractionate according to conjugation before HPLC of each group in isocratic mode.

3.7.3 Sample Injection

Extracts are usually dissolved in the mobile phase. The addition of a suitable macromolecular matrix, e.g. polyethylene glycol to the extracting solvent prior to evaporation improves the recovery of steroids (Culbreth and Sampson, 1981) suggesting that the steroids dissolve poorly in the mobile phase alone. Injectors which use rubber septa should be avoided. At the high instrument sensitivities often used for the analysis of steroid hormones, such septa may lead to the production of spurious and irreproducible peaks in the chromatogram. These may reflect the action of injected solvents on the septum. Septumless injection valves (e.g. JADE™ injectors – see www.asapanalytical.com/pdf/inlet.pdf) are, therefore, preferred.

3.7.4 Detection

The α,β-unsaturated ketone in the A-ring of naturally occurring steroid hormones absorbs ultraviolet light with maximum around 240 nm and molar extinction coefficients of 12,000–20,000. Isolated carbonyl groups absorb with a maximum around 280 (275-285) nm and molar extinction coefficients of 17–155. The natural

oestrogens have peak absorption at 280 nm due to the aromatic A-ring. Underivatised phenolic steroids can be detected with sensitivity limits of 100–10 pg/mL. Although steroids can absorb UV below 200 nm, in practice, at this wavelength it is difficult to achieve a clear signal distinguishable from noise without a reduction in sensitivity particularly when solvent gradients are used to elute the steroids. With some gradient elution systems, it is necessary to correct for base-line variation by comparison of the response of the eluate from the analytical column with the flow of solvent alone through a reference cell. An interesting paper (Agnus et al., 1994) described a system for the measurement of non-UV absorbing steroids by the so-called "indirect" photodetection using an immobilised β-cyclodextrin column using testosterone as a probe added to the mobile phase (methanol:water, 65:35, v/v). The sensitivity of detection of pregnanolone (2 nmol) by this system was comparable to that previously reported (Agnus et al., 1991) using a C4 RP column and adding β-cyclodextrin to the stationary phase and using progesterone as the probe. Sensitivity of this method is not superb but does represent nearly an order of magnitude improvement in sensitivity over that achieved with direct detection and it may be possible to improve this further.

The detection and quantitative determination of nanogram quantities of steroids has also been realised by the use of fluorescence (Seki and Yamaguchi, 1984), refractive index (Satyaswaroop et al., 1977) and electrochemical detectors (Watanabe and Yoshizawa, 1985). C-3 and C-16 conjugated oestriol glucuronides have been converted to fluorescent derivatives and separated using column-switching between two columns (Iwata et al., 1997) and by LC-ES-MS/MS Yang et al., 2003). In some cases, it has been necessary to react the steroids in the eluate with reagents to form UV-absorbing derivatives. Post-column derivatisation methods are, however, restricted to very fast reactions limiting the scope of application (Seki and Yamaguchi, 1984). Most of the urinary steroid metabolites do not have natural absorbance in the UV region. Reactive groups have been utilised in order to make derivatives for spectrophotometric detection. HPLC has thus been used to separate individual oxo-steroids after conversion to phenylhydrazone derivatives. 3α-Hydroxysteroids can be detected using a post-column 3α-hydroxysteroid dehydrogenase reactor (Lam et al., 1988) similar to that used in bile acid analysis (see below). In general, however, the most successful methods for enhancing detection are pre-column formation of fluorescent or chemiluminescent derivatives (e.g. formation of 2-(4-carboxyl)-5,6-dimethylbenzimidazoles (Katayama and Taniguchi, 1993) for oestrogens, 9-anthroyl cyanide (Neufeld et al., 1998; Shibata et al.,1998) derivatives of glucocorticoids). Many other such derivatives have been used and these are summarised in Table 3.2. There are two good reviews summarising this approach up to 2001 (Shimada et al., 2001; Appelblad and Irgum, 2002). Increase in detection sensitivity and specificity can clearly be achieved using hyphenated LC-MS technology, which is discussed later in this chapter.

Flow-through radioactivity detectors are potentially useful for examining the products of reactions with labelled substrates (Kessler, 1983; Lundmo and Sunde, 1984). The short dwell time of the sample components in the counting chamber limits sensitivity. Several laboratories have demonstrated the variety and complexity of intermediates and products formed when radioactive steroids are incubated with

steroid metabolising tissues. Current detection limits for tritium are 10,000 dpm with flow cells incorporating scintillant (around 1% efficiency) to 1,000 dpm (50% efficiency) when the column effluent is mixed with liquid scintillant before passing through a cell.

A UV absorbance detector is relatively insensitive to bile acids and because of the variety of UV-absorbing compounds in serum there is a risk of interference. Methods based on pre- and post-column derivative formation have overcome many of these problems, although sample clean-up is still required. Bile salts in bile are present in sufficiently high concentrations for detection with a refractive index or UV detector in the range 195–215 nm. Sensitivity can be improved by the use of a fluorescence detector (Andreolini et al., 1988; Kamada et al., 1983). A highly sensitive and selective fluorescence method of indirect detection is achieved using the enzyme 3α-hydroxysteroid dehydrogenase which oxidises the bile acids after their HPLC separation. The overall reaction involves conversion of the 3α-hydroxy group on the steroid nucleus to a keto group with concomitant reduction of NAD to NADH which is detected fluorimetrically. The enzyme is expensive and in its crude form may not be specific. In order to reduce the cost of the enzyme, it can be immobilised on an aminopropyl support in a column in series with the analytical column. Detection of the compounds after the post-column reaction is not uniform; the primary bile acids yield somewhat smaller quantities of NADH than the secondary bile acids. This is influenced by the time the bile acids spend in the reactor and the affinity of the enzyme for the range of bile acids encountered in biological fluids. Quantification of a number of bile acids requires optimisation and control of flow rates as well as calibration curves to compensate for the differences in response. Over a period of time the baseline and response characteristics of the enzyme column will vary.

LC methods often show chromatographic peaks that are difficult to reproduce and can be hard to identify. Artefact peaks can arise because of contamination by the injector septum and sampling equipment leading to misinterpretation of impurities and erroneous quantification. A number of investigations may be needed to locate the source of the problems (Strasser and Varadi, 2000).

3.7.5 Identification

The identification of material in chromatograms is usually assumed from a homogenous peak with elution time which coincides with that of the reference compound under similar conditions. This assumption may, however, be dangerous since it is not always possible to recognise homogeneity by inspection. The use of a photodiode array detector has a considerable advantage in this context in that it allows inspection of the peak to ensure homogeneity. A good example of this is given in the vitamin D Chapter (Chapter 11) in this book. Since the detectors currently in use are not selective for distinct classes of substances, some further demonstration of specificity is required. 3-Keto-4-ene steroids can be distinguished from other possible compounds

eluted from the column by monitoring the UV absorption at further wavelengths using a photodiode array detector (Fell et al., 1983). The sample can also be analysed separately with a different column (preferably of differing polarity or selectivity) or a different gradient elution system. Should elution times in each system coincide with those of a standard, it is highly probable that each chromatogram reflects the same steroid content. These criteria have not been rigidly applied in the published work relating to steroids. Retention indices have been widely used for recording and comparing retentions, for identifications and as the basis for prediction methods (Kuronen et al., 1998). 1-[4-(2,3-Dihydroxypropoxy)phenyl]-1-alkanone standards have been used in RP-LC for steroids with photodiode array detection (Kuronen et al., 1998). In some work, identification is enforced by a second separation of quite different selectivity (orthogonal separation). This may sometimes reveal a second peak that was masked in the first separation (Pellett et al., 2006). Eluate fractions can of course be collected and subjected to GC-MS after appropriate derivatisation.

The ultimate system for on-line identification is LC-MS/MS and considerable advances have been made over the last 10 years in improving methods of linking the eluent from an LC column to MS instruments as well as improved ionisation procedures. While LC-MS is more selective, the advent of tandem LC-MS/MS, with collision cells in between MS1 and MS2 has improved selectivity even further to the extent that the extra expense of acquiring LC-MS/MS rather than simply LC-MS is fully justified. Applications of LC-MS and other types of mass spectrometry are discussed below (see Section 3.8)

3.7.6 Quantitation

The height or area of the chromatographic peak is measured manually or with the aid of an integrator and ideally the response of the analyte is compared to the response of an appropriate internal standard. The ratios of response for the analyte to the signal from the internal standard are plotted for the concentration range of interest. The concentration of an unknown amount of steroid in the sample is determined from a calibration line. There are a large number of synthetic steroids available which can be used as internal standards. Since a number of steroid-based drugs are widely used in hospital patients, the use of two very different internal standards, for example, 19-nortestosterone and 6α-methyl prednisolone, prevents erroneous results in the case of medication by either one of the steroids selected as internal standard provided they behave in a similar way to the analyte during the analytical procedure. When internal standards are not used, the extraction and injection must be carefully controlled before peak response can be reliably derived from a calibration line using injected standards. A deferred standard technique can be adopted in which a known amount of the analyte is injected in pure form some time after but during the chromatographic run of the unknown sample.

The availability of switching valves which can be operated automatically has enabled the use of multiple columns, automatically switching selected peaks

from one column to another – so-called "column switching" or "heart cutting". Schoneshofer et al. (1983) described such a system for the measurement of triamcinolone in urine and later adapted the procedure for the measurement of urinary free cortisol (Schoneshofer et al., 1985) and for 20-reduced metabolites of cortisol and cortisone in urine (Schoneshofer et al., 1986). Henion and Lee (1990) commented that despite the obvious value of such column switching techniques, they were then not widely used. Henion and Lee (1990) also reported preliminary results using column switching techniques demonstrating that the anabolic steroid dianabol could be isolated from horse urine by such techniques and subsequently analysed by on-line M/MS. As an illustration of this technique, readers are referred to the paper by McLaughlin and Henion (1990) which describes the estimation of dexamethasone in bovine liver and muscle. After extraction of the tissues, the sample was injected onto a phenyl HPLC column. The fraction containing dexamethasone was then diverted onto a SIL column which retained the steroid. The SIL column was then back-flushed (i.e. column flow was reversed) onto a third (cyclopropyl) column from which clean extracts of dexamethasone were obtained. These techniques can be of considerable use as a means of automating assays involving HPLC and in addition offer a convenient method of measuring steroids present in trace amounts, by concentrating the analyte on the first column, "heart cutting" onto the second column, and finally by back-flushing, the third and final analytical column is presented with a tight band of concentrated analyte, without non-specific material from the matrix, which has been largely removed by the first column. An automated coupled-column HPLC system has been described for the measurement of melengesterol acetate in bovine tissues (Chichila et al., 1989) and serum cortisol has been measured using pre-column de-proteinisation and on-line extraction using column-switching (Vogeser et al., 2001). A similar method was employed to measure three androgens in cell culture medium (Chang et al., 2003). Further examples in the steroid field have been described (e.g. Magnusson and Sandstrom, 2004 (*in vitro* generated hydroxyl testosterones), Watabe et al., 2006 (estradiol-17β in river water), Rauh et al., 2006 (17-hydroxyprogesterone, androstenedione and testosterone measurement in 100 μL aliquots of serum, plasma and/or saliva from neonates with and without CAH) and Cho et al., 2006 (DHEA sulphate in plasma from patients with Alzheimer's disease).

3.8 Mass Spectrometry

3.8.1 Introduction

Simplistically, a mass spectrometer consists of an "ion source", a "mass analyser", a "detector" and a "data system". Sample molecules are admitted to the "ion source" where they are vaporised and ionised; the ions are separated according to their mass-to-charge ratio (*m/z*) in the "mass analyser" and are then detected. The resulting signals are transmitted to the "data system" and a plot of ion

abundance against *m/z* corresponds to a mass spectrum. In many cases, a "separating inlet" device precedes the ion-source so that complex mixtures can be separated prior to admission to the mass spectrometer. Today, the "separating inlet" device is usually either a capillary gas chromatography (GC) column or a high-performance liquid chromatography (HPLC) column, although capillary electrophoresis or thin layer chromatography can also be interfaced with mass spectrometry.

For steroid analysis, a number of different types of ionisation methods are used to generate gas-phase ions and include; electron ionisation (EI), chemical ionisation (CI), electrospray (ES), atmospheric pressure chemical ionisation (APCI), atmospheric pressure photoionisation (APPI), and the recently introduced, desorption electrospray ionisation (DESI) technique. Other ionisation techniques used, but to a lesser extent, are: fast atom bombardment (FAB), liquid secondary ion mass spectrometry (LSIMS), matrix-assisted laser desorption/ionisation (MALDI) and desorption/ionisation on silicon (DIOS). The selection of the appropriate ionisation mode is one of the key decisions for the analyst to make, and thus, we discussed the most important ionisation modes in some detail in Chapter 2.

3.9 Liquid Chromatography–Mass Spectrometry

3.9.1 Choice of LC-MS Interface

The decision on what form of LC to couple with mass spectrometry depends on the desired application, and it may in fact be preferable to negate an LC separation step all together when, for example, screening clinical samples for metabolic errors of cholesterol metabolism (Bove et al, 2004). Conventional LC encompasses both normal-bore (3–4.6 mm i.d., 0.5-3 mL/min) and narrow-bore (1–2 mm i.d., 20–300 µL/min) columns, while capillary LC includes micro-bore (150–800 µm i.d., 2–20 µL/min) and nano-bore columns (20–100 µm i.d., 100–1,000 nL/min). The earliest generation of electrospray (ES) interface (which is in use even today) is well-matched with flow rates of the order of 5–100 µL/min, with maximum sensitivity being achieved at the lower end of this flow-rate range, and is most compatible with narrow-bore or micro-bore columns. It is possible to interface normal-bore LC columns to such ES interfaces, but with the requirement of a post-column split. Pneumatically assisted ES, sometimes called ion-spray or turbo-ionspray, has been developed to allow the direct coupling of normal-bore columns with the ES interface which is modified to receive flow rates of up to 1 mL/min. APCI interfaces are also capable of operating at this flow rate and receiving eluate from normal-bore columns.

Theoretically, a reduction in column diameter produces a higher concentration of sample in an eluting peak (Abian et al., 1999). As ES is also a concentration dependent process, this dictates that maximum sensitivity can be achieved by using miniaturised LC, and has lead to the increasing popularity of capillary LC-MS and capillary LC-tandem mass spectrometry (MS/MS) in biological mass spectrometry.

A new generation of micro-ES interfaces have been developed which perform optimally at low flow rate (<1 μL/min) and thereby provide maximum sensitivity when coupled with capillary column LC. Despite providing maximum sensitivity, capillary LC performed at low flow rate has its limitations. Although the concentration of sample in an eluting peak is dependent on the reciprocal square of column i.d., the column loading capacity and optimum injection volume also follow a similar relation (with respect to column i.d.). This creates problems in terms of sample injection and column overloading, particularly for columns of i.d. < 300 μm where optimum injection volumes are less than 1 μL (Tomer et al., 1994). The problem of low injection volume can be overcome simply by on-line sample pre-concentration on a trap column arranged in series with the analytical column. Sample pre-concentration is performed at microlitre per minute flow rates on the trap column, which is then flushed, and sample separated on the analytical column. However, the problem of column loading capacity still exists. The best solution to column overloading is to include a group separation step prior to capillary LC. This is illustrated in the work performed by Yang et al. (1997), in which a urine extract from a child with cholestatic liver disease was separated into four fractions according to acidity on an anion-exchange column. Each fraction was analysed by capillary-LC-ES-MS and MS/MS in a 1 h run, allowing the partial characterisation of over 150 bile acids and conjugated bile alcohols. The remaining drawback with capillary column LC, is one of analysis time. For example, in the study performed by Yang et al. (1997), each LC run took 60 min precluding the possibility of high throughput analysis. However, with the development of ultra high pressure liquid chromatography (UPLC – a Waters trademark, 15,000 psi, see review by Swartz, 2005) using smaller particles (around 2 μm), or alternatively monolithic columns operated at lower pressure, it is likely that the time constraint associated with capillary chromatography will be overcome (Wang G. et al., 2006; Licea-Perez et al., 2007; Touber et al., 2007; Wang and Zhang 2007).

While capillary column LC combined with micro-ES will provide the maximum sensitivity for steroid analysis, and is preferable for the profiling of conjugated steroids and bile acids isolated from biological samples, many screening and quantitative studies require high throughput as their main priority, in which case narrow-bore chromatography combined with ES or APCI is often the method of choice.

3.9.2 Derivatisation for LC-MS

The ionisation properties of unconjugated steroids and bile acids can be improved by derivatisation. A good derivatisation reaction should be simple to perform, give a high yield and generate a minimum of side products. Preferably, the reaction will be performed under mild conditions to avoid decomposition of the target analyte. Finally, the derivative should give a higher ion yield than the target analyte, and ideally fragment upon collision-induced dissociation (CID) in an informative

manner generating abundant product ions suitable for multiple reaction monitoring (MRM) studies. Derivatives have been designed with APCI (Singh et al., 2000; Higashi et al., 2002,, 2003, 2006a), ES (Shackleton et al., 1997; Griffiths et al., 2003, 2006; Higashi et al., 2005a, b, 2007c; Nishio et al., 2007; Yamashita et al., 2007a) and MALDI (Khan et al., 2006; Wang Y. et al., 2006) ion sources in mind. There is a useful review on this derivitisation topic (Higashi and Shimada, 2004).

Girard Hydrazones are useful derivatives of carbonyl groups. The Girard reagents, i.e. Girard P (GP) hydrazine, 1-(2-hydrazino-2-oxoethyl)pyridinium chloride, and Girard T (GT) hydrazine, trimethylammonium acethydrazide chloride, are quaternary ammonium salts and effectively tag the steroid with a positively charged group, thereby greatly improving the sensitivity of analysis by ES-MS and MALDI-MS (Griffiths et al., 2003, 2006). Additionally, upon CID these derivatives usually give a prominent neutral loss of 79 and/or 107 Da for GP hydrazones, and 59 and/or 87 Da for GT hydrazones, suitable for high-sensitivity MRM or neutral-loss scans.

Shackleton et al. (1997) were the first to use the GT derivative for ES-MS in their analysis of testosterone and testosterone esters. More recently, Lai et al. (2002) have used the GP derivative to enhance the ionisation of 17-hydroxyprogesterone, while Griffiths et al. (2003) demonstrated the sensitivity gains provided by the GP and GT derivatives for the analysis of a panel of oxosteroids using both ES and MALDI. Johnson (2005) has described LC-MS/MS of a serum 17-hydroxyprogesterone, cortisol and androstenedione panel following derivatisation with the Girard T reagent. To the evaporated supernatant of a diethylether/hexane (9:1, v/v, 1 mL) extract of 100 µL of serum, Johnson added 140 µL of GT reagent (10 mM in methanol containing 1% acetic acid). After 15 min at room temperature, solvent was removed under nitrogen and 150 µL of acetonitrile/water/formic acid (50:50:0.025, v/v/v) and hexane 1 mL were added. After vortexing and centrifugation, the supernatant was removed and the aqueous layer analysed by LC-ES-MS/MS. Double derivatives can be prepared by substituting trifluoroacetic acid for acetic acid, and performing the reaction at 75 °C.

Griffiths and colleagues have used GP derivatisation for oxysterol profiling in brain and blood samples (Griffiths et al., 2006; Wang Y. et al., 2006). As many oxysterols do not posses a ketone(oxo) group, the oxysterol extract was treated with cholesterol oxidase which will convert 3β-hydroxy-5-ene and 3β-hydroxy-5α-hydrogen groups to 3-oxo-4-ene and 3-oxo groups, respectively, which are suitable for GP derivatisation. Sterols were oxidised with cholesterol oxidase essentially as described by Brooks et al. (1983). Cholesterol oxidase was from either *Brevibacterium* or *Streptomyces* sp. The enzyme from *Brevibacterium*, also catalyzes the oxidation of 3β-hydroxy-5-ene and 3β-hydroxy-5α-hydrogen steroids of the C_{19} and C_{21} series (MacLachlan et al., 2000). Reference sterols or those extracted from brain or blood were dissolved in 50 µL of isopropanol, and 10 µL of cholesterol oxidase from *Brevibacterium* (1 mg/mL, 20 U/mg protein) or 2 µL of cholesterol oxidase from *Streptomyces* sp. (2 mg/mL, 44 U/mg protein) in 1 mL of buffer (50 mM KH_2PO_4, pH 7) added, the mixture was incubated at room temperature (25°C for 2–12 h when using the enzyme from *Brevibacterium* or 37°C) for 60 min when using the enzyme from *Streptomyces* sp.) and subsequently used

as the starting solution for reaction with the GP reagent. The oxidation mixture, 1 mL (~50 mM phosphate buffer, 5% isopropanol, 10 or 4 µg enzyme and sterols) was diluted with 2 mL of methanol to give a ~70% methanol solution, and 150 mg of GP hydrazine and 150 µL of glacial acetic acid were added. The mixture was left at room temperature overnight. The GP reaction mixture (3 mL, 70% methanol) after overnight incubation was directly applied to a Sep-Pak C_{18} bed (1 × 0.8 cm in a glass column) followed by 1 mL of 70% methanol and 1 mL of 35% methanol. The combined effluent (now 5 mL) was diluted with 4 mL of water. The resulting mixture (now 9 mL in 35% methanol) was again applied to the column followed by a wash with 1 mL of 17% methanol. To the combined effluent, 9 mL of water was added. The sample was then in 19 mL of about 17.5% methanol. This was again applied to the column followed by a wash with 10 mL of 10% methanol. Now, all the GP derivatives are extracted by the column. They were then eluted with two 1 mL portions of methanol followed by 1 mL of chloroform/methanol, 1:1 (v/v). The three fractions were analysed separately by ES mass spectrometry. The derivatisation protocol has been applied to mixtures of oxosteroids on the µg–ng level and is suitable for the low-level (pg) derivatisation of neutral steroids extracted from tissue (Griffiths et al., 2006; Wang Y. et al., 2006).

2-Hydrazino-1-methylpyridine provides an alternative charged derivative to the GP reagent (Higashi et al., 2005c). The derivatisation reagent requires in-house synthesis, but reacts quantitatively with oxosteroids at 60°C in 1 h. The resulting derivatives of mono-oxosteroids provide 70- to 1,600-fold higher sensitivity than the underivatised steroids in LC-ES-MS experiments. Surprisingly, the derivative appears unsuitable for dioxosteroids, providing little improvement in sensitivity for androstenedione and progesterone.

Dansyl Chloride. Estrone, estradiol and ethinylestradiol are readily derivatised with dansyl chloride. Anari et al. (2002) have shown dansyl derivatives to give an enhanced response in ES-MS experiments and Nelson et al. (2004) have used these derivatives with APCI in a method for estrone and estradiol measurement in human plasma. After adding [$^2H_4$]estrone and [$^2H_5$]estradiol to 0.5 mL of serum, steroids were extracted with 6 mL of methylene chloride. After evaporation of solvent, 50 µL of sodium hydrogen carbonate (100 mM, pH 10.5) and 50 µL of dansyl chloride (1 g/L) were added. The samples were analysed by LC-APCI-MS after heating at 60°C for 3 min.

Oximes. Liu et al. (2000) derivatised oxosteroids in 1 mL of 70% methanol with 50 mg hydroxyammonium chloride. After heating at 60°C for 3 h, the solution was concentrated to near dryness. One millilitre of 10% methanol was added and steroid oximes extracted on a C_{18} column. After a wash with 2 mL of water, oximes were eluted with 1 mL of methanol. No, or incomplete derivatisation was achieved for the 11-oxo group and oxo groups hindered by two adjacent hydroxyl groups. For those steroids, the reaction was carried out in 100 µL of pyridine at 60°C for 1 h with 10 mg of reagent as described by Thenot and Horning (1972). Liu et al., (2000) found that oxime derivatisation gave an improvement of 20-fold in ES-MS sensitivity and applied this methodology to the LC-ES-MS profiling of neurosteroids in rat brain (Liu et al., 2003b). Neurosteroids were derivatised with 2-nitro-4-trifluoromethyl

phenylhydrazine for LC-EC-APCI-MS (Higashi, 2006). In the same paper, LC-ESI-MS is used to detect steroids after reaction with 2-hydrazino-1-methylpyridine to introduce a charged moiety. For oestrogens, picolinyl derivatives have been detected with LC-ESI-MS (Yamashita et al., 2007b). The process of derivative formation may be speeded up with the assistance of microwaves (Zuo et al., 2007).

3.9.3 Applications of LC-MS/MS to Steroid Analysis

With the ever increasing maturity of LC-MS/MS instrumentation and methodology, many commercial and clinical laboratories are embracing this technology for high sensitivity, high throughput analysis of steroids.

Internal Standards. This question has been discussed earlier in this chapter (see Section 3.1), but should perhaps be reiterated here as proper use of internal standards (IS) in MS systems is fundamental for accurate quantitative measurements. Ideally, the IS should not differ in structure from the analyte, so stable-isotope analogues with a mass difference of 3 or greater Da from the analyte are desirable. The following companies supply internal standards (mainly deuteriated) and will undertake custom synthesis: Cambridge Isotope Laboratories, www.isotope.com; CDN Isotopes, www.cdnisotopes.com; Medical Isotopes, www.medicalisotopes.com; Isotec, www.sigmaaldrich.com. Internal standards should always be added to the sample before extraction.

Sample Introduction. The major bottleneck in clinical steroid analysis by MS/MS is sample extraction, so the development of automated extraction techniques is vital. Quest Diagnostics in California (www.questdiagnostics.com) are leaders in the use of an on-line extraction system based on turbulent flow principles, and through this have overcome the rate-limiting step impeding high throughput LC-MS/MS analysis. They use a Cohesive technologies (www.cohesivetech.com) TX4 multiplexing high turbulence liquid chromatography (HTLC) system for on-line extraction of steroids from 150 µL of sera previously treated with 1% trichloroacetic acid (TCA) (Dr. Nigel Clark, personal communication). The HTLC system is divided into two functions: (1) Solid-phase extraction using a large particle size (50 µm) column and high flow rate. The high flow rate causes turbulence inside the column, which ensures optimised binding of steroid to the large particles and the passage of residual protein and debris to waste. (2) Following a loading step from 96-well plates, the flow is reversed and the sample is eluted off the loading column and onto the analytical column. Typically, sequential sample injections can be made every 1–2 min.

3.9.4 Steroid Hormones

The relevance of steroid hormone assay in the clinical environment has been reviewed by Holst et al. (2004).

Steroid Hormone Profiles. Guo et al., (2004) have published a method for the profiling of nine steroids from 760 µL of serum with minimal work-up, comprising acetonitrile protein precipitation. An API-3000 tandem quadrupole instrument (Applied Biosystems) was used with APPI source in the positive-ion mode. The MRM transitions utilised are shown in Table 3.3. The lower level of sensitivity was 100 pg/mL for each steroid, but the authors suggest that the use of an API 4000 instrument would allow a tenfold improvement in sensitivity. The latest tandem quadrupole from Applied Biosystems, the API 5000, would be expected to give an even greater improvement in sensitivity (Guo et al., 2008). The one drawback of their methodology, if used commercially, is the long chromatography time of 14 min. This method has been further improved now encompassing 12 steroids, using only 200 µL of serum with a run time of 8 min (Guo et al., 2006) and has been applied to the investigation of steroid levels in pregnancy (Soldin et al., 2005) and causes of adrenal insufficiency (Holst et al., 2007).

17-Hydroxyprogesterone is the preferred analyte for the diagnosis of 21-hydroxylase deficiency (Fig. 3.14). Investigators at the Mayo Clinic measured a panel of three relevant steroids (17-hydroxyprogesterone, cortisol and androstenedione) in blood spots as a follow-up test for newborn screening (Lacey et al., 2004; Minutti et al., 2004). The mass spectrometry was carried out on an API 3000 tandem quadrupole instrument (Applied Biosystems) with a Turbo-ionspray source operating in the positive-ion mode. LC was conducted on a narrow-bore C_{18} column (50×2.1(i.d.) mm) with a methanol/water solvent system operating in gradient mode (250 µL/min). The internal standard for the three steroids analysed was [$^2H_8$]17-hydroxyprogesterone. Steroids were analysed by MRM. The vast majority of false-positive cases had 17-hydroxyprogesterone levels < 10 µg/L accompanied by a substantial cortisol peak. Samples from confirmed cases characteristically lacked cortisol but had increased 17-hydroxyprogesterone and androstenedione. The reproducibility of the assay was good with inter- and intra-assay CVs of about 20% at the lowest level of 1.9 µg/L, improving to a mean of about 5% at 50 µg/L. The authors admit that some methodological improvement is needed before the method is ready for use as a primary screen. While the analytes of interest elute in 2–4 min, a filter paper contaminant requires a 12 min run to remove. This would translate to maximum daily run of 120, without allowing time for servicing or cleaning. However, 4min runs are possible with serum so the method can already be used for high-throughput analysis by commercial laboratories.

In two other publications, investigators have chosen to use derivatives in order to improve sensitivity. Whether this is needed will depend on the instrument used since sensitivity did not appear to be a problem in the Mayo studies. Lai et al. (2002) use the Girard P reagent with 6α-methylprednisolone as internal standard. They had partially automated the extraction and derivatization by employing 96-well technology and used an API 2000 tandem quadrupole instrument (Applied Biosystems) with a Turbo-ionspray source. The collision gas was nitrogen, chromatography was narrow-bore C_{18} (50 × 2 (i.d.) mm) with an acetonitrile/water (1:1) solvent at a flow rate of 50 µL/min. Each analysis took 3 min and 300 could be carried out before instrument cleaning was required. Intra-assay and inter-assay CVs were <12%. The maximum number of samples analyzed daily was 192 for one technician and one instrument, an equivalent workload to the RIA assay employed in their laboratory. Johnson (2005)

described LC-MS/MS of a serum 17-hydroxyprogesterone, cortisol and androstenedione panel following derivatisation with the Girard T reagent. He reported that preparation of the derivative resulted in a tenfold improvement in sensitivity compared to analysis of the steroids with underivatised ketones. This method was then at an initial stage of development since automation of sample handling was not addressed. Sample size was 100 μL and the linearity of response was over a 1–1,000 ng range. Johnson did address the issue of internal standards, deciding that employing a stable isotope analogue of each analyte would improve the accuracy by reducing the importance of variable instrument performance. As well as the [$^2H_8$]17-hydroxyprogesterone standard; he utilized [$^2H_3$]androstenedione and [$^2H_3$]cortisol. He also addressed an important issue which does not get enough attention, the instability of deuteriums in some currently available labelled standards. When subject to a harsh Girard T derivatisation, deuterium was lost from the [$^2H_8$]17-hydroxyprogesterone since this standard is originally prepared by proton/deuterium exchange. However, a mild derivatisation was developed that prevented loss of deuterium.

Redor-Goldman et al. (2005a, b) report a 17-hydroxyprogesterone, progesterone and androstenedione panel on 150 μL of serum. They transfer samples to 96-well plates, add labelled internal standards and 150 μL formic acid. After 30 min incubation, 70 μL is injected using HTLC (TX4, Cohesive Technologies) on a 50 × 1 mm extraction column followed by chromatography on a 75 × 3 mm analytical column. Mass spectrometry is on a Thermo Electron TSQ quantum tandem quadrupole with an APCI source operated in the positive-ion mode. The total run time is 1.5 min per analyte.

21-Deoxycortisol is the key analyte overproduced in congenital adrenal hyperplasia (CAH). This is the precursor of the urinary metabolite pregnanetriolone, which has long been considered a hallmark analyte for confirming CAH in infancy (Fig. 3.14).

Cristoni et al. (2004a) report the analysis of 21-deoxycortisol by ES-MS and APCI using a Thermo Finnigan LCQ ion-trap instrument operated in the positive-ion mode. They found that ES afforded greater sensitivity. They addressed the problem of the possible interference of the 21-deoxycortisol isomer, 11-deoxycortisol, itself the analyte for diagnosis of 11β-hydroxylase deficiency. While the steroids share many fragmentations, some of these are specific for each steroid and can be used for MRM. In addition, these two compounds are resolved by reversed-phase chromatography (C_{18} 250 × 2.1 (i.d.) mm, 200 μL/min). Cristoni et al. (2004a) maintain that 21-deoxycortisol is a good analyte for identifying heterozygous individuals for 21-hydroxylase deficiency, and certainly their measurement of 11-deoxycortisol will in the future allow diagnosis of 11β-hydroxylase deficiency. The report is preliminary in that they did not use internal standards and their chromatography time of 9 min would have to be shortened before a truly routine analysis could be validated. A further publication from this group describes the use of surface-activated CI ion-trap mass spectrometry for the analysis of blood 21-deoxycortisol (Cristoni et al., 2004b).

Cortisol and Related Compounds (see chapter 5). The measurement of these compounds in urine is essential for the study of Cushing's disease, glucocorticoid remediable aldosteronism (GRA), apparent mineralocorticoid excess (AME) syndrome, and related conditions. Taylor et al., (2002) at the Mayo Clinic published a method for the simultaneous analysis of urinary cortisol and cortisone. Measuring both these steroids allows the diagnosis of Cushing's disease and the AME syndrome.

Table 3.3 Unconjugated steroids and steroid sulphates of clinical importance in human serum: Reference ranges (classical techniques or LC-MS), LC-MS monitored ions and MRM transitions. Modified from Shackleton (2008) with permission [*Copyright 2008 Springer*]

Steroid	Parent ions and MRM transitions	Adult range (μg/L)	Child (μg/L) [where given age in years]	Males (μg/L)	Females (μg/L)	Follicular (μg/L)	Luteal (μg/L)	Reference (MS method)
Pregnenolone	(317)[a]	0.1 – 2.3	≤0.1	0.1 – 2.0	0.1–2.3			
Progesterone	315 → 109	0.1 – 2.5	≤0.2	**≤0.3**[b]		**<0.14**[b]	**<0.31**[b]	Guo et al. (2004)
d_9 – labeled	324 → 100							Redor – Goldman et al. (2005b)
17 - OH – progesterone	331 → 97	0.5 – 5.0	<1 year, 0.15 – 4.2	**0.32 – 3.07**[b]		**<1.85**[b]	**<2.85**[b]	Guo et al. (2004)
d_8 - labeled	339 → 100		<10 years, 0.15 – 0.8					Redor – Goldman et al. (2005b)
			>10 years, 0.15 – 3.7					Minutti et al. (2004)
17 - OH – pregnenolone	(333)[a]	0.4 – 4.5	<1.0	0.4 – 4.5	0.2 – 4.0			
21 - Deoxycortisol	347 → 311							Cristoni et al. (2004a)
	347 → 293							
Desoxycorticosterone (DOC)	(331)[a]		0.01 – 0.1	0.035 – 0.11		0.015 – 0.08	0.035 – 0.13	
Corticosterone	347 → 121	0.6 – 12.9	0.8 – 20.3					
Aldosterone	361 → 325	**0.3 – 1.6**[b]	**5 – 9** years, **<0.9**[b]					Redor – Goldman et al. (2005b)
	361 → 315		**1 – 17** years, **<3.5**[b]					
	359 → 331[c]	**0.03 – 0.16**[b]						Fredline et al. (1997)
11 – Deoxycortisol	347 → 97	**0.2 – 1.3**[b]	**0.1 – 2.0**[b]					Guo et al. (2004)
d_2 – labelled	349 → 97							
Cortisone (serum, total)	361 → 163	**6 – 27**[b]	2.3 – 17.7					Kushnir et al. (2004)
	361 → 105							

Cortisone (urine free, µg/24 h)	361 → 163	**23 – 195**[b,d]				Clarke and Goldman (2005), Palermo et al. (1996)[e]
				17 – 141[b,d]	**15 – 122**[b,d]	Taylor et al. (2002)
Cortisol (serum, total)	363 → 121 363 → 97	**20 – 240**	**20 – 230**			Guo et al. (2004)
d_4 labelled	367 → 121					Kushnir et al. (2004)
Cortisol (urine free, µg/24 h)	363 → 121 363 → 97	**3.0 – 55**	1 – 4 years, **0.9 – 8.2**			Clarke et al. (1996) Palermo et al. (1996)[e]
d_4 labelled	367 → 121		5 – 14 years, **1.0 – 45**			
				4.2 – 60	**3.0 – 43**	Taylor et al. (2002)
18 - OH – cortisol (urine, µg/24 h)	379 → 121	**43 – 515**		**43 – 295**	**51 – 515**	Clarke et al. (1996) Palermo et al (1996)[e]
DHEA	271 → 213	1.3 – 12.5		1.8 – 12.5	1.3 – 9.8	Guo et al. (2004)
DHEA sulphate	367[c]	**1,000 – 2,850**	<5 days, 100 – 2,540 <5 years, 10 – 400 10 – 17 years, 150 – 5,550	**1,800 – 3,450**	**670 – 3,700**	Shackleton et al. (1990)
Testosterone (total)	289 → 109 289 → 97		<10 years, F, **≤0.35** < 10 years, M, **≤ 0.4**	**1.2 – 11.1**	**0.03 – 0.46**	Redor – Goldman et al. (2005a)
d_5 - labelled	294 → 112 294 → 99		>10 years, F, **≤0.40** >10 years, M, **0.21 – 10.0**			
Analyte d_3 - labelled	289 → 97 292 → 97					Starcevic et al. (2003)
Dihydrotestosterone (DHT, total)	291 – 255	0.05 – 0.75		0.25 – 0.75	0.05 – 0.3	Zhao et al. (2004)

(continued)

Table 3.3 (continued)

Steroid	Parent ions and MRM transitions	Adult range (μg/L)	Child (μg/L) where given age in years	Males (μg/L)	Females (μg/L)	Follicular (μg/L)	Luteal (μg/L)	Reference (MS method)
Androstenedione	287 → 97	**0.2 – 2.85**		**0.4 – 2.2**		**0.2 – 0.75**	**0.6 – 2.85**	Guo et al. (2004)
d_7 - labelled	294 → 100							Minutti et al. (2004)
								Redor – Goldman et al. (2005b)
Estriol[c]	287 → 171	<0.1		<0.1	<0.1			Guo et al. (2004)
d_2 - labelled	289 → 147							
Estrone (total)	504 → 171[f]			**0.01 – 0.06**	**0.017 – 0.20** PM[g] <0.05	0.015 – 0.25	0.015 – 0.2	Nelson et al. (2004)
Estradiol	506 → 171[f]		>10 years, 0.005 – 0.06 10 – 17 years, F, 0.005 – 0.4 10 – 17 years, M, 0.005 – 0.04	**0.01 – 0.04**	**0.015 – 0.35**			Nelson et al. (2004)
Cholesterol sulphate	465[c]	**50 – 300**						Shackleton and Reid (1989)
$^{13}C_2$ - labeled	467[c]							

[a]Bracketed number corresponds to the protonated molecule if mass spectrometry was used for quantification.
[b]Figures in bold obtained by tandem MS; others by immunoassay techniques (from Quest Diagnostics manual).
[c]Negative ion.
[d]Urine μg/24 h.
[e]Data also from GC-MS.
[f]Dansyl derivative.
[g]PM is post-menopausal.

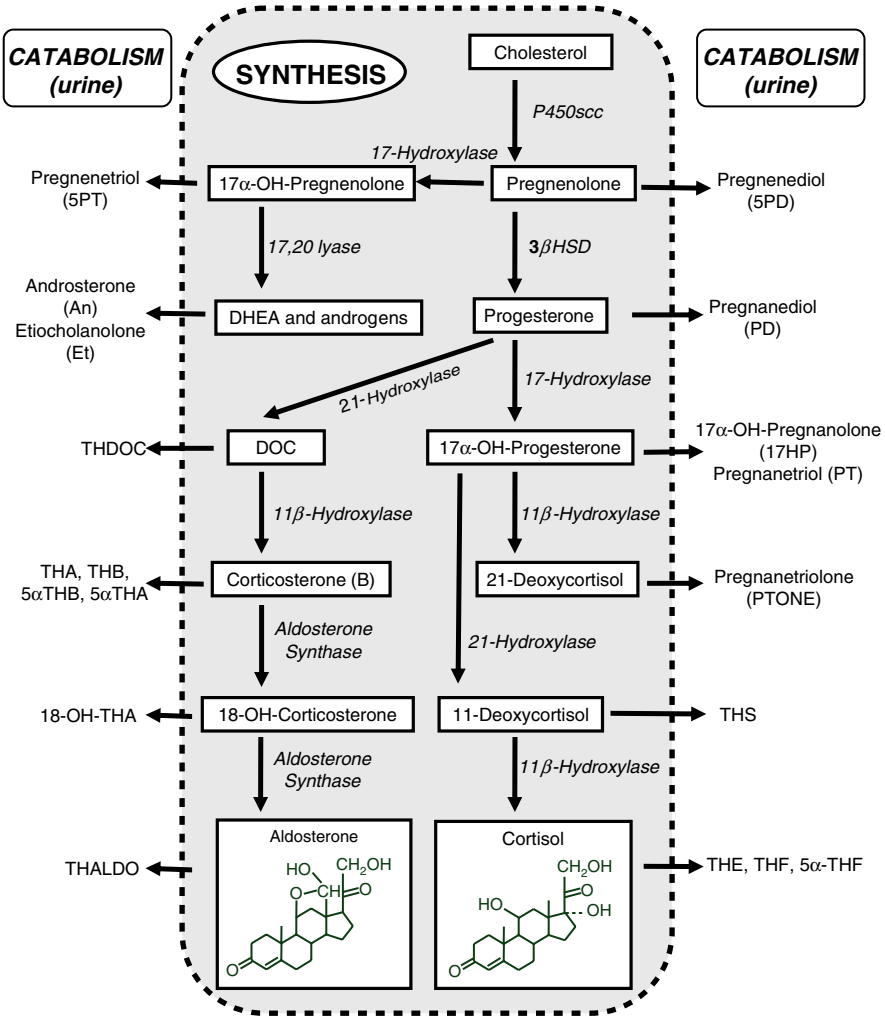

Fig. 3.14 Synthesis of adrenal steroids and major excreted metabolites. Modified with permission from Shackleton (2008)

They used [$^2H_4$]cortisol as internal standard and took a 0.5 mL urine sample. There was an extensive extraction and washing step with 4.5 mL methylene chloride. An API 2000 tandem quadrupole (Applied Biosystems) was used in the positive-ion mode with MS/MS. Chromatography was conducted on a normal-bore C_{18} column (33 × 4.6 (i.d.) mm) with C_{18} pre-column (4 × 2 mm). An isocratic methanol/water solvent system was used at a flow rate of 1 mL/min. The source was of the Turbo-ionspray type, and using a column splitter, 200 μL/min was introduced to the source. MRM was conducted in the positive-ion mode monitoring *m/z* 363 → 121 for cortisol, 367 → 121 for [$^2H_4$]cortisol and 361 → 163 for cortisone. Cortisol and

cortisone were separated and both were eluted within 2 min. Inter- and intra-assay variation for both compounds was <9% for amounts above 2 μg/dL.

Quest Diagnostics use an LC-MS/MS panel for diagnosing cortisol-related disorders. This panel was designed to diagnose Cushing's syndrome and the hypertensive conditions, AME syndrome and GRA. The panel quantifies cortisone, cortisol, 6β-hydroxycortisol and 18-hydroxycortisol. The Quest analysis uses [$^2H_4$]cortisol as an internal standard and HTLC for on-line extraction. This panel has replaced the RIA and HPLC methods previously used by this commercial laboratory. A recent publication describes MS/MS of cortisone and cortisol in serum using APPI and similar conditions and MRM transformations to those listed above (Kushnir et al., 2004).

11-Deoxycortisol. CAH caused by 11β-hydroxylase deficiency is diagnosed by finding elevated 11-deoxycortisol. This steroid is included in the panel of Guo et al., (2004, 2006), reported above, monitoring the transitions *m/z* 347→97 for analyte and 349 → 97 for [$^2H_2$] internal standard.

Aldosterone. Fredline et al. (1997) report an MRM negative-ion ES method for aldosterone using flumethasone as internal standard. The MRM transition used was 359 → 331. The assay was linear over a 15–500 pg/mL range and the limit of quantitation 15 pg/mL. A manual extraction was used and recovery and accuracy were excellent. Quest Diagnostics use APCI in the positive-ion mode monitoring the transitions 361 → 325 and 361 → 315 (Dr. Nigel Clarke, personal communication).

Testosterone and Dihydrotestosterone (DHT). Testosterone assays have been notoriously inaccurate, particularly at the low levels found in women and children. Excess testosterone in women is often a cause of infertility, hirsutim, amenorrhoea, and obesity and accurate measurement is essential for evaluating the causes of these disorders. Accurate measurement in small sample volumes is essential in pediatrics. Starcevic et al. (2003) report the LC-MS/MS of serum testosterone using an API tandem quadrupole mass spectrometer (Applied Biosystems) in the positive-ion mode. [$^2H_3$]Testosterone was the internal standard. The run time is 1.25 min. They monitored the transition m/z 289 → 97 for analyte and 292 → 97 for internal standard. These investigators achieve excellent reproducibility and linearity. Sensitivity down to 10 pg/mL allows them to readily measure the hormone in samples from females and children. Data produced using this new method correlates perfectly with data produced by classical methodology. Quest Diagnostics have established a routine system for testosterone, which has now replaced their other methods of analysis (Clarke and Goldman, 2005; Redor-Goldman, 2005a, b). The analysis is carried out on a Thermo Finnigan TSQ Quantum Ultra tandem quadrupole operated with an APCI source in the positive-ion mode. They use two transitions 289 → 109 and 289 → 97, for analyte and 294 → 112 and 294 → 99 for [$^2H_5$] labelled internal standard. On-line extraction and short retention times allow them to assay several thousand samples per month per instrument. Higashi et al. (2005b & c) use an ES-MS/MS method for measuring testosterone and DHT with derivatization with 2-hydrazino-1-methylpyridine to improve sensitivity. Further studies using a similar approach in rat brain and serum (Higashi et al., 2006b) and prostate (Higashi et al., 2005b, 2006c) have been described.

Androstenedione has been measured as part of a CAH panel by Minutti et al. (2004), using the transition 287 → 97, and by Quest Diagnostics using HTLC and APCI (Clarke and Goldman, 2005; Redor-Goldman, 2005a, b). LC-ESI(+)-MS has been used to measure androstenedione in rat brain (Higashi et al., 2007a, c, 2008).

Estrone and Estradiol. The routine measurement of estrone and estradiol by immunoassay techniques has also given rise to the familiar problems of poor sensitivity, cross-reactivity and poor inter-method reproducibility. Most automated methods cannot measure these steroids in sera of children and men. Development of LC-MS/MS methods has also proven challenging as many investigators have found that estrone and estradiol are poor ionisers. Thus, desired sensitivity has not been achieved. The Mayo group (Nelson et al., 2004) published the LC-MS/MS quantification of estradiol and estrone using dansyl chloride derivatives. These derivatives are easily prepared, provide excellent sensitivity in positive-ion mode APCI, and produce a prominent product-ion suitable for MRM. The internal standards used were [$^2H_5$]estradiol and [$^2H_4$]estrone. Methods for measuring estrone and estradiol after derivatisation using LC-ESI(+)-MS (Nishio et al., 2007) and LC-electron capture APCI-MS (Higashi et al., 2006a) have also been described.

Using the best of modern instrumentation dansyl derivatization may not be necessary to reach sensitivity requirements for estradiol measurement. Estradiol is an analyte within the Guo et al. (2004) panel. Using an API 3000 tandem quadrupole (Applied Biosystems) with an APPI source in the positive-ion mode, they measure the *m/z* 255 → 159 transition for estradiol and *m/z* 259 → 161 for [$^2H_4$] estradiol. With the API 3000 instrument, they could not measure estradiol below 100 pg/mL, although preliminary studies showed that the API 4000 (Applied Biosystems) could achieve sensitivity of 10 pg/mL. The Applied Biosystems Company have demonstrated reproducible estradiol measurement at concentrations down to 0.1 pg 'on-column' on their API 5000 instrument with an APCI source. The linearity was excellent over the range 0.001–10 ng/mL (company demonstration data). Guo et al. (2008) describe an LC-MS/MS method for the analysis of serum estrogens in 200μL of serum without derivatisation but do not specify the source of the serum samples.

Estriol. This steroid is also part of the Guo et al., (2004) panel, monitoring the negative-ion transitions m/z 287 → 171 for estriol and m/z 289 → 147 for [$^2H_2$]estriol.

DHEA and DHEA Sulphate. Guo et al., (2004) analyzed these in the positive-ion mode with APPI using the transitions *m/z* 271 → 213 for DHEA and 273 → 213 for the [$^2H_2$] internal standard. DHEA sulphate is more suited to the measurement by negative-ion ES mass spectrometry as demonstrated in an early study by Shackleton et al. (1990a). DHEA has also been measured in human saliva using LC-ESI(+)-MS after derivatisation with 2-hydrazino-1-methylpyridine (Higashi et al., 2007d).

Neurosteroids. LC-ESI(+)-MS has been described in a number of publications from Shimada's group with (Higashi et al., 2005, 2006b, 2007a) and without (Higashi et al., 2008) prior derivatisation to improve the ESI response.

3.9.5 Steroid Metabolites and Precursors

GC-MS is the most powerful technique for steroid profile analysis, and with just a few exceptions all disorders of steroid synthesis and metabolism first had their metabolome defined by GC-MS analysis of urine. The coupling of GC to an MS system also provides unparalleled opportunity for identification of the structures of unknown steroids, especially when linked to different derivative formation prior to GC. GC-MS for steroid analysis is thus discussed in detail in Section 3.10. Here, discussion will continue on LC-MS applications. LC-MS has the potential for steroid profile analysis, however, to date; LC-MS has been mostly used for the analysis of individual metabolites or panels of metabolites as discussed above.

Early studies on bile acids, steroid sulphates and glucuronides found in urine or plasma were performed using FAB-MS by Shackleton and colleagues, amongst others (Shackleton and Straub, 1982; Shackleton, 1983; Shackleton et al. 1983; Gaskell et al., 1983; Clayton et al. 1987). Today's API techniques, in particular ES, have supplanted FAB as the method of choice for the ionisation of bile acids and steroid conjugates.

Steroid Sulphates. Acidic steroids are preferably ionised in the negative-ion mode. Pioneering work on the ES analysis of steroids was performed by Henion and colleagues in the late 1980s (Weidolf et al., 1988). They combined narrow-bore HPLC (C_{18} 100 × 1 (i.d.) mm, 40 µL/min) with negative-ion ES for the analysis of steroid sulphates. By performing selected ion monitoring (SIM) studies on standard compounds, they were able to obtain the very impressive detection limit of 10 pg on-column. When they operated their tandem quadrupole instrument in the MRM mode, which offers great gains in selectivity, they obtained an on-column detection limit of 300 pg. Shackleton and colleagues were the first to use ES-MS and ES-MS/MS for the analysis of steroid sulphates in plasma (Wong et al., 1992). Despite using off-line HPLC, they were able to achieve low nanogram detection limits. Steroid sulphates can also be analysed by ES operated in the positive-ion mode. Bowers and Sanaullah (1996) recorded positive-ion ES-MS and MS/MS spectra of a series of steroid sulphates. They achieved a detection limit of 65 pg by SIM. The MS/MS spectra were found to contain major fragment ions corresponding to $[M + H - 80]^+$, $[M + H - 98]^+$ and $[M + H - 116]^+$. 17-Keto(oxo)steroid sulphates (Higashi et al., 2007b) and DHEA (Higashi et al., 2007d) have been determined in human saliva by LC-ES-MS after extraction with RP-Strata-X cartridges. Strata columns have also been used to extract metabolites of DHEA and 4-androstenedione from EpiskinTM incubation media prior to LC-APCI-ion trap MS (Luu-The et al., 2007).

Nano-ES, without LC separation, has been used for the analysis of steroid sulphates. Chatman et al. (1999) used negative-ion nano-ES in combination with MS/MS for the analysis of steroid sulphates in plasma. By recording the product-ion spectra, they were able to identify testosterone sulphate ($[M - H]^-$, *m/z* 367) in the urine of a primate. Their results were based on the observation of MS/MS fragment ions which included *m/z* 177 which is characteristic of testosterone sulphate.

Chatman et al. (1999) also exploited precursor-ion scanning for *m/z* 97 (HSO_4^-), and were able to obtain a very impressive detection limit of 200 amol (~70 fg) for steroid sulphates added to cerebrospinal fluid. For the analysis of neutral steroids, Chatman et al. (1999) used a derivatisation method in which alcohol groups are converted to sulphate esters, and then the resulting steroid sulphates are analysed by negative-ion nano-ES. This method was also used by Sandhoff et al. (1999) for the quantification of free cholesterol in CHO (Chinese hamster ovary) cells and Golgi membranes.

Nano-ES, again without LC separation, has been used to study the fragmentation of neurosteroid sulphates (Griffiths et al., 1999) at high collision-energy (4 keV–400 eV) on a hybrid magnetic sector – TOF instrument (Micromass). Complete structural information could be obtained from 1 ng (~3 pmol) of steroid sulphate (e.g. pregnenolone sulphate), while fragment ions characteristic of the sulphate ester group, i.e. *m/z* 80 (SO_3^-) and 97 (HSO_4^-) could be obtained from 3 pg (10 fmol) of sample. In this work, the advantage of high-energy MS/MS was evident.

APPI has been used for the analysis of steroid sulphates. This was demonstrated by Guo and colleagues who interfaced a C_{18} column (330 × 3 (i.d.) mm) to an APPI source (Guo et al., 2004). With eluent flow rate of 0.5 mL/min a 20-min chromatographic run was performed going from 100% solvent A (2% methanol) to 89% B (100% methanol). The dopant used was toluene at a flow rate of 50 µL/min. In the positive-ion mode DHEA sulphate fragments in the ionisation process with loss of the sulphate ester group to give the $[M + H - H_2SO_4]^+$ ions at *m/z* 271, while free DHEA gives $[M + H - H_2O]^+$ ions also at *m/z* 271 (Table 3.3). However, the retention times on the C_{18} column for these two steroids were 7.46 and 13.99 min, respectively. By performing MRM studies (*m/z* 271 → 213 for both steroids), the sensitivity of the method was of the order of 100 pg on-column.

Steroid Sulphate Profiles. Liu et al. (2003a & b) have used capillary LC combined to low-flow-rate ES to analyse steroid sulphates from biological samples. They used a C_{18} capillary column (350 × 0.1 (i.d.) mm) preceded by a short trap column, and performed isocratic and gradient elution at flow rates of 0.2–0.3 µL/min. Liu et al. (2003a) illustrated the potential of combining low flow rate LC with MS/MS for steroid profile analysis, where numerous isomeric compounds require separation and identification, by analysing steroid sulphates in plasma. Briefly, 5 µL of plasma was diluted with 1 mL of 70% ethanol containing an isotope-labelled standard. The mixture was centrifuged to remove precipitated proteins, and the supernatant passed through a bed of Bondasil C_{18} (~30 mg) packed in a pasture pipette to remove cholesterol and non-polar lipids. The effluent was collected, dried and redissolved in 100 µL of 10% methanol, and finally an aliquot of this solution was injected onto the column. Shown in Fig. 3.15 are total ion current (TIC) and reconstructed ion chromatograms (RICs) for steroid sulphates expected to be found in plasma. Twenty microlitres of the sample corresponding to 1 µL of plasma was injected onto column. The peak at 17.76 min in the RIC of *m/z* 367 corresponds to the $[M - H]^-$ ion of DHEA sulphate, and that at 32.97 in the RIC of 395 to the $[M - H]^-$ ion of pregnenolone sulphate. Using an isotope-labelled internal standard, the concentration of these two sulphates were determined to be 4.1 and 0.22 µM (1.5 and 0.08 mg/L), respectively (cf. Table 3.3). Sulphate esters of androsterone,

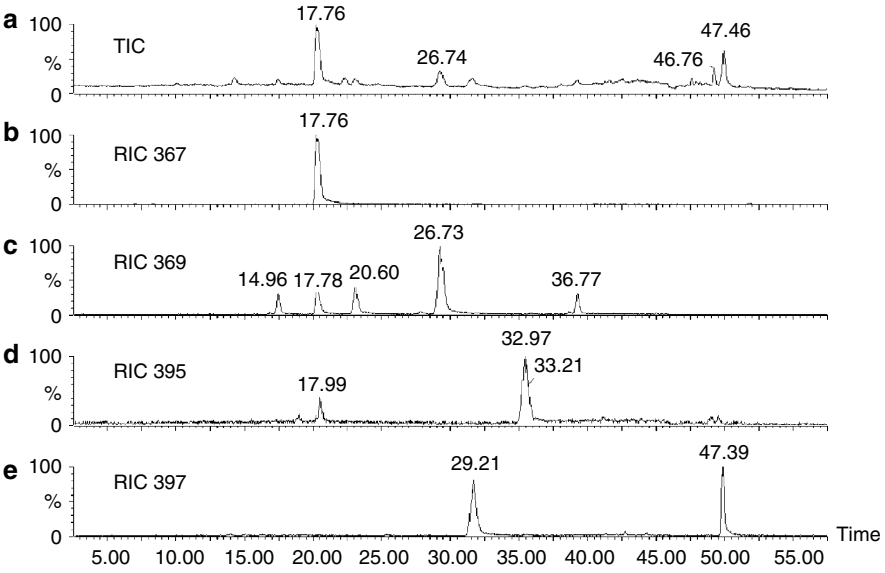

Fig. 3.15 LC-MS of steroid sulphates in human plasma. Total ion current chromatogram (TIC) and RICs obtained from an analysis of a human plasma sample. (**a**) TIC, (**b**) RIC of *m/z* 367, (**c**) RIC of *m/z* 369, (**d**) RIC of *m/z* 395, (**e**) RIC of *m/z* 397. Twenty microlitres of sample solution, corresponding to 1 μL of plasma, was injected onto the pre-column. After an initial desalting period of 20 min with solvent A (10% methanol containing 10 mM ammonium acetate), elution was performed with a gradient starting at 50% solvent B (80% methanol containing 10 mM ammonium acetate) going to 70% in 40 min, maintained at this level for 20 min and then increased to 100% solvent B in 10 min. Data acquisition was started 34 min after the start of the gradient. Time is given in minutes. Identification of the peaks is detailed in text, which also takes data from Fig. 3.16. From Liu et al., Griffiths & Sjövall (2003a) with permission)

epiandrosterone, and androstenediol all give $[M - H]^-$ ions of *m/z* 369. Shown in Fig. 3.15c is the RIC for this *m/z*, and in Fig. 3.16 are high-energy (400 eV) MS/MS spectra for the five peaks observed in the RIC. High-energy (400 eV) MS/MS spectra contain information that not only indicates the nature of the conjugating group but also its location. This is illustrated for the $[M - H]^-$ ions of *m/z* 369 in Fig. 3.15c. The predominant ion in RIC for *m/z* 369 (Fig. 3.15c) gives the MS/MS spectrum illustrated in Fig. 3.16d. The peak at *m/z* 191 indicates that the sulphate group is on the A-ring and that the C-5–C-6 bond is saturated, and comparison of this MS/MS spectrum with that of authentic androsterone sulphate indicates that this compound is androsterone sulphate. The peak at 20.60 in the RIC shown in Fig. 3.15c gives the MS/MS spectrum shown in Fig. 3.16c. The MS/MS spectra in Figs. 3.16c and d are very similar; however, the $[M - H - 80]^-$ ion at *m/z* 289 is less abundant in Fig. 3.16c. This subtle difference, and also the earlier elution of this compound, suggest it is epiandrosterone sulphate. The chromatographic peak eluting at 36.77 min is too late to be another stereoisomer of androsterone sulphate; however, the fragment ions at *m/z* 191 and 259 in its MS/MS spectrum (Fig. 3.16e) indicate that the sulphate group is on the A-ring and that the B-ring is saturated.

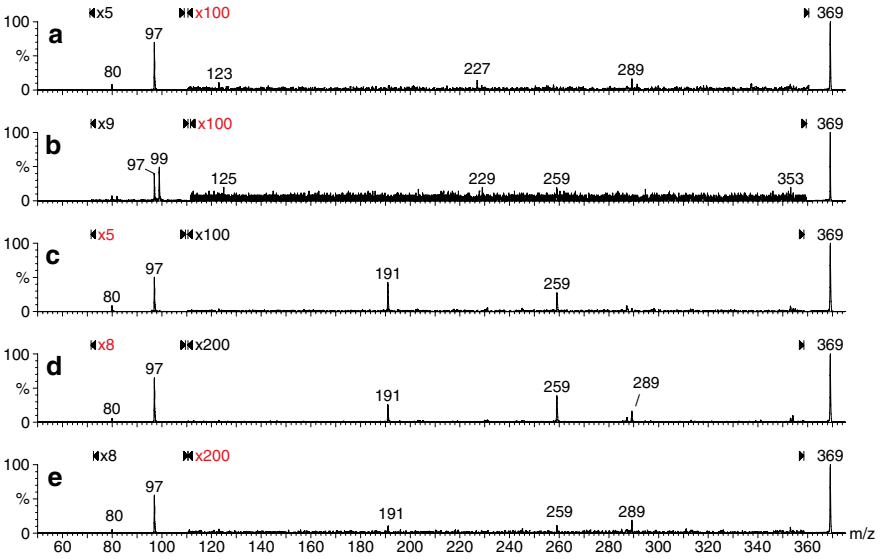

Fig. 3.16 MS/MS of steroid sulphates from human plasma. High-energy (400 eV) CID spectra of ions of *m/z* 369 from the human plasma sample in Fig. 3.15. The spectra were recorded on an AutoSpec-OATOF hybrid magnetic sector – OATOF instrument. The CID spectra of the five individual peaks in the RIC of *m/z* 369 in section c are shown in the order of their retention times (**a**-**e**). The spectra are magnified by a factor of 5, 8 or 9 between *m/z* 70 and 110 and by factors of 100 (**a**-**c**) and 200 (**d**&**e**) between *m/z* 110 and 358. Methane was used as the collision gas at a pressure which attenuated the precursor ion beam by 75%. Chromatographic conditions are given in Fig. 3.14. From Liu et al. (2003a), with permission.

The peak at 14.96 in the RIC gave the MS/MS spectrum in Fig. 3.16a. The absence of a fragment ion at *m/z* at 191, but the presence of one at *m/z* 227 indicate that this steroid has the same AB-ring structure as in DHEA sulphate, and this compound is probably androst-5-ene-3β,17β-diol-3-sulphate. Finally, the peak at 17.78 min corresponds to the ^{34} S-isotopic peak of DHEA sulphate. Liu et al. (2003a, b) investigated the sensitivity of their low-flow-rate chromatographic system, and established an on-column detection limit of 3 pg (7.5 fmol) for steroid sulphates in spectra recorded over a small *m/z* range (360–416); this was improved to 0.2 pg (500 amol) by performing SIM and 0.1 pg by monitoring the $[M - H]^- \rightarrow 97$ ($[HSO_4]^-$) transition in an MRM experiment. The above data clearly demonstrate the advantages of performing low-flow-rate LC-MS and LC-MS/MS when small quantities of biological samples are available, and the levels of endogenous metabolites are low. 17-Keto(oxo) steroid sulphates have also been measured in human plasma by LC-ES-MS (Higashi et al. 2007b).

Steroid Glucuronides can be analysed by negative- and positive-ion ES (Bowers and Sanaullah, 1996; Borts and Bowers, 2000; Kuuranne et al., 2000). When positive-ion ES spectra are recorded with an ammonium acetate buffer, steroid 17-*O*-glucuronides with a 3-oxo-4-ene structure tend to give $[M + H]^+$ and [M +

$NH_4]^+$ ions, while 3-*O*-glucuronides tend to give just $[M + NH_4]^+$ ions. This can be explained by the high gas-phase basicity of the 3-oxo-4-ene structure favouring protonation at the C-3 ketone group. Low-energy MS/MS spectra of the $[M + H]^+$ or $[M + NH_4]^+$ ions show $[M + H - 176]^+$, $[M + H - 194]^+$ and $[M + H - 212]^+$ fragment ions. This series of fragment ions is analogous to the series of major fragment ions observed in the spectra of steroid sulphate $[M + H]^+$ ions. Interestingly, 17-*O*-glucuronides with a 3-oxo-4-ene structure give steroid-skeleton fragment ions at *m/z* 97 and 109 which confirms that protonation occurs at the C-3 ketone group. In a study of anabolic steroid glucuronides aimed at identifying the most promising ionisation method for subsequent MS/MS studies, Kuuranne et al. (2000) concluded that the fragment-ion patterns generated by $[M + H]^+$ and $[M + NH_4]^+$ ions are more structurally informative than those generated from $[M - H]^-$ ions, and that positive-ion ES-MS/MS is the most promising method for further development of LC-MS/MS based methods for anabolic steroid glucuronide analysis.

Oxysterols are formed in the first step of cholesterol metabolism, and have traditionally been analysed by GC-MS. However, LC-MS methods are now gaining popularity. To achieve maximum sensitivity, Griffiths and colleague use a derivatisation strategy which also involves an enzymatic conversion of 3β-hydroxy-5-ene or 3β-hydroxy-5α-hydrogen sterols to 3-oxo-4-ene or 3-oxo sterols (Higashi et al., 2005a), respectively, then treatment with the GP reagent to give GP hydrazones (Griffiths et al., 2006; Khan et al., 2006; Wang et al., 2006). Using this methodology, Griffiths and colleagues have profiled the oxysterol content of rodent brain and human blood. They used a micro-bore (150 × 0.18 (i.d.) mm) C_{18} column with a methanol/water gradient and confirmed 24S-hydroxycholesterol to be the major oxysterol in rat brain. Other oxysterols identified in rodent brain include 24,25-, 24,27-, 25,27-, 6,24, 7α,25-, and 7α,27-dihydroxycholesterols. In addition, 3β-hydroxy-5-oxo-5,6-secocholestan-6-al and its aldol, two molecules linked to amyloidogenesis of proteins, were characterised in rat brain (Wang et al., 2007).

Burkard et al., (2004) have used APCI in combination with reversed-phase HPLC for the analysis of oxysterols in plasma. Chromatography was performed on a C_{18} column (125 × 2 (i.d.) mm) with a methanol/acetonitrile/10 mM ammonium acetate gradient run at 250 µL/min. The run time was 35 min, and 24S-hydroxycholesterol separated from 27-hydroxycholesterol. In the absence of protonated molecules, SIM was performed on the $[M + H - H_2O]^+$ ions at *m/z* 385. The quantification limit for plasma samples of 0.5 mL was 40 µg/L for 24S-hydroxycholesterol and 25 µg/L for 27-hydroxycholesterol, and the levels of these oxysterols in healthy volunteers were 64 ± 14 and 120 ± 30 µg/L, respectively.

Oxysterols can also be formed by cholesterol ozonolysis. Pulfer et al. (2004) separated cholesterol ozonolysis products on a C_{18} column (250 × 4.6 mm) using an acetonitrile/water/methanol 1 mM ammonium acetate gradient (1 mL/min), with a 40 min run time. Eluent was split to ES-MS (50–100 µL/min) and either a scintillation detector or fraction collector. Using negative-ion ES $[M + CH_3CO_2]^-$, adducts of oxysterols were observed while in the positive-ion mode $[M + NH_4]^+$ ions were detected. By collecting fractions from the LC column and performing MS/MS and GC-MS on these, the major cholesterol ozonolysis product was identified as 5-hydroperoxy-B-homo-6-oxa-choles-

tane-3β,7-diol or 7-hydroperoxy-B-homo-6-oxa-cholestane-3β,5-diol. Pulfer and Murphy (2004) used this methodology further to identify the major cholesterol-derived product from the treatment of lung surfactant with ozone, as 5β,6β-epoxycholesterol. 5β,6β-Epoxycholesterol was found to be metabolised by lung epithelial cells to small amounts of the expected metabolite, cholestan-3β,5α,6β-triol (Sevanian et al., 1991) and more abundant levels of an unexpected metabolite cholestan-6-oxo-3β,5α-diol. Other workers have used negative-ion ES-MS analysis of 2,4-dinitrophenylhydrazone derivatives of cholesterol ozonolysis products. By performing LC separations on a C_{18} column using acetonitrile/water/methanol (75:20:5), Wentworth et al. (2003) have observed 3β-hydroxy-5-oxo-5,6-secocholestan-6-al in arterial plaques.

3.10 Gas Chromatography–Mass Spectrometry

Despite major advances in capillary liquid chromatography and API sources, GC-MS is still the preferred method for steroid metabolite profile and structural analysis. The metabolic transformations of steroid hormones and their precursors are summarised in Fig. 3.17. About 5–10% of steroids are excreted as free compounds. 3β-Hydroxy-5-ene steroids are almost exclusively excreted as sulphate esters, while ring-A reduced steroids are excreted as glucuronides. Profiling of steroid metabolites is usually conducted on urine samples, although similar methodology can be used on serum and amniotic fluid. As an example, the measurement of pregnanolones in third trimester pregnancy plasma has recently been described using GC-MS of *O*-methyl-oxime-trimethylsilyl ether derivatives as described below (Hill et al., 2007) and perfluorobenzyl derivatives of *in vivo* DHEA metabolites (Labrie et al., 2007) – there are many similar applications.

3.10.1 Sample Preparation

Samples should be frozen on collection and may be shipped with an ice-pack or on dry ice. Alternatively, samples may be shipped after solid-phase extraction (SPE) on a C_{18} cartridge.

Urine (or serum) 1–2 mL is extracted on a C_{18} SPE cartridge, e.g. Sep-Pak from Waters. First the cartridge is washed by passing of 4 mL of methanol then 4 mL of water, and then loaded with 1–2 mL of urine. After washing with 4 mL of water, the steroids are eluted with 4 mL of methanol (Shackleton, 1986, 1993). The methanolic extract is dried, and 3 mL of 0.1 M acetate buffer pH 4.5 (0.2 M sodium acetate: 0.2 M acetic acid, 3:2, v/v) added. After adding 10 mg of sulphatase (type H1 from Sigma), and 12 μL β-glucuronidase/aryl sulphatase (Roche Diagnostics), hydrolysis proceeds for 3 h at 55°C. Steroids are re-extracted on a C_{18} cartridge. Some steroid sulphates are not hydrolyzed enzymatically, e.g. sulphates of C-17 (in C_{19} steroids) and of C-20 hydroxyls. This is significant in one disorder, oxidoreductase deficiency (ORD), as prominent excretion products are pregnenediol

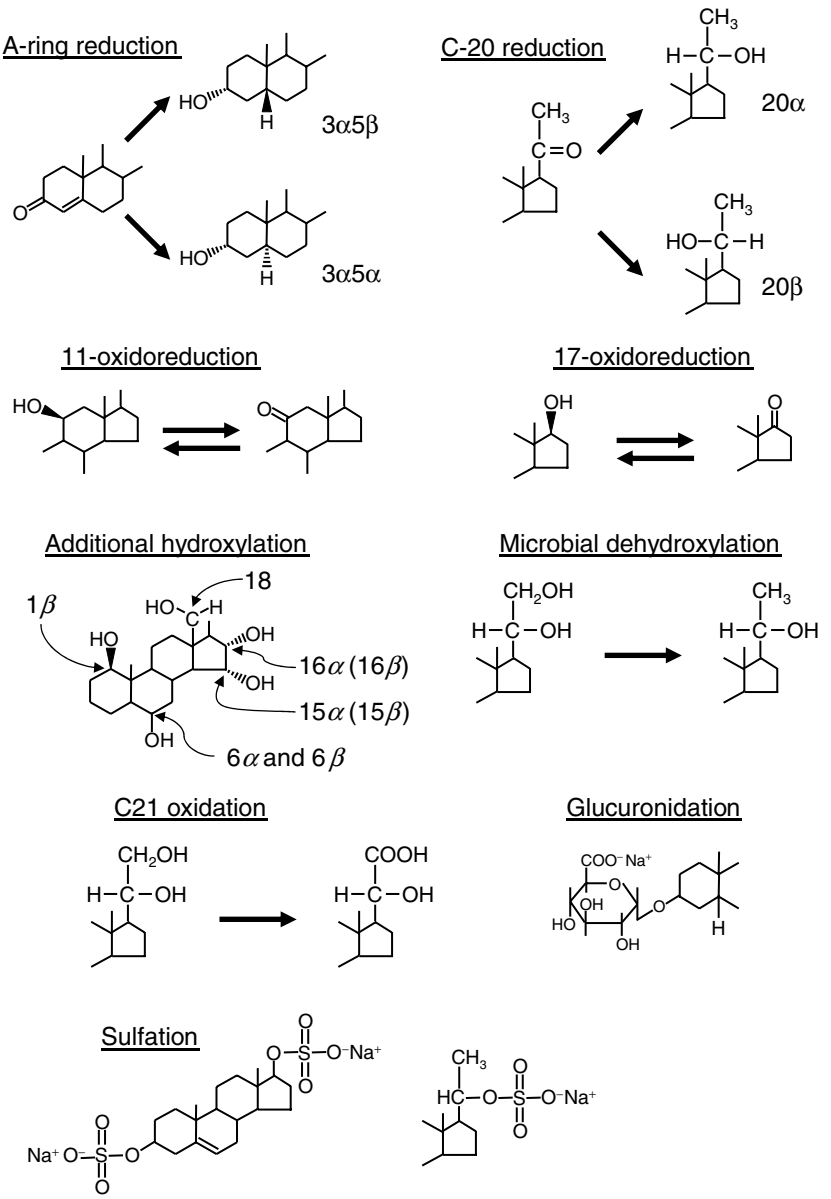

Fig. 3.17 Summary of the major metabolic transformations of hormonal steroids and precursors. (from Shackleton, 2008, with permission) [*Copyright 2008 Springer*]

disulphate in children with the disorder and epiallopregnanediol disulphate in mothers carrying an ORD fetus. It so happens that these steroids undergo desulphation and dehydration during derivatisation and can be quantified as $\Delta^{17\text{-}20}$ pregnandienol and pregnenol, respectively (Griffiths et al., 2005).

After extraction, and de-conjugation, steroids are derivatised. To the methanolic eluate from the C_{18} cartridge, methoxyamine hydrochloride (100 µL, 2% in pyridine) is added and derivatisation allowed to proceed for 60 min at 55°C. Pyridine is blown off, and 50 µL of trimethylsilylimidazole (TMSI) added. Silylation proceeds for 16 h at 100°C. The reaction can be performed for 4 h by increasing the temperature to 120°C. TMSI is involatile and must be removed prior to GC-MS. This is usually achieved by chromatography on Lipidex columns (Shackleton, 1986), but a more efficient method has recently been devised by Dr. Norman Taylor (personal communication). One millilitre of cyclohexane is added to the reaction tube while still hot. TMSI is removed by adding 500 µL of water, vortexing, centrifugation and discarding the bottom layer. The tube is vortexed after addition of a further 500 µL of water, and the top layer is transferred to an injection vial taking care not to transfer any of the aqueous layer.

3.10.2 Apparatus and Scanning

Non-polar capillary columns (DB1 type) are almost universally used for steroid separations. Mass analysis is often performed on a single-stage quadrupole with an EI source, although magnetic-sector instruments can be used for high-resolution work. Ion-trap instruments provide an excellent alternative for profile analysis. The ability of these instruments to perform MS^n can be exploited to increase the amount of structural information, or to reduce background signal giving an improvement in detection limit.

Separation of derivatised steroids can be achieved on DB-1 cross-linked methyl silicone columns, 15 m × 0.25 (i.d.) mm, film thickness 0.25 µm (J & W Scientific), using helium carrier gas at constant pressure. A 1–2 µL aliquot of the final derivatised extract is injected onto the system in the split-less mode, and the GC temperature ramped as follows: initial 50°C, held for 3 min, increased to 230°C at 30°C/min, thereafter increased to 285°C at 2°C/min. The injector and transfer line are kept at 260°C and 280°C, respectively. The *m/z* range scanned is 90–650 or 1,000.

Mass spectra of methyloxime (MO)-trimethylsilane (TMS) ether derivatives of steroids typically show the following fragments: $[M - 31]^+$(loss of CH_3O from the methyloxime), sequential losses of 90 Da (trimethylsilanol), loss of the primary TMS group $[M - 103]^+$and combinations thereof (see Chapter 2 and Griffiths et al., 2005). Often, the choice of ion for SIM or in an extracted ion chromatogram (EIC) (also called reconstructed ion chromatogram, RIC), is one of the above fragments. It should be noted that methyloximes give *syn*- and *anti*-forms which may or may not be resolved by GC. If they are resolved, for quantitative measurements it is preferable to determine the area of both peaks.

Scanning. Ion traps are usually operated in the scanning mode and EIC drawn for the ions of interest. For true profile analysis, quadrupole instruments are also operated in this mode; however, to gain additional sensitivity SIM is performed. Shackleton (2008) has developed profile analysis based on SIM of selected analytes, trying to ensure that components of every steroid class of interest is included. Table 3.4 shows a list of steroids used for adult profiles, with ions monitored, amount of steroid in calibration mixture and excretion ranges.

3.10.3 Quantification, Internal and External Standards

Quantification of all steroids can be performed by relating the intensity of specified ions to that of the internal standard stigmasterol ($[M - 90]^+$, *m/z* 394). Instrument calibration is achieved by running an external standard daily.

Capillary GC tends to discriminate against higher mass long retention time components. The degree of discrimination of individual components throughout a run is easily determined by including internal standards which elute before and after the steroids of interest. Almost all normal urine steroids elute in a window defined by 5α-androstane-3β-ol and cholesteryl butyrate internal standards. Quantification is performed against stigmasterol, but in an acceptable run the other two standards should give almost identical quantitative values. If this is not the case, calibration using the external standard should be repeated.

Internal Standards The three principle internal standards used are 5α-androstane-3β-ol, stigmasterol and cholesteryl butyrate. Stigmasterol is used for quantification. 5α-androstane-3β-ol and cholesteryl butyrate bracket the urinary steroids of interest and are used for monitoring column performance and temperature-dependent discrimination.

External (Calibration) Standards. Table 3.4 lists steroids included in the external calibration standard used in Shackleton's laboratory (Shackleton, 2008). The table also gives information on the steroids routinely measured, the ions chosen, and the expected adult ranges, and typical retention times.

Quality Assurance (QA). In Shackleton's laboratory, a QA sample is run with every batch of samples and the results should not differ daily by more than 15%. For profile analysis, quantification for each analyte may be required against a five point standard curve. In practice, a series of calibrants with identical amounts of internal standard, but increasing amounts of reference analyte, typically covering a 100-fold dynamic range are prepared. The QA sample is a urine pool prepared by mixing equal proportions of 24 h samples from men and women.

3.11 Summary

Prior to 1980, GC-MS was the only mass spectrometric technique for steroid analysis, and profile analysis was important but seldom practiced. Almost all assays were carried out by RIA and related techniques. Now, mass spectrometric steroid analysis is common but has divided into two categories; steroid profile analysis which is still preferentially carried out by GC-MS, and analysis of discrete analytes, or small panels of analytes, for which LC-MS/MS is becoming the only viable technique. These techniques should be viewed as complementary, rather than competing. Clearly, what is not viable economically is the use of GC-MS for the analysis of discrete analytes; protracted work-up procedures and long GC-MS runs make the technique overly expensive. The great advantage GC-MS has is in chromatographic

resolution, and unless a multitude of analytes are determined there is little point in using this approach. For an unknown disorder or steroid mixture, there is no technique more superior. Characterization of a steroid by GC-MS fragmentation is much more readily achieved because of the large data base of documented fragmentations.

The present and future of routine clinical steroid analysis is LC-MS/MS. This is a "new technology" and the methods used still lack consistency. Some groups favour derivatisation to improve sensitivity, and others find it is not required. This may be largely a factor of the age of the instrument and form of ionisation. Several different ionisation sources are in use; ES, APCI, APPI, to name a few. Investigators in the field have differing opinions on their relative sensitivity for steroid analysis. The biggest need, however, is for reliable extraction and work-up methodologies. The HTLC system, as used in analysis mentioned above (Section 3.9.3), appears good for on-line operation, but other systems no doubt will evolve. Another concern is the continuing use of non-labelled internal standards in analyses of some compounds. This is inherently a problem as ion-suppression effects, with any source type are less easy to control when an internal standard of different structure is used. This is in addition to having less control over steroid recovery. The use of labelled steroids has its own possible pitfalls. Some of the labelled steroids currently used have deuteriums in less-stable positions. Analysts must be rigorous in establishing that their methodologies do not result in proton–deuterium exchange. Tai and Welch and their colleagues have published a series of papers in the period 2004–2007, describing candidate reference methods using isotope dilution LC-MS/MS – in the main, validating their methodology by comparison with certified reference material from EC Institute of Reference Material and Measurement (formerly BCR, Brussels) or the Australian National Measurement Institute (Pymble, Australia) All the methods used ESI(+) and the steroids studied were not derivitised, except for oestradiol where dansyl derivatives were formed prior to LC-MS/MS (Tai and Welch, 2005). Tri-deuteriated internal standards were used except for a tetra-deuteriated standard for urine 19-nor-androsterone (Tai et al., 2006a) and a di-^{13}C label for serum progesterone (Tai et al., 2006b). Simple solvent extraction with ethyl acetate after Sep-Pak C18 extraction was used for serum cortisol extraction (Tai and Welch, 2004) and hexane replaced ethyl acetate for serum extraction of the less-polar testosterone (Tai et al., 2007).

GC-MS may eventually be replaced by LC-MS/MS for profile analysis, but this may be still some way off; however, the advantage provided by LC-MS/MS for conjugate analysis is indisputable. Further, the development of LC-MS/MS data bases, such as provided by the LIPID MAPS consortium (http://www.lipidmaps.org/about/index.html) will allow easier interpretation of MS/MS spectra. Yet, at the time of writing, GC-MS is still the favoured technique for studying known rare disorders and describing the metabolome of new ones. GC-MS (or GC-MS-MS) is, however, widely used even today (e.g. Arroyo et al., 2007; Biddle et al., 2007; Courant et al., 2007; Gambelunghe et al., 2007; Hajkovba et al., 2007; Hill et al., 2007; Hsing et al., 2007; Knust et al., 2007; Li et al., 2007; Martello et al., 2007; Meffre et al., 2007; Noppe et al., 2007; Stopforth et al., 2007a, b; Zuo et al., 2007) and still retains a role in the investigation of structural aspects of steroids under investigation (see Chapter

Table 3.4 The comprehensive urine steroid GC/MS profile. From Shackleton, 2008, with permission [*Copyright 2008 Springer*]

Component	Retention time	Specific ion Monitored	Calibration mixture amount (μg)	Excretion Males μg/24 h(n = 17)	Excretion Females μg/24 h(n = 17)
5α-Androstan-3β-ol (IS)	11.28	333	2.5	–	–
3β5βTHAldo (IS)	21.34	506	0	–	–
5P-3β,17,21-ol-20-one (IS)	21.03	594	0.5	–	–
Stigmasterol (IS)	25.27	394	2.5	–	–
Underiv.	24.50	412	0		
Cholesteryl butyrate (IS)	28.77	368	2.5	–	–
Androsterone (An)	13.09	270	2.5	798–4,705	373–3,414
Etiocholanolone (Et)	13.23	270	2.5	689–3,252	450–2,900
11–Oxo-Et	14.28	269	2.5	79–1,026	57–916
11β-OH-An	15.21	268	2.5	500–1,733	191–854
11β-OH-Et	15.38	268	2.5	18–1,034	14–687
5α-Androstan-3α,17β-diol	13.20	331	0.5	48–578	15–147
DHEA	13.83	268	2.5	5–1,476	20–1,139
5-Androstene-3β,17β-diol (5AD)	14.03	239	2.5	45–954	28–201
16α-OH-DHEA	15.68[a]	266	2.5	40–796	35–655
5-Androstene-3β,16α,17β-triol (5AT)	17.31	432	2.5	42–710	40–540
5,17-Pregnadien-3β-ol (5PD)	12.91[b]	372	0.5	10–50	10–50
5-Pregnene-3β,20α-diol (5PD)	17.17	372	0.5	10–150	10–150
5-Pregnene-3β,17α,20α-triol (5PT)	19.49	433	0.5	28–1,062	44-342
Estriol	17.90	504	0.5	1–16	2–32
17-OH-Pregnanolone (17HP)	15.19	476	2.5	41–728	32–657
Pregnanetriol (PT)	16.63	435	2.5	186–1,505	87–1,311
Pregnanetriolone (PTONE)	18.48	449	0.5	4–37	1–77
THS[c]	17.54	564	0.5	10–109	17–117
THDOC[c]	17.19	476	0.5	2–38	1–157
THA[c]	19.70	490	2.5	104–554	76–596

5αTHA[c]	20.33	490	2.5	52–277	38–298
THB[c]	19.96	564	2.5	32–238	26–262
5αTHB[c]	20.27	564	2.5	135–588	49–447
(18-OH-THA)	21.37	457,578	0	45–184	25–207
THAldo	21.12	506	0.2	10–58	6–63
Cortisone (E)	24.39[a]	531	0.5	92–366	49–215
Cortisol (F)	25.73[a]	605	0.5	35–168	25–115
THE[c]	19.38	578	2.5	1365–5,788	727–3,815
THF[c]	20.39	562	2.5	942–2,800	458–1,907
5αTHF[c]	20.61	562	2.5	796–2,456	142–1,589
(18-Oxo-THF)	20.40	594,420	0	1–10	1–10
α-Cortolone	20.99	449	2.5	449–2,044	457–1,564
β-Cortolone	21.60	449	2.5	231–1,534	216–814
β-Cortol	21.46	343	2.5	196–880	124–690
α-Cortol	22.38	343	2.5	96–509	122–365
6β-OH-F	26.53	513	0.5	122–487	53–416

[a]These steroids give two peaks both of which are integrated.

[b]This steroid is an artifact of 5-pregnene-3β,20α-diol disulphate.

[c]Abbreviations not given in table: THS, tetrahydrosubstance S, (3α,17α,21-trihydroxy-5β-pregnane-20-one); THDOC, tetrahydrodeoxycorticosterone, (3α,21-dihydroxy-5β-pregnane-20-one); THA, tetrahydro-11-dehydrocorticosterone (3α,21-dihydroxy-5β-pregnane-11,20-dione); 5αTHA, (3α,21-dihydroxy-5α-pregnane-11,20-dione); THB, tetrahydrocorticosterone (3α,11β,21-trihydroxy-5β-pregnane-20-one); 5αTHB, 5α-tetrahydrocorticosterone (3α,11β,21-trihydroxy-5α-pregnane-20-one); THE, tetrahydrocortisone (3α,17α,21-trihydroxy-5β-pregnane-11,20-dione); THF, tetrahydrocortisol (3α,11β,17α,21-tetrahydroxy-5β-pregnane-20-one); 5αTHF (3α,11β,17α,21-tetrahydroxy-5α-pregnane-20-one).

11) as the usual method of ionisation (EI+) provides detailed structural information not always available from ionisation methods used in LC-MS. There is a recent review on steroid hormone analysis by LC-MS/MS (Soldin and Soldin 2009).

The most important feature of new MS technologies for steroid measurement is the impact this will have on patient care. More cost effective analyses will encourage more detailed evaluation of patients with, for example, endocrine disorders, and the sensitivity and accuracy of measurements will make diagnosis more definitive and monitoring of the effectiveness of therapeutic manoeuvres more reliable.

Mass spectrometry will continue to be a major method for identification and structure determination of steroids with known and unknown functions. Mass spectrometry will be very important for the analysis of chemical fractions with biological activities and for detection of ligands to orphan receptors (by classical mass spectrometry combined with detection of biological activity using cells with reporter gene constructs). There are already examples of ES mass spectrometry of receptor–ligand complexes, and the future may give us methods for structural definition of ligands bound to their receptors. Metabolic studies with multiply and specifically labelled compounds will become increasingly important. Increased sensitivity and resolution can be achieved with Fourier transform mass spectrometry. One may hope to detect new ligands by screening of biological extracts mixed with receptor populations, and elucidate complex metabolic pathways in cells by specific isotope labelling. The future for mass spectrometry in the era beyond the proteome is bright.

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Chapter 4
Immunoassay of Steroids

M.J. Wheeler and G. Barnard

4.1 Introduction

Prior to 1970, few chemical methods existed that were appropriate for the determination of nanomolar and picomolar concentrations of steroid hormones in biological fluids. Nevertheless, by the end of the 1960s, several technologies were being proposed for the measurement of steroids in serum and urine, which included gas–liquid chromatography (Collins et al., 1968), double isotope derivatization (Kliman and Peterson, 1960; Gandy and Peterson, 1968), spectrophotometry and fluorometry (Brown et al., 1968).

By the early 1970s, there were several reports of competitive binding assays for the measurement of steroids, using specific binding proteins including serum-binding proteins (Mayes and Nugent, 1970; Reeves et al., 1970) and specific receptors (Korenman et al., 1970). However, it was the introduction of immunoassay for the measurement of steroids that provided the appropriate analytical tools. The immunoassay of steroid hormones in biological fluids has been reviewed (Abraham, 1974; Cameron et al., 1975; Pratt, 1978) and methodologies for specific steroids may be found in Wheeler and Hutchinson (2006).

In fact, the development of radioimmunoassay (RIA) has proved to be one of the most significant advances in clinical chemistry (Yalow and Berson, 1960). Variations of the technique have made possible the assay of haptens and proteins and have resulted in a millionfold increase in sensitivity over previous methods. In the same year, Ekins reported wider implications of the approach and developed an assay for thyroxine which involved the use of a plasma protein as the specific binding reagent (Ekins, 1960). Furthermore, he introduced the terms ‘limited reagent method’ and ‘saturation analysis’ to indicate that the concentration of the binding protein was insufficient to bind all the analyte (labelled and unlabelled). Accordingly, the percentage

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of radioactive analyte bound to the antibody (or protein) was inversely related to the concentration of unlabelled analyte present in the standard or sample.

If it is assumed that

(i) The binding of the antibody (Ab) and antigen (Ag) obeys the law of mass action
(ii) The antibodies have identical independent binding sites
(iii) The antigens have single, identical reactive antigenic sites

then the equilibrium reaction may be expressed as

$$\mathrm{Ag} + \mathrm{Ab} \underset{K_{-1}}{\overset{K_{+1}}{\rightleftharpoons}} \mathrm{AgAb}$$

where k_{+1} is the association reaction rate constant, k_{-1} is the dissociation reaction rate constant, Ab is unoccupied antibody-binding sites, Ag is the unbound antigen, and AgAb is the antibody–antigen complex. The equilibrium or affinity constant K_a is given by:

$$K_a = \frac{k_{+1}}{k_{-1}} = \frac{[\mathrm{AgAb}]}{[\mathrm{Ag}] + [\mathrm{Ab}]}$$

where square brackets denote molar concentrations. In addition:

$$[\mathrm{Ab}^*] = [\mathrm{Ab}] + [\mathrm{AbAg}]$$

or

$$[\mathrm{Ab}] = [\mathrm{Ab}^*] - [\mathrm{AgAb}]$$

where [Ab*] is the total molar concentration of antibody-binding sites in the system. Thus substituting

$$K_a = \frac{[\mathrm{AgAb}]}{([\mathrm{Ab}^*] - [\mathrm{AbAg}]) + [\mathrm{Ag}]}$$

$$K_a([\mathrm{Ab}^*] - [\mathrm{AbAg}]) = \frac{[\mathrm{AbAg}]}{[\mathrm{Ag}]}$$

which shows that, under ideal equilibrium conditions, the ratio of the bound to free antigen is a linear function of the concentration of the bound antigen. The limit of sensitivity of a competitive immunoassay is set by two factors: (i) the relative error in response at zero dose and (ii) the reciprocal value of the equilibrium constant, which is temperature-dependent (Jackson and Ekins, 1986). This is illustrated in Fig. 4.1.

4.2 The Antibody

An antibody is a serum protein belonging to a family called the immunoglobulins (Ig), of which there are five classes: IgG, IgM, IgA, IgD, and IgE. The immunoglobulin class of antibody used in immunoassays is IgG although in the past some antibodies have been developed using IgM. The general structure of IgG has been

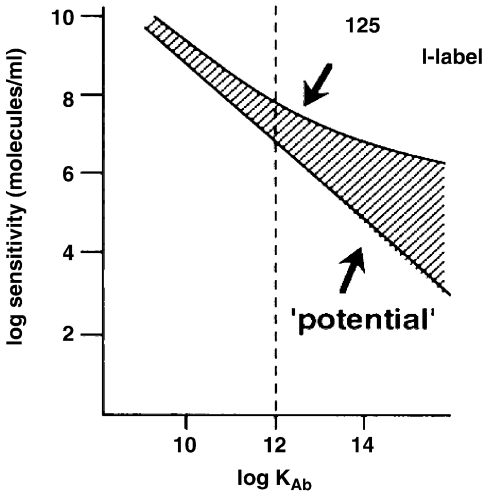

Fig. 4.1 Factors affecting the sensitivity of competitive immunoassays (after Jackson and Ekins, 1986). Optimal sensitivity = $CV_R o/K$ where $CV_R o$ is the relative error in response at zero dose and K is the affinity constant, e.g. $0.01/10^{12} = 10^{-14}$

elucidated by electron microscopy and by differential cleavage of the molecule by specific reagents (Kabat, 1981).

The results of these studies show that the IgG is composed of two pairs of polypeptide chains, two heavy (440–450 residues) and two light (210–220 residues). The variable regions of the immunoglobulin molecule comprise approximately 110 amino acid residues of both the heavy and light chains which combine through noncovalent interaction to produce specific antigen recognition sites. Nevertheless, studies involving X-ray crystallography have indicated that relatively few of these amino acids are directly involved in antigen contact. In addition, the surface of a protein antigen will be covered with overlapping and spatially separated antibody recognition sites called epitopes.

It has been estimated that between five and ten amino acids in clusters (sequential or spatial) will constitute an epitope. Consequently, an antiserum produced by immunization with a complex antigen will contain a great variety of immunoglobulins of diverse characteristics. Such an antiserum is appropriately termed polyclonal, since its immunoglobulin content has arisen from many antibody-secreting B-lymphocytes, each recognizing a separate epitope, circulating in the blood system of the host animal.

Steroids and other low molecular weight analytes (<1,000 kDa) are too small to elicit a measurable immune response and are called *haptens* – they are, of course, antigens in that the steroid is able to bind to an antibody developed to recognize it. Such compounds may be rendered immunogenic, however, by conjugation to larger carrier-protein molecules, such as bovine serum albumin and keyhole limpet hemocyanin. In the case of bovine albumin up to 20 molecules of hapten may be linked to a molecule of protein whereas almost 200 molecules of hapten may be linked to

a molecule of hemocyanin. It has not always been shown whether this makes any difference to the success of antibody production but the choice of the latter protein is obviously to enhance chances of success. Antibodies will be produced to the hapten molecule (Erlanger, 1981), parts of the protein molecule and, in many cases to the bridging link between the hapten and the protein. The antibodies that are additional to the antibody that recognizes the hapten are usually of no consequence as they will not participate in the reaction between the hapten and the antibodies to it. There is, however, evidence that antibodies that recognize the bridging link can interfere in hapten assays where the tracer is linked to the signal molecule, e.g. an enzyme using the same bridge in the same position on the hapten. In such cases a different bridge or a bridge in a different position on the hapten can be used to produce the tracer as will be discussed later.

4.2.1 Immunogen Production

The synthesis, purification, and application of steroid-protein immunogens has been described (Nieschlag and wickings 1975; Aslam and Dent, 1998). Many immunogen parameters have pronounced effects on antibody characteristics. These include (i) method and position of linkage on the smaller molecule, (ii) length of the spacer arm between the hapten and the carrier protein, (iii) number of haptens linked to the carrier molecule, and (iv) tertiary structure of the immunogen. The linkage is generally a peptide bond between a carboxyl group on the small molecule and a free amino group (e.g. from lysine) on the carrier protein. If the steroid does not contain a free carboxyl group an acidic derivative will need to be prepared. Various methods have been used to form peptide bonds between the hapten and the carrier molecule via existing or manufactured oxo or hydroxyl groups on the steroid, including carbodiimide and mixed anhydride reactions. Some commonly used steroid derivatives are shown in Fig. 4.2.

4.2.2 Immunization Protocols

A wide variety of immunization procedures and regimes have been reported for the production of polyclonal antibodies recognizing steroid molecules (Harlow and Lane, 1998). These include subcutaneous, intra-dermal and intra-muscular injections as well injections directly into the lymph gland and footpad. The initial injection was commonly carried out in an oily medium containing *Escherichia coli* (Freunds Complete adjuvant) which caused an abscess at the injection site. Because of the discomfort caused by this procedure the UK Home Office has limited the procedure, in the first instant, to subcutaneous injection using Freunds Incomplete adjuvant, i.e. with no *E. coli*. A simple procedure is (1) inject the animal at about eight sites subcutaneously across the back using

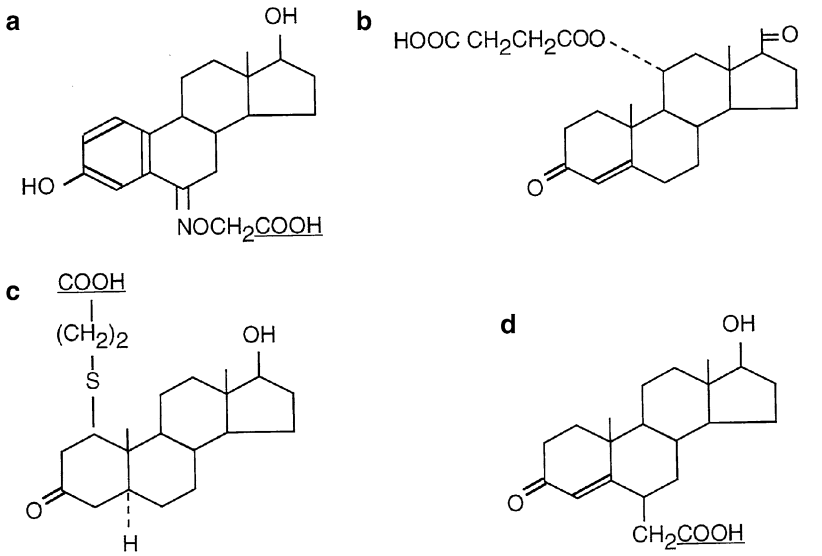

Fig. 4.2 Steroid derivatives used to form immunogens (**a**) O-carboxymethyl oximes, e.g. oestradiol-6-O-(carboxymethyl)oxime, (**b**) hemisuccinates, e.g. progesterone-11α-hemisuccinate, (**c**) mercaptoproprionate, e.g. 5α-dihydrotestosterone-1-carboxyethyl thioether, and (**d**) carboxymethyl steroids, e.g. testosterone carboxymethyl

a 1:1 emulsion of antigen (0.5–1.0 mg) in saline and Freunds Incomplete adjuvant, (2) at 4 weeks take a small blood sample by ear-bleed to check if there is antibody production and give a subcutaneous booster injection as before, (3) take monthly ear bleeds to monitor antibody avidity and titer. Up to about 4 weeks after immunization antibodies are mostly of the IgM subclass and have low affinity for the immunogen. After this period IgG antibodies are mostly produced by the proliferating B-lymphocytes through a complex process known as 'class switching'.

4.2.3 Monoclonal Antibody Production

Kohler and Milstein (1975) reported the successful fusion of specific lymphocytes with myeloma cells to yield hybrid cells (hybridomas) that were stable *in vitro*. In culture, the hybridomas continue to secrete specific antibody predefined by the original lymphocyte parent. A brief description of monoclonal antibody production is now described but more in-depth information can be obtained from dedicated publications (Goding, 1996; Zola, 1999). Briefly, several mice (or rats) are immunized by conventional procedures and the sera tested for the presence of appropriate antibodies. Subsequently, the best animals are sacrificed and the

spleens homogenized to give cell suspensions containing the antibody-secreting B-lymphocytes. Lymphocytes and lymphocyte-compatible myeloma cells are fused in the presence of polyethylene glycol.

The cells are then placed in a medium containing hypoxanthine, aminopterin, and thymidine (HAT medium). The aminopterin blocks *de novo* synthesis of DNA and RNA. Lymphocytes have an alternative pathway for nucleotide production that employs the enzyme hypoxanthine guanine phosphoribosyl transferase (HGRPT). The hybrid cells inherit this pathway from the parent lymphocytes but the unfused myeloma cells are deficient in this enzyme and are unable to survive. The unfused lymphocytes also die due to their inherently short life span of a few hours. The hybrid cells inherit immortality from the myeloma cells and grow and divide in the HAT medium. Surviving hybridomas are repeatedly diluted out into microtitre wells and cultured to give single-cell clones. These clones are grown and the culture medium tested for the presence of antibodies. Positive clones are recloned, cultured, aliquoted, and frozen in liquid nitrogen.

The production of useful monoclonal antibodies recognizing steroid haptens has not been straightforward. In general, monoclonal antibodies have displayed lower affinity than their polyclonal counterparts and improved specificity is not guaranteed. This may be due in part to the co-operativity between immunoglobulins from different clones contained in a polyclonal antiserum. Nevertheless, despite these difficulties many commercial assays use monoclonal antibodies. For example, Siemens, Beckman, Roche, and Tosoh all use a mouse anti-testosterone monoclonal antibody in their automated testosterone assays (Lamph et al., 2003b).

4.2.4 Other Methods of Saturation Analysis

For the analysis of major steroids in biological fluids, immunoassay has superseded procedures using serum-binding proteins and receptors as the binding reagent. Some steroid assays have been developed relatively recently due to the lack of high affinity, high specificity antibodies, e.g. vitamin D metabolites and secosteroids. IDS, Ltd. have developed both RIA and EIA for 25-hydroxyvitamin D and 1,25-hydroxyvitamin D assays. Recently they have launched a fully automated chemiluminescent system for 25-hydroxyvitamins D.

Saturation analysis, using labelled steroid and receptor binding, has been applied to the measurement of steroid receptors. A satisfactory receptor assay must (i) measure only the cellular receptor and not steroid-metabolizing enzymes or plasma-binding proteins, (ii) reflect the biological specificity of the ligand, and (iii) be quantitatively reproducible. An account of the procedures is beyond the scope of this chapter but a useful practical reference is that of Leake and Habib (1987). Further details and applications can be obtained from more recent reviews (Stavreus-Evers and Cekan, 2001; Bai et al., 2003).

4.3 Antibody Characterization

By definition, an immunoassay is a procedure to measure the concentration of an analyte by using the unique properties of an antibody. Consequently, the quality of the method will be mainly dependent on the characteristics of the antibody. It is obvious, therefore, that a reagent with poor specificity and affinity will never yield a method that has good specificity, sensitivity and precision. It is essential to characterize available antibodies carefully in order to select appropriate reagents for further immunoassay development.

4.3.1 Screening Assay

In order to detect the presence of antibodies in polyclonal antisera or monoclonal antibody culture supernatants, an appropriate screening assay has to be established. If a labelled antigen is available, a simple screening system may be devised using a second (species-specific) antibody immobilized to a solid matrix (e.g. particles, tubes, or microtitre plates). The second antibody (e.g. anti-rabbit, anti-sheep, anti-mouse IgG, etc.) is selected depending on the species of animal used in raising the antisera. After washing the solid phase, serial dilutions of the antiserum or the culture supernatant are added to the coated solid phase in the presence of a constant amount of labelled antigen. After an incubation, excess reactants are washed away and the presence of the labelled antigen is detected.

4.3.2 Antibody Titer

An appreciation of antibody titer can be obtained by plotting the signal which is a measure of specific antigen-antibody interaction against the antiserum dilution in the presence and absence of excess competing analyte. This is illustrated in Fig. 4.3. It is essential that the analyte is able to compete for binding and, thereby, reduce the signal detected. If it cannot compete (i.e. there is no signal reduction, even in the presence of a relatively high concentration of analyte), the use of this particular antibody-label combination will be totally inappropriate. Maximum displacement indicates greatest theoretical sensitivity but optimal antibody dilution will also depend on precision of analysis, amount of signal at a given antibody dilution, and desirable working range of the assay.

An appreciation of the potential sensitivity of the method can be obtained by incubating an appropriate dilution of the antibody in the presence and absence of a minimum concentration of the competing analyte. A significant reduction in the signal obtained in the presence of a minimum concentration of analyte would indicate that the method might have sufficient sensitivity. Nevertheless, it is appropriate

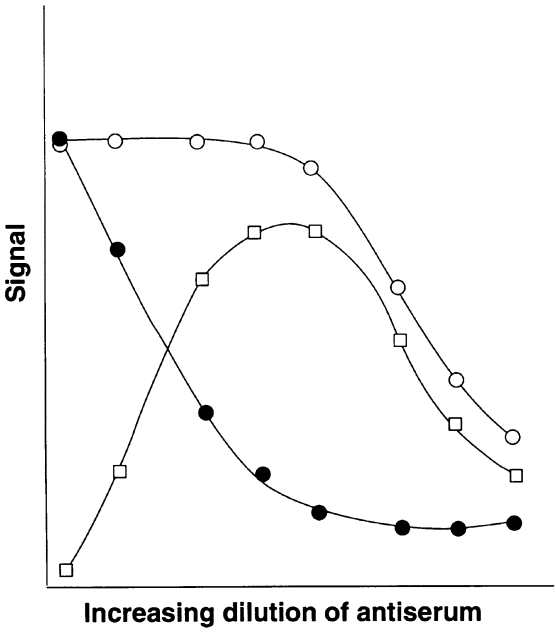

Fig. 4.3 Curves showing the displacement of tracer from antibody following the addition of a fixed amount of analyte. o, Initial binding of tracer with antibody in the absence of analyte; •, binding in the presence of analyte; and □ degree of displacement of tracer by added analyte

to be cautious because the performance of the method in buffer solution may have little comparison to its performance when applied to biological material, because of unexpected matrix phenomena.

4.3.3 Affinity and Avidity

There is some confusion between the terms affinity and avidity in antibody characteristics. Affinity is basically a thermodynamic expression of primary binding reaction between a single antigen-binding site and a single epitope. In general, this is confused in an immunoassay. For example, using a polyclonal antiserum and a complex antigen, there will be a multiplicity of binding reactions. These binding phenomena are more accurately characterized in terms of avidity, which is the summation of the effects of many single binding reactions expressed in a given assay system.

Without doubt, use of antisera demonstrating the greatest avidity for the overall binding reaction will facilitate the development of immunoassays that demonstrate maximum sensitivity. A measure of the affinity-avidity of a competitive immunoassay can be obtained by Scatchard analysis (Scatchard, 1949).

4.3.4 Specificity

In an ideal assay system, the antibody would react only with the compound under investigation (i.e. the analyte), but in practice, many substances of similar structure may compete with the labelled antigen for binding. This phenomenon of cross-reactivity is due to a combination of two factors. Firstly, if the antiserum is polyclonal it may contain many antibodies that react with substances closely related to the analyte. Even a monoclonal antibody may react to steroids very closely related to the analyte. Secondly, although the antibodies may have a lower avidity for other compounds, the relative concentrations of the potential cross-reacting substances may be very much higher than the concentration of the analyte. For example, the mean concentration of cortisol and 11-deoxycortisol in human serum is about 300 nmol/L and 7 nmol/L, respectively. If antibodies to these two steroids both have a cross-reaction to the other steroid of 1% then, in an immunoassay of cortisol, the 11-deoxycortisol will contribute 0.07 nmol/L to the cortisol result of 300 nmol/L and be insignificant whereas, in an 11-deoxycortisol assay, the cortisol will contribute 3 nmol/L increasing the 11-deoxycortisol result from 7 nmol/L to 10 nmol/L, an increase of almost 43%.

In the case of a steroid, three classes of cross-reactant will be of interest in the investigation of antibody specificity: (i) the authentic steroid (e.g. C), (ii) the derivatized steroid tracer (e.g. C-X, where X may be an enzyme etc.), and (iii) in the case of a direct (non-extraction) assay, any metabolite of the authentic steroid (e.g. C-Y, where Y might be a hydroxyl group, sulfate, or glucuronide).

4.3.5 Bridge Recognition Problems

If an immunogen has been synthesized using a derivative of the steroid conjugated to a protein carrier, an antiserum may be produced that binds much more avidly to the derivative than to the authentic compound. This is due to the chemical bridge linking the steroid to the protein being involved in the binding reaction with the antibody. If the same derivative is used to produce a labelled steroid it may be impossible to develop a sensitive immunoassay. Several strategies have been used to overcome this problem. Kellie et al. (1975) reported that a glucuronide bridge had high cross-reaction with free steroid. When Corrie et al. (1981) used this system for a progesterone radioimmunoassay the sensitivity was similar to an assay using tritium as label but the standard curve was shallow and unusable. A better standard curve was obtained using a heterologous link system where the steroid was linked to protein via an 11α-hemisuccinate bridge and the iodinated tyramine linked via 11α-glucuronide bridge (Corrie 1983). Similarly other workers have used the same position on the steroid molecule and modified the bridge; others have linked the steroid to protein using one position on the steroid and a bridge on another position to link the label. The latter approach may lead to loss of specificity due to two sites being masked. Corrie et al. (1981) showed that a glucuronide bridge was poorly

recognized allowing progesterone to be linked to both protein and the label via a bridge at the same site on the steroid molecule.

4.3.6 *Cross-Reaction with Closely Related Compounds and Metabolites*

A common convention for calculating the cross-reaction of an antiserum to one analyte with a closely related analyte is to prepare two separate standard curves of the two analytes, usually in buffer. Antiserum and label solutions of the first analyte are added to the reaction. After incubation and separation, the signal is measured and two standard curves, plotted as B/B_0 are drawn. The concentration at 50% B/B_0 is calculated and the cross-reaction calculated in the following way.

$$\%\text{Cross-reaction} = \frac{\text{Concn of specific analyte at 50\% signal}}{\text{Concn of cross-reactant at 50\% signal}} \times 100$$

This is illustrated in Fig. 4.4. Although this procedure may not mimic the real situation in biological fluids it does provide a standardized procedure allowing easy comparison of different antisera. Cross-reactions can also be examined in serum or another matrix by adding known amounts of the cross-reactant to a sample and calculating the increase in measured analyte compared to the 'un-spiked' sample.

It should be appreciated that a relatively low cross-reactivity with a substance will be important if the expected concentration of the cross-reactant is significantly higher than the specific analyte. In addition, it should be pointed out that overall

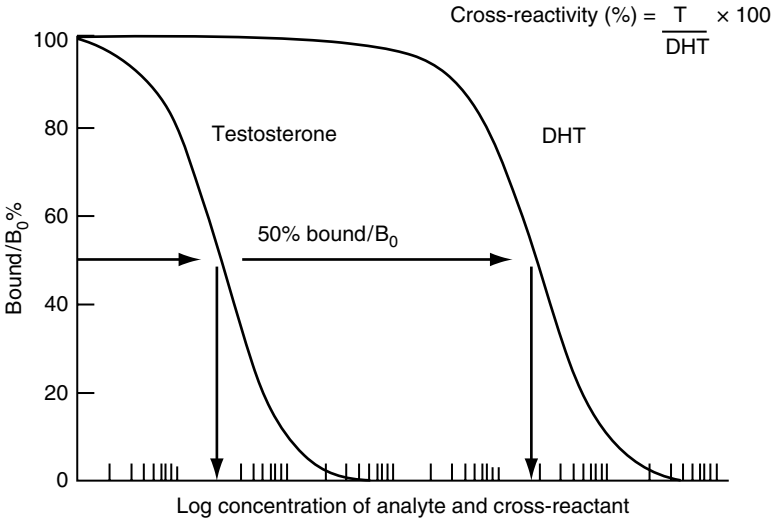

Fig. 4.4 Assessment of cross-reaction using 50% displacement of tracer

Table 4.1 Examination of the cross-reaction of a testosterone antiserum with DHT showing how the label and separation system can affect the apparent cross-reaction in an immunoassay

Label	Separation system	Cross-reaction with DHT (%)
Tritium	Dextran-coated charcoal	30.0
Iodine	Dextran-coated charcoal	10.8
Tritium	PEG + second antibody	3.0
Iodine	PEG + second antibody	3.3

assay specificity and sensitivity may be affected by altering the assay configuration. Table 4.1 shows how an antiserum to testosterone had a very low cross-reaction to 5α-dihydrotestosterone in two different immunoassay formats but a high cross-reaction in another assay format.

4.4 The Label

A variety of different labels have been used in the development of steroid immunoassay. To date, at least ten labels have been advocated, and these include (i) radioisotopes – beta and gamma emitters, (ii) enzymes, substrates and cofactors, (iii) chemiluminescent compounds, and (iv) fluorescent compounds.

4.4.1 Radioisotopes

Traditionally, tritium was the most commonly used label in the immunoassay of steroids. The primary reason has been the availability of tritium-labelled steroids from suppliers such as Amersham International plc (Amersham, UK) and New England Nuclear Corporation (Boston, MA, USA). Labelled steroids with high specific radioactivity are available for many compounds with four or six tritium atoms included in the steroid nucleus. The main disadvantage to the use of the high specific activity tracers is increased radiolysis. Nevertheless, labelled steroids have a long half-life and can be purified chromatographically if necessary. With the fall in use of tritiated steroids, particularly in immunoassays, some products have been withdrawn. Tritiated forms of most common steroids are still available and companies such as Amersham International plc will produce specific tritiated steroids to order if required.

Iodinated steroid tracers have replaced tritiated tracers in many radioimmunoassays. The half-life of ^{125}I is relatively short (only 60 days) and in addition, the steroid cannot be iodinated directly, since the incorporation of iodine into the steroid nucleus is likely to result in conformational changes, or even complete destruction of the steroid molecule, with the loss of immunological activity (Tarle et al., 1978; Sweet et al., 1979; Ali et al., 1987). Consequently, compounds which may be iodinated, such as histamine, tyramine, or tyrosine methyl esters, are commonly attached to the steroid through a bridge. The method of Nars and Hunter (1973) has been widely used.

Ideally, the chemical linkage used for the preparation of the tracer should be different from the bridge used to prepare the immunogen, since antibodies may be directed against the bridge as well as the steroid ring. This 'bridge recognition' problem is common to all labels that are external to the steroid nucleus, unlike tritiated labels that have no bridge. The application of heterologous assay systems for the measurement of steroid hormones has been described (van Weeman and Schuurs, 1975; Corrie, 1983).

The main advantages of iodine labels over the use of tritiated steroids include (i) higher specific activity, (ii) cheaper and more robust assays, (iii) gamma emitters can be counted without the use of costly liquid scintillant or counting vials, (iv) shorter counting times, and (v) lower quenching.

The problem of quenching can be understood when it is appreciated that scintillation counters are very sensitive luminometers, detecting light emitted from appropriate fluors that have been excited by the absorption of energy released during irreversible radioactive decay. The excited fluor emits light as its energy level returns to ground state (e.g. solid and liquid scintillation). Low-energy beta particles given out by tritium are highly subject to quenching (the light is absorbed by constituents of the matrix, particularly water), and this imposes limitations on how counting can be accomplished. On the other hand, gamma emission, which has a higher energy level and is minimally affected by quenching has a number of advantages. Counting times are shorter and no scintillant is required which simplifies the separation of bound from free label and gives more flexibility in assay design.

Consequently, the growing use of iodinated steroids encouraged Amersham International and New England Nuclear to offer many iodinated steroids commercially. In addition, several diagnostic kits exploiting the use of iodinated tracers are available commercially for the measurement of steroids (e.g. Siemens Healthcare Diagnostics, New York, USA and Immunodiagnostic Systems, Ltd., Bolden Business Park, Tyne and Wear, NE35 9PD, UK). More recently, with the introduction of automated direct immunoassays using non-radioisotopic labels, availability of iodinated steroids has diminished and fewer companies produce radioimmunoassay kits.

4.4.2 Non-radioisotopic Labels

The increasing use of radioisotopic immunoassays has exposed several difficulties which have been both well documented and overstated. Nevertheless, a major impediment is the radioactive half-life and radiolysis of the labelled material, which reduces potential assay sensitivity and imposes a time limit on the usefulness of a valuable reagent. In addition, the potential health hazards, together with the legislation and bureaucracy associated with the use and disposal of radioactive compounds and the solvents and photofluors necessary for liquid scintillation counting, are encouraging a reassessment of conventional laboratory practice. Consequently, there is an increasing trend to adopt non-radioisotopic detection systems (Kricka 1994).

Over the last 20 years, many alternative systems for the measurement of steroids in biological fluids have been proposed. Of these, three main approaches have been successful commercially, involving the use of enzymes, chemiluminophores and fluorophores. Enzymes are prone to interference that reduces enzyme activity and fluorescent assays are prone to interference from naturally fluorescing components of serum such as protein and bilirubin. Therefore all but one of the major commercial companies that have automated methods use chemiluminescent labels.

4.4.2.1 Enzymes

Enzymes have proven to be sensitive and versatile labels in various immunoassay systems (Ngo and Lenhoff, 1982). Sensitivity is achieved by the amplification of the signal by prolonged incubation and catalytic turnover. Versatility is due to the variety of substrates and end-points that can be used. Factors affecting the choice of enzyme include (i) the turnover number of the pure enzyme, (ii) the purity of the enzyme preparation, (iii) the sensitivity, ease and speed of product detection, (iv) the absence of interfering factors in the test fluid, (v) the presence of potentially reactive groups for coupling, (vi) the stability of the enzyme and conjugate, and (vii) the availability and cost.

The enzymes that have been used most extensively are horseradish peroxidase, alkaline phosphatase from calf intestinal mucosa, β-D-galactosidase from *E. coli*, glucose oxidase from *Aspergillus niger*, urease, penicillinase and luciferase. For example, alkaline phosphatase, a dimeric glycoprotein (140,000 kDa) is widely available, is very stable and has a high catalytic turnover number. In addition, it can be used with a variety of substrates, examples of which together with alternative endpoints include (i) colorimetric endpoint: 5-bromo-4-chloro-3-indoyl phosphate or nitroblue tetrazolium, (ii) chemiluminometric endpoint: 3-(2′-spiroadamantane)-4-methoxy-4-(3′-phosphoryloxy)phenyl-l,2-dioxetane (AMPPD), (iii) bioluminometric endpoint: D-luciferin orthophosphate, and (iv) fluorometric endpoint: 4-methylumbelliferyl phosphate.

There are some difficulties specific to the use of enzymes in immunoassay. These include (i) the relatively large label, which can interfere with the antibody-binding reaction, (ii) the synthesis of labelled antigen, which is difficult to control, and the need for product to be characterized for enzyme- and antibody-binding activities, and (iii) the end-point determination, which is more complex and time-consuming than for RIA. Notwithstanding these difficulties, several commercial enzyme immunoassays were introduced.

4.4.2.2 Chemiluminescence

In chemiluminescence, excitation is effected by a chemical reaction (usually an irreversible oxidation reaction). The chemical reaction may be mediated by enzymes in a biological system in which case the luminescence is commonly

known as bioluminescence. If the chemical reaction is initiated by an increase in the temperature of the reactants then the resulting luminescence may be accurately described as thermochemiluminescence. If the chemical reaction is initiated by an electric charge applied to the reactants then the resulting luminescence may be termed electrochemiluminescence.

Many organic compounds are chemiluminescent upon oxidation. These include the cyclic hydrazides such as isoluminol (6-amino-2,3-dihydro-phthalazine-l,4-dione) and its derivatives, aminonaphthyl hydrazides acridinium esters and acridans, dioxetans and active oxalate esters (McCapra, 1974).

Isoluminol and Its Derivatives

Derivatives of isoluminol have been synthesized and studied in a variety of oxidation systems, and their use as labels to monitor specific protein-binding reactions has been reported (Schroeder and Yeager, 1978). The chemiluminescent reaction of isoluminol derivatives is illustrated in Fig. 4.5. Although the steroid-isoluminol conjugates have been shown to be extremely stable, they have a relatively low quantum yield. Consequently for high sensitivity, the end-point measurement is usually preceded by an incubation at high temperature and alkaline pH. In addition, the oxidant (e.g. hydrogen peroxide) is added by rapid injection when the assay tube is situated in front of the photodetector, because the rate of the chemiluminescent reaction is too fast for the reagent to be added outside the instrument.

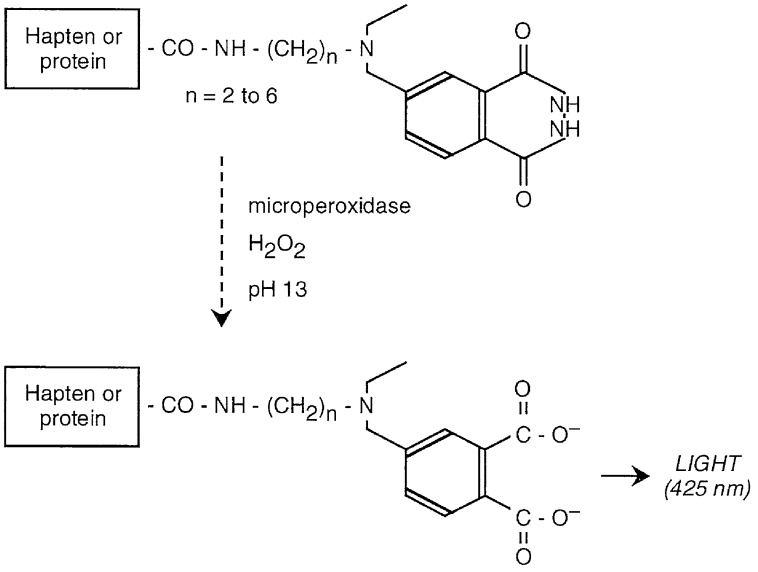

Fig. 4.5 Chemiluminescence reaction of isoluminol derivatives

Alternative oxidation systems would be preferable that generate constant, stable light emissions (cf. bioluminescent reactions) without sacrificing sensitivity. Nevertheless, in systems where the chemiluminescent moiety is in limited concentration (i.e. the label is chemiluminescent), this would require an increase in chemiluminescence quantum yield of several orders of magnitude.

Acridinium Derivatives

Various acridinium salts can be stimulated to produce light in the presence of dilute alkaline hydrogen peroxide and in the absence of a catalyst (Simpson et al., 1981; Weeks et al., 1982). Such compounds include derivatives of acridine, possessing a quaternary nitrogen centre which can be derivatized to produce a labile phenyl ester. The chemiluminescent reaction involves the hydrolysis of the ester bond, intermediate dioxetenone production by hydroperoxide ions, and the formation of electronically excited *N*-methylacridone. The chemiluminescent moiety is liberated prior to electronic excitation, minimizing quenching from the sample. This results in restoring a relatively high quantum yield without resort to more complicated chemical hydrolytic procedures. Thus the oxidation reaction is relatively simple, and this is illustrated in Fig. 4.6.

For example, Richardson et al. (1985) developed and evaluated a solid-phase CIA for the measurement of progesterone in extracts of peripheral plasma using a

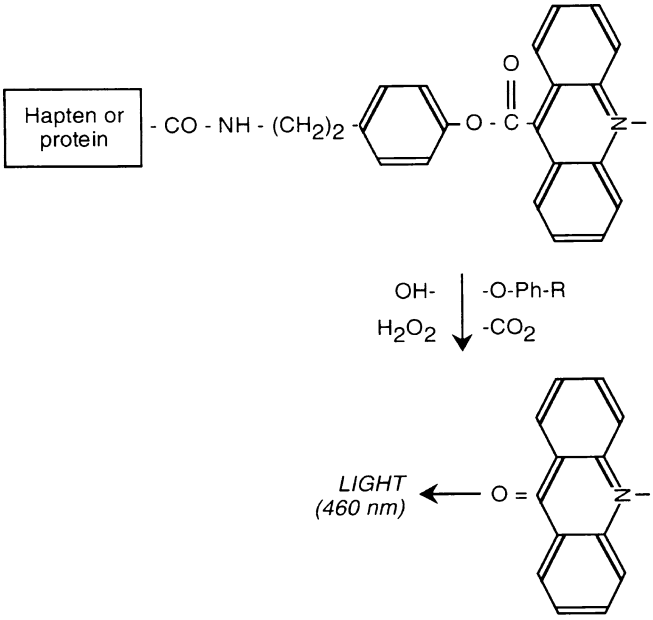

Fig. 4.6 Chemiluminescence reaction of acridinium esters

novel hapten-acridinium ester conjugate, 11-progesteryl *N*-succinoyl tyramine 4-(*N*′-methyl) acridinium-9-carboxylate. The application of acridinium esters in immunoassay has been successfully commercialized and automated, e.g. Siemens Healthcare Diagnostics, New York, USA. Different companies have introduced different modifications. For example, Abbott Diagnostics, Illinois, USA use a patented sulfopropyl acridinium carboxamide that they claim has better solubility and stability than traditional N^{10}-methylacridinium-9-carboxylic acid phenyl esters (Quinn, 2005).

Chemiluminescence Enzyme Immunoassay

Horseradish peroxidase may be used in association with an enhanced chemiluminometric endpoint using luminol as substrate. Thorpe et al. (1985) reported the finding that many compounds (e.g. substituted phenols) enhance the light emission from the horseradish peroxidase catalysed oxidation of luminol. The enhancement effect produces a light emission that decays slowly (glowing for several minutes) and its intensity may be 1,000 times greater than the basic reaction.

The enhanced system facilitates the rapid, sensitive assay of peroxidase conjugates and is appropriate for the development of competitive binding immunoassays of steroids, using steroid-peroxidase or antibody-peroxidase conjugates. This system has been exploited commercially, originally as Amerlite (Amersham International plc, Amersham, Bucks, UK) and, more recently, by Ortho-Clinical Diagnostics, High Wycombe, Bucks, UK. An alternative chemiluminescence enzyme immunoassay system is used on the Immulite® family of instruments (Siemens Healthcare Diagnostics, New York, USA). The chemiluminescent substrate is adamantyl dioxetane phosphate and the label alkaline phosphatase. An unstable adamantyl dioxetane anion is created that breaks down leading to chemiluminescence. The signal is enhanced one hundred times by reading through a 2A neutral density filter (Babson, 2005).

4.4.2.3 Fluorescence

Fluorescent molecules absorb light of a particular wavelength which excites their electronic field from a resting to a higher energetic state. The return to the resting condition is accompanied by non-radiative conversion, such as heat, or by radiative transition directly to ground state (fluorescence) or through a semistable triplet state (phosphorescence). The small decrease in energy, which results in the difference in wavelength between the excitation and emission energies, is defined as the Stokes shift.

In fluorescence, this shift is normally 30–50 nm; in phosphorescence, however, it may be in excess of 200 nm. The ratio between the absorbed light and emitted light is defined as the quantum yield. The fluorescence lifetime is defined as the

decay rate of the excited state. In principle, fluorescence measurement is potentially a very sensitive procedure. In practice, however, sensitivity is restricted to a concentration range of 10^{-9} to 10^{-12} mol/L because of a high background signal due to light-scattering or fluorescent compounds in the sample, reagents and cuvettes. In addition, fluorescent molecules are often very sensitive to changes in their environment. Minor changes in temperature, pH, polarity, oxidation state or the proximity of quenching groups can alter quantum yield or wavelength of emitted light. Inner filter effects occur when there is close proximity of two fluorescent probes on a protein which can be self-quenching.

Developments in fluoroimmunoassays have been reviewed (Barnard, 1988; Hemmila, 1991; see also relevant sections in Wild, 2005). The fluorescent molecules used most frequently include (i) fluorescein, (ii) rhodamine, (iii) umbelliferone, and (iv) rare earth chelates. The structures of some of these compounds are shown in Fig. 4.7.

An ideal fluorophore should possess the following characteristics: (i) a high quantum yield of fluorescence (i.e. number of emitted photons approximately equal to the number of exciting photons), (ii) a high Stokes shift (i.e. absorption and emission maxima widely spaced), (iii) an ability to be excited by common light sources, (iv) the emission to be detected by conventional photomultipliers, (v) a simple labelling procedure, and (vi) stability and immunological activity. In particular, it has been demonstrated that the fluorescent half-life of complex chelates of certain lanthanide elements (e.g. europium, samarium and terbium) is up to six orders of magnitude longer than conventional fluorescence labels (Hemmila, 1991). Consequently, the emission from these lanthanide chelates can be distinguished from the background fluorescence with a short decay time by using a time-resolved fluorometer with appropriate delay, counting, and cycle times. This is illustrated in

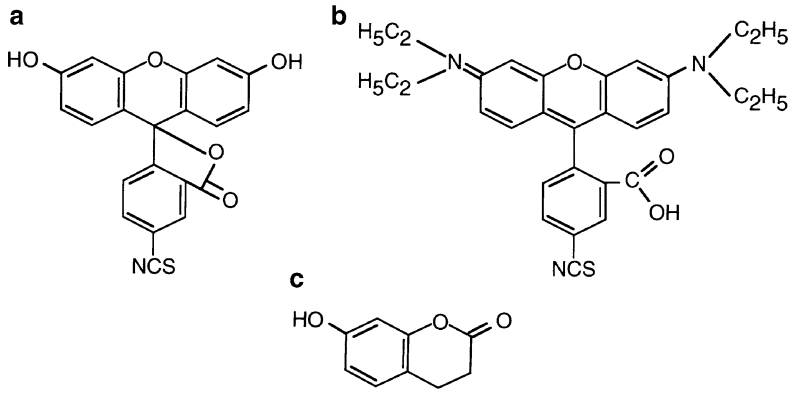

Fig. 4.7 Structure of flurophores: (**a**) Fluorescein isothiocyanate, (**b**) rhodamine isothiocyanate, and (**c**) umbelliferone

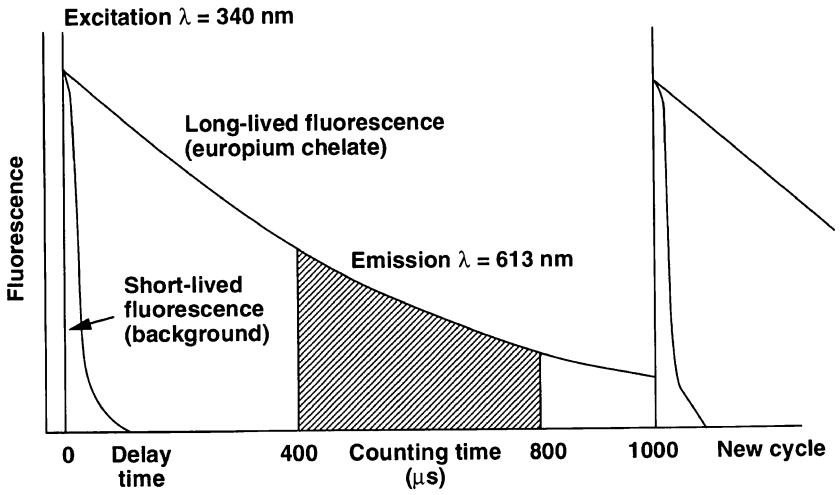

Fig. 4.8 An example of time-resolved fluorescence using europium chelate

Fig. 4.8. Time-resolved fluorescence is used in the DELFIA immunoassay system marketed by Perkin Elmer, Wellesley, MA, USA.

In order to use the lanthanide as a label in immunoassays, the ion has to be strongly bound to one of the immunoreactive components. Derivatives of polycarboxylic acids (e.g. EDTA, EGTA), with functional groups for linkage to the free amino groups of proteins and appropriate steroid haptens, have been used as chelators (Mikola et al., 1993). The conjugates are very stable at pH 7 but are non-fluorescent. The proposed structure of a europium-labelled oestradiol conjugate is shown in Fig. 4.9.

In order to measure fluorescence with high sensitivity it is necessary to dissociate the lanthanide from the immunoreactive components. This process is achieved simply by lowering the pH to 2–3 by the addition of an appropriate buffer (phthalate). A β-diketone (e.g. 2-naphthoyltrifluoroacetate) chelates the lanthanide ions at low pH and possess the required properties to produce chelates with high-intensity fluorescence. In addition, it is necessary to expel water from the complex to obviate quenching, and this is accomplished by addition of detergent (Triton X-100), which dissolves the sparingly soluble organic component in a micelle. The complex is further insulated from water in its micelle by the addition of a synergistic agent (trioctylphosphine oxide).

4.5 The Assay

Over the last 10 years a variety of radio- and non-radio-isotopic immunoassays have been introduced for the measurement of steroid hormones in unextracted serum or plasma. These procedures enjoy an almost unprecedented popularity as

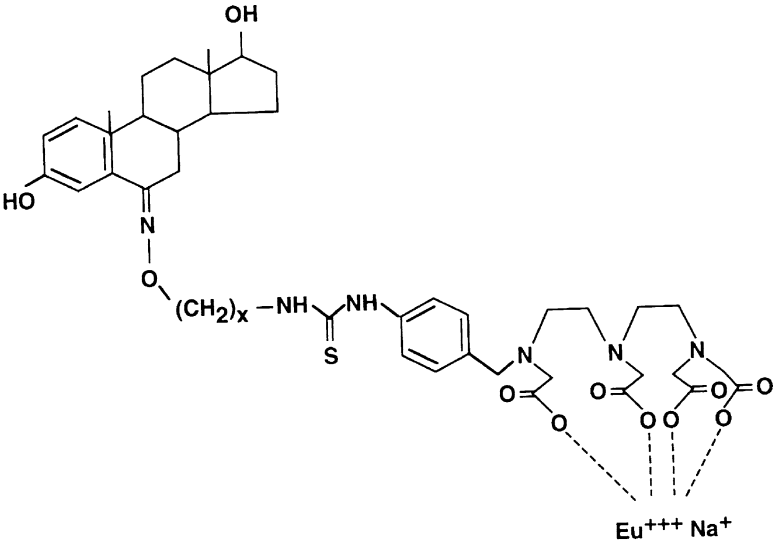

Fig. 4.9 Proposed structure of a europium-labelled oestradiol conjugate (after Mikola et al., 1993)

the methods of choice in the clinical laboratory. This is all the more remarkable since these procedures can never provide an absolute measurement and accuracy will be always dependent on a variety of factors that include (i) antibody specificity, (ii) assay standardization, and (iii) particularly matrix effects.

4.5.1 Sample Preparation

In the pioneering days of steroid immunoassay great care was taken to ensure that the immunoassay of any particular steroid was as accurate as possible. Invariably, this involved complex and, in some cases, very labour-intensive methods of sample preparation.

4.5.1.1 Extraction and Chromatography

In order to obviate plasma protein interference in the immunoassay, various methods have been used to extract and purify steroids from biological materials (Breuer et al., 1976). These include (i) solvent extraction with diethyl ether or light petroleum to remove conjugates and proteins and (ii) chromatography (thin-layer or simple column) for the separation of specific steroids or steroid groups. In addition, internal standards of tritiated steroid were added to the sample prior to treatment in order to correct for recovery.

4.5.1.2 Direct Assays

Compared with conventional extraction assays, the direct approach offers major advantages of improved precision, increased batch size and opportunity for automation. Nevertheless in many cases, and for a variety of reasons, a number of non-extraction serum assays have been criticized in particular clinical situations. These include (i) the measurement of ovarian hormones in postmenopausal women (Key and Moore, 1988), (ii) the measurement of steroids in patients receiving hormone replacement therapy (Ratcliffe et al., 1988; Nahoul et al., 1989), (iii) the measurement of oestradiol in males and neonates (Diver, 1987), and (iv) the measurement of testosterone in females (Slaats et al., 1987; Wheeler and Lowy, 1987; Herold and Fitzgerald, 2003). Criticism has included poor accuracy, poor precision, poor specificity, and lack of sensitivity.

In direct assays, steroids must be quantitatively displaced from natural-binding proteins before or during the assay procedure and a number of methods have been employed to achieve this. Major steroids (e.g. progesterone and testosterone) bound in serum to corticosteroid binding globulin (CBG) and sex hormone binding globulin (SHBG) respectively may be displaced by protein-binding agents such as 8-anilino-l-naphthalene sulphonic acid (ANS) and salicylate, by proteolytic enzymes, by low pH, high concentrations of urea or by heat treatment. In addition, danazol, dexamethasone and cortisol, which have high affinities for CBG, have been used in direct assays for progesterone (Ratcliffe, 1983; Barnard et al., 1988). A disadvantage of some of these displacing agents is that they will partially reduce the specific binding of the antibody at their effective blocking concentration. In addition, conditions employed for displacing steroids bound to sex hormone-binding globulin (SHBG) in direct assays for testosterone and oestradiol also place great demands on the specificity of the antiserum, and these exogenous steroids are potential sources of cross-reaction (De Boever et al., 1986; Roda et al., 1986). Addition of high concentrations of displacing steroids have caused problems in automated systems by binding to tubing and subsequently slowly leaching off and interfering with the measurement of that steroid in following analyses. The complex mixtures of displacing agents put direct immunoassay at a distinct disadvantage, and performance in terms of sensitivity may be affected significantly (Barnard et al., 1988; Taieb et al., 2003).

The concentration of the displacing agent that can be added may be a compromise between loss of sensitivity and interference in the assay system while trying to block binding to specific binding proteins over a wide concentration range. For example, it has been shown that both recovery and precision are affected by the concentration of SHBG in testosterone assays (Wheeler et al., 1996; Boots et al., 1998).

In addition, there is a more critical requirement for improved antibody specificity in non-extraction procedures because of the presence of relatively high concentrations of steroid conjugates (glucuronides and sulphates) in unextracted serum. This is particularly true in the case of samples from patients undergoing hormone replacement therapy, which may involve the administration of oestradiol, progesterone and/or testosterone in a variety of combinations and by a variety of routes (e.g. intramuscular, oral, nasal, intravaginal or percutaneous). The route of administration profoundly

affects the metabolism of the hormone and leads to the formation of particular profiles of steroid conjugates, some of which may cross-react significantly in the immunoassay (Nisbet and Jomain, 1987; Nahoul et al., 1989). In many cases, commercial direct procedures may be totally inappropriate for use in these situations.

4.5.2 Sample Type

4.5.2.1 Serum

Another difficulty in the development of direct assays for the measurement of steroids is the importance of selecting a suitable serum matrix for the preparation of the calibrators to equalize any interference arising from the presence of serum (Ratcliffe, 1983; Bangham, 1988). Calibrator performance may be adjusted by varying the concentration of (i) protein, (ii) detergent, (iii) a suitable competing steroid, and (iv) the actual calibrator in the matrix. At best this approach is a compromise, and there will always be clinical samples where the matrices behave quite differently in the immunoassay, leading to positively or negatively biased results. Figure 4.10 shows how the linearity on dilution was affected by sample matrix in a commercial oestradiol assay. In this particular assay it appeared that the manufacturer had made up their calibrators in a matrix that did not mimic serum. Good linearity was only obtained when the manufacturer's kit QC was diluted.

Direct and/or commercial assays should ALWAYS be evaluated, before routine use, in clinical situations in which it is intended to use them i.e. assays which are designed for use in adults should not automatically be applied in paediatrics. It may, however, not be immediately apparent in what context the original assay has been developed/evaluated.

4.5.2.2 Alternative Biological Fluids

Because of the difficulties inherent in the measurement of steroids in serum or plasma, alternative biological fluids have been investigated.

Saliva

There is good evidence of a strong correlation between the free concentration (non-protein bound) of a steroid such as cortisol or progesterone and its concentration in saliva (Riad Fahmy et al., 1982; Read et al., 1990). Conventional competitive immunoassays using high-avidity antisera are just capable of the sensitivity necessary to measure salivary cortisol and progesterone. Furthermore, the techniques have the advantage for sequential measurements where repeated venepuncture is inappropriate. Nevertheless a great deal of care must be taken in sample collection. For example, saliva contains enzymes involved in steroid metabolism, and exogenous

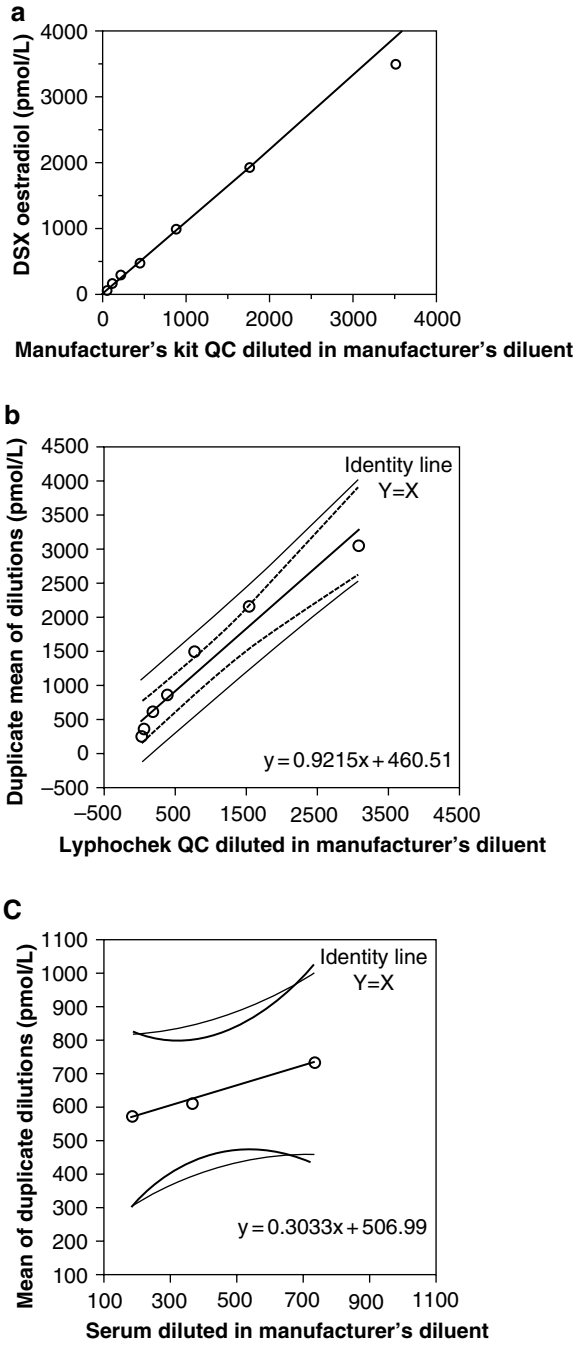

Fig. 4.10 Linearity studies showing matrix effects when (**a**) the manufacturer's QC (**b**) Lyphochek QC or (**c**) human serum is diluted with the manufacturer's diluent (Lamph et al., 2003a)

materials such as toothpaste and food particles can interfere with the antibody–antigen reaction.

Furthermore, harsh brushing of the teeth may lead to blood leakage into the saliva, which may invalidate the approach due to the much higher concentrations of total steroid in blood. However, Granger et al. (2007) found that, in their study of 363 children, blood contamination was very rare: they measured transferrin concentrations in saliva samples as a marker of blood contamination. They examined the outlier results for cortisol, testosterone and DHEA, measured by immunoassay, and found that only one DHEA result was associated with a high transferrin result; this suggests that blood contamination does not contribute significantly to the majority of elevated results for these steroids. Several saliva ultrafiltration collection devices have been described which may obviate these problems (Wade and Haegele, 1991). One of the most popular devices today is the Salivette® from Sarstedt, Ltd., Leicestershire, UK. This device has a small 5 mL plain tube, containing a cotton pad, which sits in a larger conical polystyrene tube. The cotton pad is placed in the mouth until saturated and replaced into the inner tube. The whole device is placed in a freezer until steroid measurement. It is then defrosted and centrifuged. The inner tube has a hole in its bottom and clear saliva is pulled out of the cotton pad into the bottom of the outer conical tube. The inner tube plus the cotton pad is discarded. It has been reported that the cotton pad interferes with the measurement of steroids (Cook et al., 1992; Wood, 2009) and IBL, Hamburg, who supply kits for the measurement of salivary steroids, recommend and supply ultra-pure polypropylene collection tubes. They strongly advise against the use of Salivettes® and even suggest recycled polypropylene tubes adsorb steroids and lead to inaccurate results. This seems to be little appreciated since the use of Salivettes® to collect saliva is still very common (Yaneva et al., 2004). However, data to support the comments from IBL seem to be very limited. This is an area that needs further investigation as the evidence for the above interference is very limited. Another possible problem in saliva steroid measurement is the use of commercial kits designed to measure serum. Investigators usually modify these kits to provide additional sensitivity but details are limited and accuracy unproven. Sensitive tandem mass spectrometry methods now provide a means for these methods to be validated.

Urine

With the advent of immunoassay, there was a move away from measuring steroids and their metabolites in urine. In many cases this was unfortunate, since the immunoassay of steroid urinary metabolites often yields similar clinical information to that obtained from the measurement of the primary steroids in serum. For example, results from studies of ovarian function and steroid metabolism suggest that measurement of urinary metabolites of oestradiol (estrone-3-glucuronide, EG) and progesterone (pregnanediol-3-glucuronide, PG) are suitable to assess ovarian activity (Kesner et al., 1992). Accordingly, immunoassay methods have been developed to measure these steroid metabolites in diluted urine and to investigate the usefulness

of this parameter for delineating the fertile period and predicting ovulation (Usuki et al., 2000; Miro et al., 2004).

Barnard and Kohen (1998) have reported the simultaneous time-resolved fluorescence immunoassay of EG and PG in samples of early morning urine (EMU) to monitor ovarian function in women, using different lanthanide chelates as labels. The method demonstrates appropriate sensitivity and excellent precision (all CVs 5–8%) across relevant working ranges for each hormonal parameter. The technique has been applied to serial EMU samples collected from women with normal menstrual cycles and from stimulated cycles. The advantage to the patient is the ease of serial sample collection, which can be performed at home. The advantage to the clinician is an overview of hormonal activity that cannot be obtained from the results obtained from serum samples taken at infrequent intervals.

As serum is collected for other biochemistry and hematology testing, urine is used infrequently as a test medium. The main exception, apart from urine steroid profiling by HPLC and gas chromatography mass spectrometry (GCMS), is the measurement of urine cortisol as a screening method for Cushing's syndrome. Urine cortisol provides an integrated measure that reflects the free cortisol concentration in blood. Many of the companies with automated serum cortisol methods also have a protocol for urine cortisol measurement. These methods are very matrix dependent with significant cross-reaction with other glucocorticoid conjugates in the urine. Figure 4.11 shows how the result for urinary free cortisol was affected by the diluent used to reconstitute the urine extract. Therefore most methods are a compromise of a number of factors but give useful clinical information as long as reference ranges are adequately established first.

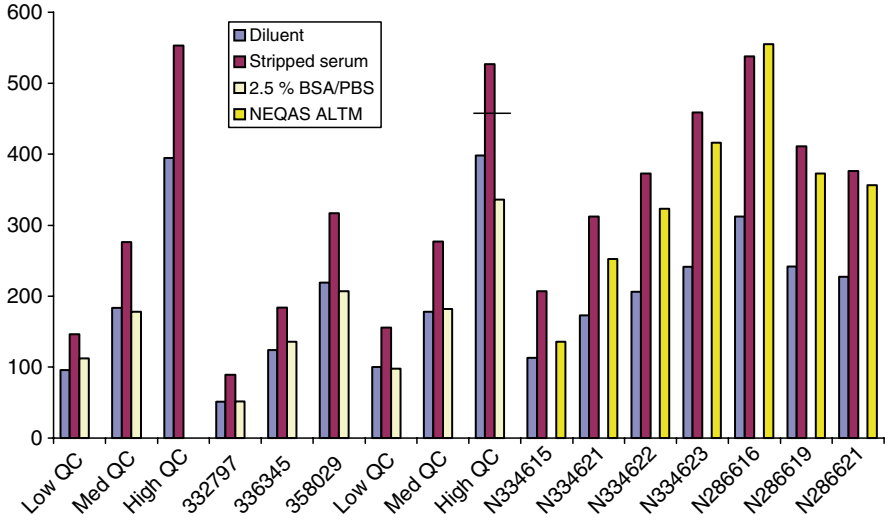

Fig. 4.11 Effect on the urinary cortisol result from diluting an extracted urine sample 1:1 with different diluents

4.5.3 Separation Methods

Where the separation of antibody-bound and free fractions is obligatory, the principal aim of the procedure is the complete separation of the two fractions without changing the distribution between them of the compound to be measured. Some separation methods that have been used in immunoassay systems are shown in Fig. 4.12.

The selection of a suitable separation system (Wild and Kusnezow, 2005) involves the consideration of many interrelated factors. For example, the chemical structure of the compound to be measured will influence its solubility in aqueous solutions, and this property affects the degree of non-specific binding obtained with different separation techniques. Furthermore, the characteristics of the antiserum (in particular, avidity) and the choice of labelled antigen will have a major influence on the extent to which the separation technique will perturb the antigen–antibody reaction.

The methods may be assigned to one of the following groups: (i) separation of unbound material (e.g. dextran-coated charcoal), (ii) precipitation of the antibody (e.g. double antibody with and without polyethylene glycol assistance), and (iii) solid-phase systems. This last category includes magnetic particles, tubes, beads and microtitre wells, as well as microcrystalline systems such as antibody-coated cellulose or Sepharose.

Historically, dextran-coated charcoal has been widely used in steroid immunoassays, particularly those using tritium as tracer. It has a large surface area in relation to its volume, and has the property of being able to adsorb small molecules such as free steroids from solution on to its surface. Antibody-bound steroids are large molecular weight complexes which are not adsorbed onto the surface of the charcoal and remain in solution. After centrifugation, the supernatant can be decanted into a scintillation vial, and the radioactivity of the antibody-bound fraction counted. Charcoal will gradually remove antigen from the antibody and so separation is carried out at 4°C for only 10–15 min.

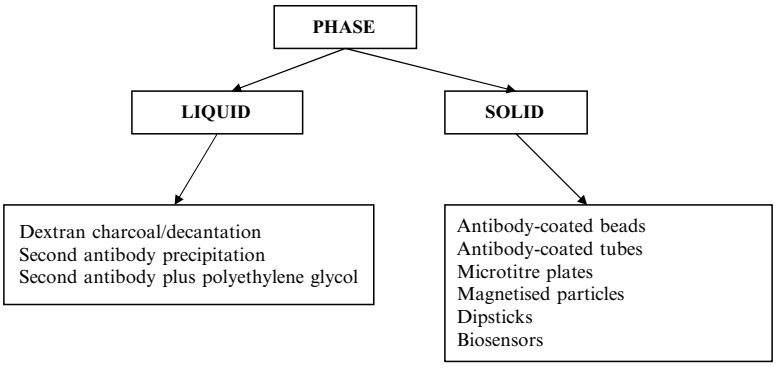

Fig. 4.12 Separation methods used in immunoassay

Second antibody assays have two great advantages over charcoal assays. Firstly, the separation is more specific and more complete, as only IgG-anti-IgG is precipitated in a second antibody reaction, whereas charcoal adsorbs all manner of substances from serum and so increases the likelihood of non-specific effects. Secondly, the second antibody technique does not disturb the equilibrium between antibody-bound and non-antibody-bound steroid so minimizing non-specific binding and false results.

In particular, use of an excess reagent coating increases the potential for greater sensitivity. In the case of labelled antigen methodology, a variety of capture reagents can be used. The use of second antibody (species specific) reagents has been described. Yet another approach is the use of biotinylated antibodies, which can be captured on immobilized streptavidin. This latter approach has the advantage of faster assays, as the antibody and antigen are in solution, and obviates agitation to keep the antibody-bound solid phase in suspension.

4.5.4 Validation and Quality Control

In order to validate an immunoassay fully, detailed data on the precision, sensitivity, inaccuracy and interference of the method must be obtained (Blockz and Martin, 2005).

4.5.4.1 Imprecision

In recent years the precision profile has become the method of choice to describe assay performance over the working range of analyte concentrations (Ekins, 1983a). The profile is essentially a smooth curve obtained when analyte concentration (x-axis) is plotted against imprecision (% CV) of the measured concentration.

In the development of an assay it is usual to assess the inter- or within-assay and the intra- or between-assay precision of an assay. Purists prefer to use the term imprecision as it is a measure of the uncertainty of measurement. Within-assay imprecision is determined by measuring a range of samples (n = 6–20) within one assay run. It is important for the range to cover the full analytical range of the assay. Between-assay imprecision is calculated by running samples (usually n = 3–5) in separate assays across the corresponding number of days. Although manufacturers provide imprecision data in their literature the concentration range is often limited and does not cover the analytical range adequately and it is therefore advisable for the investigator to establish the imprecision across the whole working range of the assay. Acceptable imprecision is always dictated by the state of the art. An intra-assay precision of <10% was deemed to be acceptable for manual assays some of which could achieve <5% precision over most of the clinically important range. Early automated assays sometimes struggled to achieve the precision of good manual assays. In recent years significant improvements in assay precision have been achieved as electronics, control of processes and temperature, and robotics

controlling precise delivery of assay tubes and reagents have greatly improved and become both more reliable and sophisticated (Wheeler, 2001).

To achieve a certain standardization for providing imprecision data the CLSI has published guidelines on the determination of imprecision. If followed, this makes comparison of manufacturer's data for different methods more meaningful. However, the guidelines are not prescriptive in the matrix to be used and this can have a profound effect on the results (National Committee for Clinical Laboratory Standards, 1992). Figure 4.13 shows how ranking and precision of oestradiol methods changed depending on whether serum or commercial QC material was used.

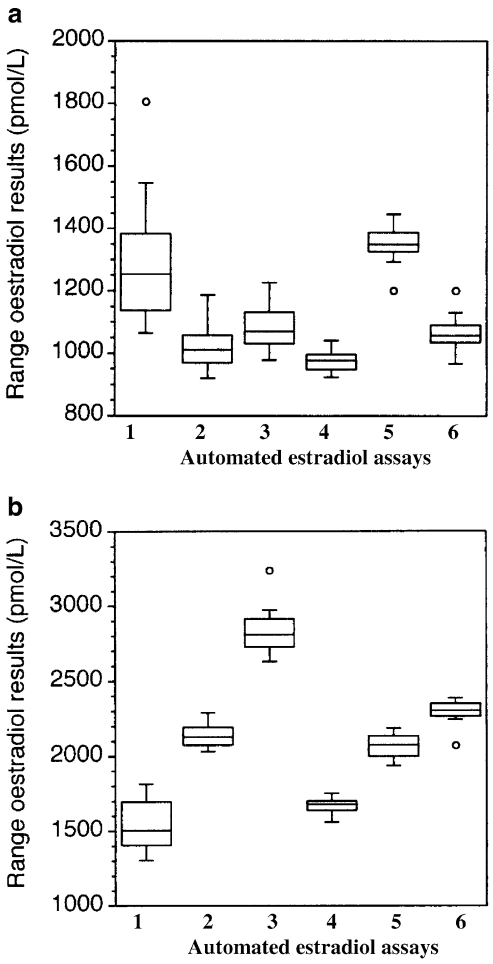

Fig. 4.13 (**a**) Serum or (**b**) commercial QC material was used to assess the precision and mean result of different commercial oestradiol methods. This resulted in changes to both due to the difference in matrix of the two QC samples (Lamph et al., 2004)

4.5.4.2 Sensitivity

It is now common to talk about analytical sensitivity and functional sensitivity based on guidelines for TSH assays (Hey et al., 1991). Analytical sensitivity is easily determined from repeated measurement of an assay tube with zero analyte. The analytical sensitivity is then taken as the mean −2.5 SD of the signal as read off the calibration curve (some developers have used −2 SD and −3 SD as well). Functional sensitivity is the concentration at the lowest acceptable imprecision. However, there is no statement as to what constitutes acceptable imprecision and, in the past, an intra-assay imprecision of <10% has been used. Nicoloff and Sencer (1990) suggested an imprecision of <20% as the cut-off for sensitive TSH assays and this has been adopted by other researchers and commercial companies. However, these authors were addressing a particular problem in the use of TSH assays and no general consensus has been adopted for the imprecision that indicates the functional sensitivity of any assay. Consideration of biological variation of an analyte (Petersen et al., 2002) also needs to be taken into account and Fraser (1992) has published data on this for common biochemical measures but there is little on steroids.

4.5.4.3 Calibration Curves

In extraction assays the matrix of the calibration curve is usually exactly the same as the samples since both will be reconstituted in assay buffer. In direct assays the matrix of the calibration curve can only mimic the matrix of the sample as it must contain substances that are not found in serum, such as displacers, often at high concentrations. Lack of linearity of diluted samples in some assays has revealed the limitations introduced by this approach (Lamph et al., 2003a). Hence it is unwise to use repeated analysis of the calibration curve to indicate imprecision of an assay.

4.5.4.4 Recovery and Bias

An estimation of recovery (bias) may be obtained by adding (spiking) known amounts of analyte to a sample. The difference between the concentrations in the unspiked and spiked samples will give the recovery. If the immunoassay demonstrates poor recovery (negative bias), this may be indicative of an interaction between the steroid and the sample matrix (e.g. binding to plasma proteins). On the other hand, recoveries greater than 100% (positive bias) may indicate that the sample matrix significantly inhibits the binding reaction. This experiment does not take into consideration any contribution of cross-reacting substances so that an assay can have good recovery but poor accuracy and a positive bias due to interference from a cross-reactant.

4.5.4.5 Inaccuracy

Accuracy has often been estimated from a recovery experiment as described above. Although recovery may be quantitative, as already explained, this experiment does not take into account any cross-reactions. Others have used the mean result from all methods for a sample (ALTM) calculated in External Quality Assessment Schemes. Although experiments have shown that the ALTM in UKNEQAS for steroids is close to the concentration measured by mass spectrometry, it does not account for any adjustments made to a calibration curve to overcome low recovery, cross-reactions or matrix interference. The only valid way to determine the accuracy of a method is by using a reference method such gas chromatography mass spectrometry (GCMS) or tandem mass spectrometry (TMS).

Isotope dilution GCMS (ID-GCMS) validated pools have been prepared for many routine steroids and studies have been carried out to compare methods using a panel of reference sera (Thienpont and De Leenheer, 1998; Thienpont et al., 2002). In the study of Taieb et al. (2003) accuracy for 16 testosterone methods varied from 68.1% to 101.8%, with 12 methods having recoveries of <90%. More recently Moal et al. (2007) compared five immunoassays with liquid chromatography–tandem mass spectrometry LC–MS/MS. They examined three manual assays, one of which employed liquid extraction, and two automated assays. All the assays showed a positive bias to LC–MS/MS and all overestimated concentrations in women and children. Many manufacturers state that the calibrators in their steroid methods have been GCMS validated. This may have no validity if the matrix of the calibrators does not behave in the same way as the matrix of the samples. Thienpont (1998) has pointed out that it is very difficult to develop valid non-extraction assays.

4.5.4.6 Interference

Interference can occur from a number of sources and at different stages during the immunoassay; Table 4.2 lists the major sources of interference. Marks (2002) examined a number of assays in a multicentre study. Surprisingly there were few

Table 4.2 Interferences that can occur in steroid immunoassay

Assay type	Interference
Extraction assays	Impure solvent
	Contaminated extract
	Matrix of reconstitution reagent
Direct assays	Haemolysis
	Lipolysis
	Icterus
	SHBG concentration
Any immunoassay	Cross-reactants
Any immunoassay with monoclonal antibody	Heterophilic antibodies

false positive results found in steroid assays from this study although other studies have indicated interference is a problem in current assays (Boots et al., 1998; Taieb et al., 2003).

Blood Collection Tube

Plastic blood collection tubes are coated with a silicon compound to aid clotting of the specimen and a surfactant to stop clots adhering to the side of the tube. There have been variable reports of interference from these additives on steroid assays (Ferry et al., 1999; Bush et al., 2001; Wiwanitkit, 2001) but recently an alert was sent out by the Medical Health products Regulatory Agency (MHRA) of the UK indicating that certain lot numbers of Becton Dickinson SSTII tubes were causing interference in immunoassays including some steroid assays (MHRA, 2004). This problem now seems to be resolved but, of course, does not mean that it could not happen again in the future. This kind of interference can be difficult to detect because of the variable nature of interference. This interference is likely to cause unexpected changes in patient results that are not confirmed by subsequent sampling.

Extraction

In extraction assays attention should be given to the cleanliness of glassware and the purity of solvent.

(i) Detergent contamination on glassware can lead to contaminants being carried over in the extract. It is not clear how these interferents act but personal experience has shown there is commonly reduced binding of the antibody to the label. A quick solvent wash of the glassware before use followed by air-drying is a helpful precaution against this type of interference.
(ii) Ether is a common solvent for steroid extractions. Peroxides can form in ether over time and, sometimes, fresh ether can be contaminated in this way. Again there is reduced binding with poor calibration curve characteristics. Although some investigators have found they are able to use Winchesters (2.5 L bottles) of ether without problems others use a fresh 500 mL bottle of ether for each assay. Residual ether is then used to solvent-wash the next batch of tubes. Yet others purify the ether before use by running the solvent down a column of silica. Because of the hazards associated with the use of ether some centres use dichloromethane as an alternative solvent.
(iii) The mode of extraction is also important. It is inadvisable to use glass screw-cap blood tubes, especially with inserts. The inserts are usually stuck into the top of the cap with glue and this is efficiently extracted by the solvent, especially if the solvent comes into direct contact with the cap; for example when using a blood-suspension mixer. The extract is milky and there is poor antibody–antigen binding.

Cross-Reactions

Some cross-reactions are well recognized, e.g. antibodies to cortisol cross-reacting with prednisolone, testosterone antibodies cross-reacting with dihydrotestosterone and oestradiol antibodies cross reacting with estrone and some synthetic estrogens. Direct assays may cross-react with steroid conjugates that would have been removed in an extraction assay. Such cross-reactions may cause spuriously high results. Dehydroepiandrosteone sulfate (DHEAS) has recently been recognized as a cross-reactant in commercial direct testosterone assays causing a positive bias. Heald et al. (2006) compared the results of 1,271 samples analysed on the Abbott Architect immunoassay analyser with those obtained from an extraction assay for the same samples. They found a median interference of 1.4 ng/mL. DHEAS was measured in 42 samples and a correlation of $r = 0.77$, $P < 0.001$ was obtained between DHAS concentration and the degree of interference. This was confirmed by Warner et al. (2006) who also found that addition of DHEAS, to either a female serum or testosterone-free assay diluent, resulted in a consistent increase in the testosterone result. DHEAS has also been added to male and female samples distributed to laboratories through the UK National External Quality Assessment Scheme (Middle, 2007). Commonly used automated immunoassay analysers such as the Beckman Access, Roche *Elecsys,* Roche *Modular* as well as the Abbott Architect were all found to have significant cross-reaction with DHEAS. A cross-reaction as low as 0.01% would have a significant effect on testosterone measurement of female and paediatric samples.

Binding Proteins

Direct assays rely on a variety of reagents to displace the steroid from the binding protein. The efficiency of the displacement may vary at different concentrations and can lead to poor agreement between assays (Boots et al., 1998).

Heterophilic Antibodies

Human antimouse antibodies can result from intensive exposure to animals such as with animal technicians or more commonly in patients receiving monoclonal antibody therapy. This interference has been associated more with protein assays than steroid assays. Nevertheless such interference can occur in steroid assays using antisteroid monoclonal antibodies since heterophilic antibody can interfere with the binding between steroid and antibody. Manufacturers add nonimmune rabbit and mouse serum to assays to block this interference but this may not remove it totally as concentrations of heterophilic antibodies in some patients can be very high.

Matrix Effects

The matrix of the calibrators in an assay can be so different from the serum matrix of the patient sample that the sample does not dilute out parallel to the calibration curve. Immunoassays are sensitive to the sample matrix and this sensitivity can vary between assays. Commercial QC material only mimics the matrix of human serum and depending on which is used for assay validation, different results can be obtained for bias and precision (see Figs. 4.10 and 4.13).

Interference can be investigated in a variety of ways. The simplest method is to dilute out the sample examining for loss of parallelism with the calibration curve. Heterophilic antibodies can be removed by using blocking tubes. Finally reanalysis in a different method can be carried out. None of these approaches is entirely foolproof (Ismail et al., 2002) and two or all of the procedures may be required to completely rule out interference.

4.5.4.7 Miscellaneous Controls

Other controls included in an assessment of assay performance may include blanks such as steroid-free plasma or serum to demonstrate that the assay does not have non-specific interference that indicates the presence of antigen when it is, in fact, absent. In addition it is also useful to understand the magnitude of 'non-specific binding' by determining the amount of label bound in the absence of antibody.

4.5.4.8 Correlation with Other Methods

It is helpful to be able to compare the values obtained in an immunoassay with an alternative reference method that measures the concentration of analyte specifically, such as high performance liquid chromatography or mass spectrometry. Comparison with other immunoassay methods does not give an indication of accuracy but agreement with market leaders. Such comparisons should be carried out with serum samples and not commercial QC material as the agreement between methods can be entirely misleading with the latter (Lamph et al., 2003a, 2004).

4.5.4.9 External Quality Assurance

Over the last 10 years, excellent external quality assurance schemes have been devised to assess objectively the performance of clinical laboratories, in-house methods and commercially available diagnostic kits. A number of external quality assessment schemes (EQAS) are available for steroid assays, e.g. UK National External Quality Assessment Scheme (UK NEQAS), Welsh External Quality Assessment Scheme (WEQAS), Randox Scheme. It is now mandatory in the UK that NHS clinical laboratories must belong to an EQAS for all clinical assays. Not only is there regular distribution

of samples to assess laboratory performance but educational exercises are also carried out where recovery, cross-reaction and interference studies are performed. UK NEQAS has also formed a Steroid Forum to discuss problems in steroid assays.

4.6 Future Developments

Over the last 25 years, steroid immunoassays have undergone continuous change in the search for methods that would be more sensitive, specific, and convenient. For example, convenience has been achieved by all major diagnostic companies through the automation of direct testosterone assays. This, however, has generally been at the expense of sensitivity. There are many publications that have indicated the limitations of current commercial steroid assays (Taieb et al., 2003; Wang et al., 2004; Wheeler and Barnes 2008) and companies still strive to develop assays with sufficient accuracy, specificity, and sensitivity to meet clinical requirements (Novotny and Wilson, 2005). It is therefore safe to anticipate further significant refinements and developments in the measurement of steroids by automated immunoassay systems over the next few years. These will be minor compared to the potential development of microarray systems that will be able to provide clinical profiles of substances including steroids, peptides, proteins, and drugs. The use of idiotypic antibodies will probably be required for steroids to be part of microarrays.

There is a growing interest in tandem mass spectrometry that could move steroid measurement away from immunoassay completely. TMS has the advantage of being a very simple technique that is rapid and highly specific. It also offers simultaneous measurement of several substances.

4.6.1 Immunometric Assays

All immunoassay techniques can be divided into two basic types, competitive and noncompetitive, typified respectively by RIA and immunoradiometric assay (IRMA). The advantages of the noncompetitive approach have been well documented and include greater sensitivity, precision, and working range of analyte (Jackson and Ekins, 1986). The sensitivity of a noncompetitive method is related to the fractional nonspecific signal (i.e. the percentage of the total signal) and the relative error with which the response at zero dose can be measured. Since the signal at low dose is very low, the relative error in its measurement is also low. Consequently, the realization of potential sensitivity is improved by reducing nonspecific binding to a negligible amount.

Optimal sensitivity = $N \cdot CV_{nsb}/K$ where N is the fractional nonspecific signal; CV_{nsb} is the relative error in response at zero dose; and K is the affinity constant, e.g. $(0.01 \times 0.01)/10^{12} = 10^{-16}$ M levels. This is illustrated in Fig. 4.14, which shows the effect of fractional nonspecific signals of 0.01 and 1%.

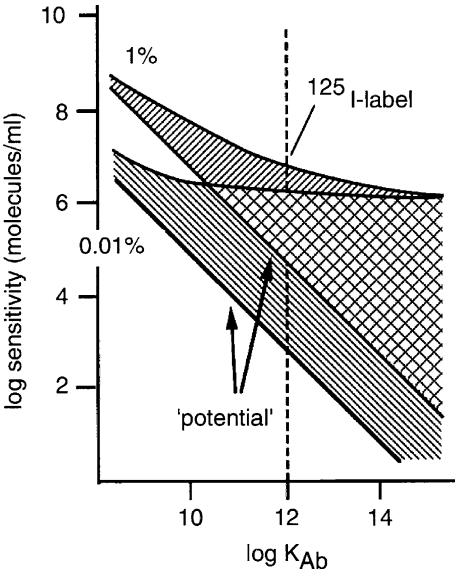

Fig. 4.14 Factors affecting the sensitivity of noncompetitive immunometric assays (after Jackson and Ekins, 1986). Optimal sensitivity = ($N{\cdot}CV_{nsb}/K$ where N is the fractional non-specific signal; CV_{nsb} is the relative error in response at zero dose; and K is the affinity constant, e.g. $(0.01 \times 0.01)/10^{12} = 10^{-16}$ M

The practical application of noncompetitive technology, however, has been the development of two-site assays for the measurement of compounds with more than one antigenic determinant (epitope). By definition, the two-site assay is unsuitable for the measurement of haptens (e.g. steroids), although it is probable that a small molecule may possess more than one epitope. Invariably, the cleft nature of the antigen binding site of one specific immunoglobulin will preclude, by steric hindrance, binding of a second antibody which might recognize an alternative epitope. Consequently, to date, noncompetitive technologies have not been applied successfully to the measurement of small molecules.

Ekins (1985) has suggested that the fundamental difference between competitive and noncompetitive procedures is based upon the detection of antibody occupancy. For example, the use of a labelled (or immobilized) antigen in a competitive immunoassay is to detect those antibody-binding sites that are not occupied by the analyte in the standard or sample.

Alternatively, the use of the labelled antibody in a noncompetitive immunometric assay is to detect the presence of the captured analyte (i.e. occupied antibody-binding sites). Accordingly, the development of a noncompetitive assay for the measurement of steroids primarily requires a method for detecting antibody occupancy.

Once this is understood, it is quite straightforward to devise various schemes that are not based on the conventional two-site approach, which requires the availability of spatially separate and distinct epitopes on the surface of the antigen. For example, the identification and application of anti-idiotypes or anti-allotypes, which bind to

the antibody only when the antigen is bound, would facilitate the development of a simple and possibly universal non-competitive technology.

Theoretically, reagent binding could be due to (i) direct antigen–antibody recognition, or (ii) binding to epitopes exposed by conformational changes in the tertiary structure of the immunoglobulin (constant or variable regions) after antigen binding.

Barnard and Kohen (1990) described three monoclonal antibody screening assays for the identification and production of two types of anti-idiotypic antibody that recognize epitopes of the variable regions of the heavy and light chains of the primary immunoglobulin molecule. The anti-idiotypes were of two types: (i) beta-types, which recognize the binding site of the antibody (paratope) and compete with the antigen for binding, and (ii) alphatypes, which recognize the framework region of the variable region of the antibody and which are not sensitive to the presence or absence of the antigen.

The availability of these reagents enabled the conception of a novel approach for the detection of antibody occupancy which has been termed idiometric assay. The method is applicable to the measurement of small and large molecules and is typified by a method for the determination of oestradiol in serum (Barnard and Kohen, 1990). In principle, the method can be adapted for the measurement of free steroid in serum and in samples of saliva taken daily throughout the menstrual cycle. The principle of steroid idiometric assay is shown in Fig. 4.15.

Independently, Self (1985, 1989) has patented various approaches of noncompetitive immunoassays for the measurement of small molecules. Most recently, his group has developed a noncompetitive assay for digoxin utilizing an antibody that recognizes the presence of digoxin in the primary antibody-binding site (Self et al., 1994).

Although methods have been published for noncompetitive methods for the measurement of small molecular weight analytes these have yet to be commercialized.

4.6.2 Microarrays

Developments in nanotechnology have led to the development of microarray assays. Assays can be carried out on minute drops of liquid of 1–10 μL (Kumar et al., 2005). Antibodies can be coated on a solid surface as a microspot and a microsample added and reacted. The binding can be detected by radioactivity or usually fluorescence. This technology has been applied widely to the detection of proteins particularly DNA and mRNA (Ewis et al., 2005). The discovery of a range of disease-associated biomarkers has led to multianalyte testing that, with suitable instrumentation, could be incorporated into routine clinical laboratory (Hadd et al., 2005). Diagnosis is based on the presence and absence of biomarkers as well as their ratio with one another. Microarray technology is able to assess many biomarkers

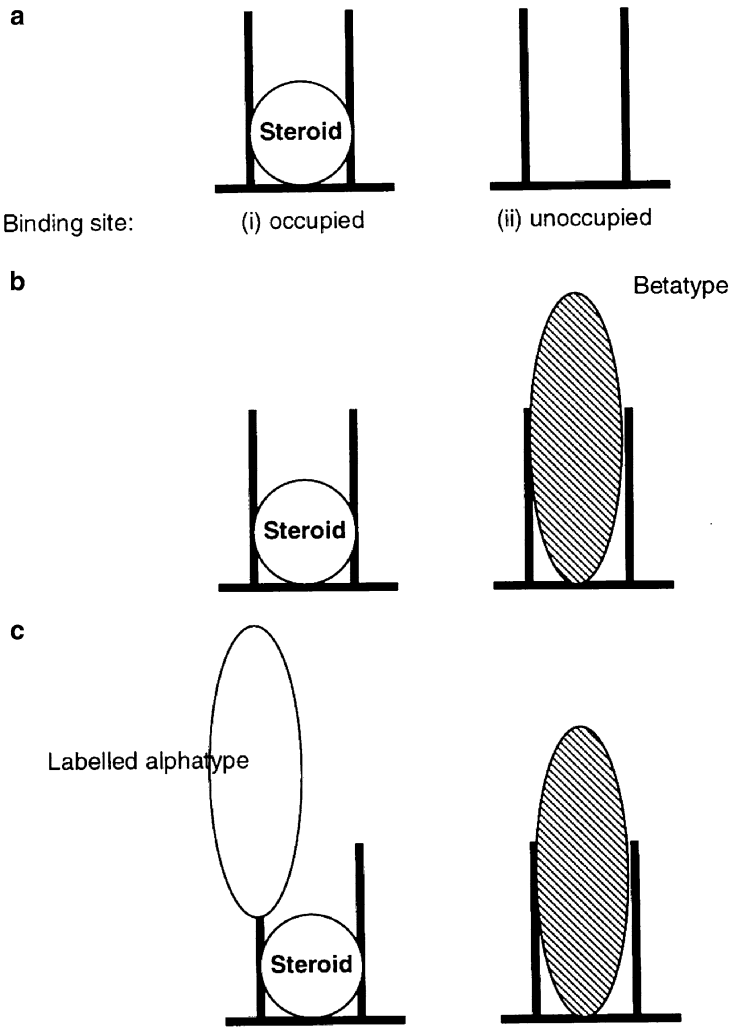

Fig. 4.15 Principle of steroid idiometric assay (after Barnard and Kohen, 1990). (**a**) Analyte capture – results in occupied and unoccupied sites, (**b**) beta-type addition – blocks un-occupied sites, and (**c**) labelled alpha-type addition – detects analyte-occupied sites

on one chip and can generate a huge amount of complex data requiring sophisticated data reduction packages.

However, Ekins has used microspot technology to develop immunoassays of high sensitivity using what he describes as the ambient analyte assay (Ekins, 2005). Although Ekins describes a TSH assay it is possible to apply this technology to steroids using anti-idiotypic antibodies described by Barnard and Kohen (1990) and Self et al. (1994). However, these developments may be overtaken by an interest in

the future to study steroid action at the cellular level rather than what circulates in the blood (Bassett et al., 2005; Titus et al., 2005) partly because of the more advanced developments in protein microarray technology and partly because cellular studies may give a clearer understanding of disease events.

4.6.3 Assays for Free (Unbound) Steroids

It is possible that the measurement of total steroid concentration in serum may not be the best parameter to measure in order to understand the clinical status of the patient under investigation. For example, in the case of thyroid hormones, measurement of free thyroxine is generally considered to be more clinically relevant than the measurement of total thyroxine in serum (Ekins, 1983b, 1992). This may also be true for steroids. It has been suggested that the successful free steroid hormone method will be based on the immobilization of a trace amount of high avidity, specific antiserum on to the surface of a solid phase (Cook and Beastall, 1987). The assay would then involve addition of plasma or serum and the antibody-binding sites occupied in proportion to the concentration of free steroid. Furthermore, it is suggested that the concentration of specific antibody should be very low to prevent the disturbance of equilibrium between the steroid and its endogenous binding protein.

After the binding reaction, plasma is removed, the solid phase washed and the unoccupied binding sites remaining on the solid phase saturated with an excess of labelled steroid. The signal, as in all competitive assays, is inversely proportional to the free steroid concentration originally present in the plasma or serum. Probably the main impediment to the development of such a method is the potential sensitivity that can be obtained from the conventional competitive immunoassay. Nevertheless, current assays for free thyroxine are able to measure down to 5 pmol/L which is below the normal range of free testosterone concentrations in women (10–50 pmol/L).

The lack of assays for free steroids indicates the difficulty associated with the development of these assays. Dynamic tests are probably more helpful diagnostically than free cortisol measurement but free testosterone measurement in plasma is useful clinically. The lack of a reliable free testosterone assay has led to the adoption of various approaches that include the use of the ratio of SHBG to testosterone concentrations (the androgen index) as well as a number of more complex calculations (see review by Wheeler, 1995). A commercial kit is available from Diagnostic Systems Laboratories Inc, USA but this method has been criticized as the results do not agree with those of the reference method, equilibrium dialysis (Rosner, 2001). Without a simple method for free testosterone measurement investigators have used alternative approaches such as measuring the non-SHBG bound testosterone or calculating the free testosterone concentration from measurement of the total testosterone and SHBG concentrations. Vermeulen et al. (1999) have suggested the latter approach is, at the moment, the most reliable. The ratio of cortisol to cortisol-binding globulin as an index of free cortisol has also been investigated (Le Roux et al., 2002, 2003).

Alternatively, developers have studied noncompetitive technology for the sensitive and specific measurement of steroids and other haptens. To date, these developments are in their infancy.

4.6.4 Tandem Mass Spectrometry

TMS is a nonimmunoassay procedure that is simple, rapid, and highly specific. A number of publications are now appearing where a single steroid has been measured by TMS either for routine investigation (Cawood et al., 2005; Maunsell et al., 2005) or as a reference method against which commercial assays are compared (Wang et al., 2004). TMS has the advantage over immunoassay methods of being potentially interference free and able to measure at sensitivities superior to commercial automated methods. It is likely to be applied to specific samples such as those from women, pre-pubertal children or where interference is suspected. Another advantage of TMS is the ability to provide a steroid profile. Guo et al. (2004) described a method using HPLC–MS/MS that measured 9 steroids in 18 min using only 760μL serum. More recently they report the development of their method to measure 12 steroids using 200μL serum (Guo et al., 2006). The assay takes just over 21 min; protein precipitation and centrifugation 10 min plus 11 min LC–MS/MS.

4.6.5 Biosensors

Government and medical interest in carrying out testing away from the laboratory and near the patient or at the patient's bedside has encouraged the development of smaller and smaller instruments. With miniaturization of electronics and improved sensitivity of detection systems it has become possible to manufacture instruments that are considerably smaller than previous analysers. Hand-held devices have been made for measuring common substances like cholesterol and glucose but developments in the immunoassay field are making it possible to develop small instruments like biosensors for hormones and other antigenic substances. As well as the medical field, biosensors are also being developed for the agri-food and environmental industries (see www.gwent.org). Immunobiosenors are of interest to medical and agricultural sectors.

4.6.5.1 Plasma Resonance Biosensors

A biosensor is an instrument that detects changes in chemical concentration, due to biochemical interaction such as between antibody and antigen, and converts them to an electrical signal. In surface plasmon resonance (SPR), photons incident on a noble metal surface cause electrons in the metal to move as a plasmon and generate an

electrical field. Changes in the chemical environment on the surface of the noble metal cause a shift in the angle or wavelength required to induce SPR thus providing a means of detecting small mass changes on the sensing surface (Hommola, 2003). Steroid or antibody may be bound to the surface and endogenous steroid can compete for antibody-binding sites so changing the antibody/antigen binding at the surface. A biosensor instrumentation is available as the BIACORE system (GE Healthcare) and has enabled the method development of steroids (Kaiser et al., 2000). This instrumentation was used by Gillis et al. (2002) to develop an assay for progesterone in bovine milk. This method had a sensitivity of 3.56 ng/mL with a precision <5%. Results were available in just over 5 min allowing rapid analysis of a large number of samples in one day. The chemical bridge to link the steroid to the biosensor surface has an effect on antibody binding and the sensitivity of the assay. The length of the chemical bridge between progesterone and ovalbumin was investigated by Wu et al. (2002). These workers found that increasing the linker from 4 atoms to 11 or 18 atoms increased antibody-binding capacity and response rate when using BIACORE SPR. Using the same instrumentation Mitchell et al. (2006) investigated the effect of forming conjugates through positions 2 and 4 of the steroid as well as the functional 3 position. They found that linking the former two conjugates to the biosensor surface gave a 9.5-fold increase in signal with a sensitivity of 25 pg/mL.

4.7 Summary

The introduction of steroid immunoassay methods has enabled clinical chemist and researchers to measure low concentrations of a large number of different compounds, which has led to a dramatic increase in our understanding of steroid physiology as well as helping to diagnose steroid-related disease more accurately. Currently many commercial radio- and non-radio-isotopic kits for the measurement of common steroids are available. In general, these methods do not require sample pretreatment, although it must be remembered that this approach may be unsuitable for application in many clinical situations.

The design of current steroid assays bears little resemblance to those developed in the early 1970s. For example, the introduction of iodinated steroid derivatives has simplified analysis, reduced the cost and improved sensitivity and precision. The same can be said for the introduction of many different nonradioisotopic detection systems, with their emphasis on antibody-coated particles, tubes and microtitre plates. In fact, there seems little doubt that fully automated nonradioisotopic immunoassay systems will grow in popularity and eventually totally replace the use of radioisotopes in the clinical laboratory.

In the future, we may anticipate steroid immunoassays with increased sensitivity that can measure free concentrations of steroid in biological fluids and tissues which may be more clinically relevant. In addition, availability of labelled steroids of ever-increasing specific activity may lead to the development of very sensitive and precise nonseparation assays that can be easily automated.

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Chapter 5
Analysis of Corticosteroids

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5.1 Introduction

The adrenal cortex is the source of a number of steroid hormones that play a fundamental role in regulating body metabolism. The elucidation of the complicated biosynthetic pathways and the metabolic fate of these hormones proceeded relatively slowly until it became possible to make reliable measurements of the concentrations of these important steroids in body tissues. Because of the complex structure of the steroids, their close chemical similarity to each other and their occurrence in low concentrations in body tissues, the development of suitable analytical techniques proved a major challenge to chemists and biochemists

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working in this field. Although many of these problems still exist, there has been significant progress in the clinical field, particularly because of the availability of radiolabelled and stable isotope-labelled steroids and the introduction of immunoassay (IA) methods for routine clinical biochemical analysis. The advent of highly selective separatory chromatographic methods (LC and GC) increasingly linked to mass spectrometers means that analytical methods are now available for all of the important adrenal cortical hormones, their major precursors and metabolites, and synthetic glucocorticoids. These methods have found wide application, especially in clinical research and investigation, where they have been used to elucidate the nature of many endocrine disease processes, and for diagnosis and treatment. These facts in themselves highlight the importance to the analyst of ensuring that the techniques used are as reliable as possible.

In this chapter, we have attempted to review the current methods available for the analysis of adrenal corticosteroids, paying particular attention to some of the analytical problems encountered, and also indicating the areas in which these analyses find their main applications. The term 'corticosteroid' is not precise and, although it might be expected to include any steroid produced by the adrenal cortex, in this chapter its use is restricted to those steroids with a structure containing 21 carbon atoms, which is further defined below. However, the adrenal cortex produces in addition a number of steroids with a 19 carbon structure, generally referred to as adrenal androgens, but these are dealt with in Chapter 6.

5.2 Structures of Corticosteroids

As indicated above, for the purposes of this chapter, the corticosteroids are a group of C_{21} steroids produced by the adrenal cortex. The following features characterise them structurally, when secreted but may be modified during catabolism:

- A double bond at C-4 and an oxo group at C-3 (they therefore belong to the class of active steroid hormones known as 4-en-3-ones).
- A side-chain at C-17 consisting of a C-21 hydroxl group with an oxo group at C-20, i.e. $–CO–CH_2OH$.
- The presence or absence of a hydroxyl group at C-17, thus giving rise to the classification of 17-oxygenated corticosteroids, such as cortisol or 11-deoxycortisol, and 17-deoxy-corticosteroids, such as 11-deoxycorticosterone (DOC), corticosterone and aldosterone, often confusingly referred to, on the basis of their prime physiological activity, as gluco- and mineralo-corticoids respectively (see below). It should be noted here that there are species differences in the roles of various steroids (e.g. serum cortisol is used as a stress marker in humans, whereas corticosterone is used in rats and other species).
- A hydroxyl or oxo group, which may or may not be present at C-11.

The structural features of the physiologically important naturally occurring corticosteroids are shown in Figs. 5.1 and 5.2.

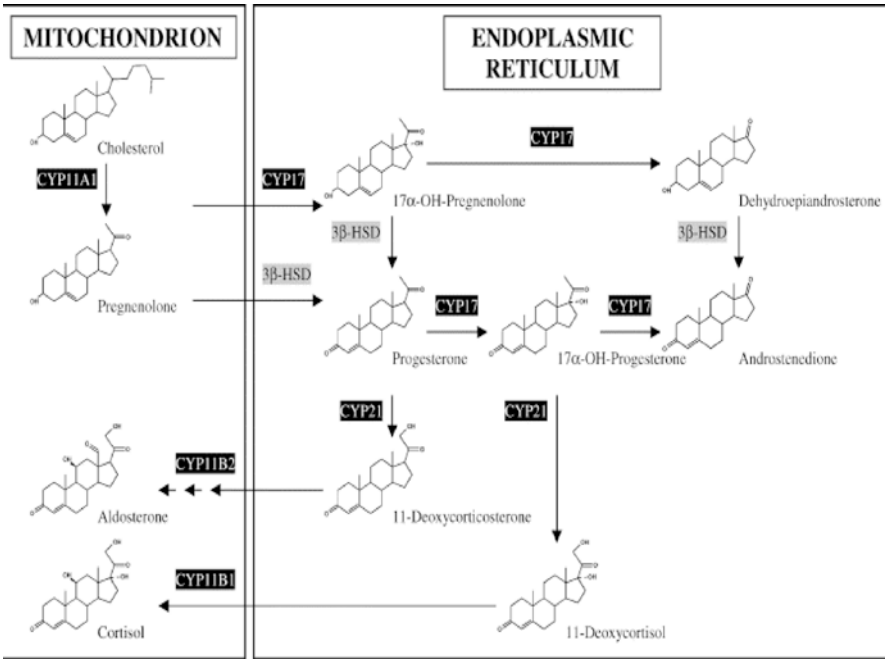

Fig. 5.1 Steroid biosynthesis in the adrenal cortex. The diagram shows the reactions leading from cholesterol to cortisol, aldosterone and androstenedione, including the names of the corresponding enzymes and the organelles in which the reactions are carried out (from Lisurek and Bernhardt, 2004, with permission; copyright Elsevier Ireland, Ltd.)

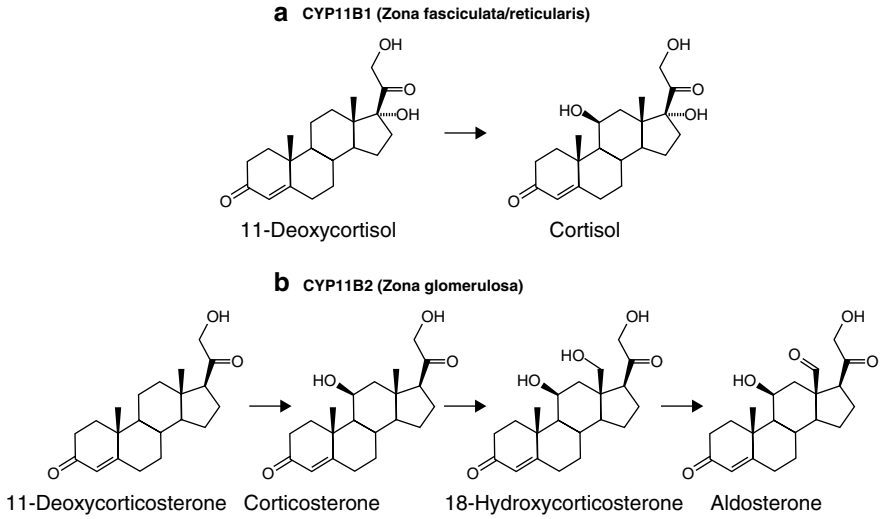

Fig. 5.2 Reactions catalysed by human CYP11B1 and CYP11B2. (**a**) CYP11B1 catalyses the 11β-hydroxylation of 11-deoxycortisol to cortisol. (**b**) CYP11B2 converts 11-deoxycorticosterone via corticosterone and 18-hydroxycorticosterone to aldosterone (from Lisurek and Bernhardt, 2004, with permission; copyright Elsevier Ireland, Ltd.)

5.3 Biological Activity

Corticosteroids may be broadly classified as 17-hydroxylated or non-17-hydroxylated (17-deoxy) corticosteroids and, although there is some overlap in physiological effects, the former group in humans possesses the more potent glucocorticoid activity. This means that they have important effects on a variety of metabolic events, including glucose and protein metabolism. In contrast, 17-deoxycorticosteroids, such as aldosterone and DOC, influence electrolyte and water balance (see later sections). The effects of the glucocorticosteroids have been dealt with in detail by Stewart (2008). A summary of this is given below.

5.3.1 Glucocorticoids

Glucocorticoids, such as cortisol, exert effects on a wide range of metabolic events in tissues as diverse as those of the liver and skeletal muscle, as well as adipose and lymphatic tissues. The primary effects are those that occur within 4 h of administration of the hormones and they include increased glycogen synthesis, increased gluconeogenesis, increased amino-acid uptake, increased hepatic RNA and protein synthesis, and promotion of lipid mobilisation. The overall effect (that is directly antagonistic to that of insulin) is to increase the blood glucose level by stimulating hepatic synthesis of glucose from amino acids. The increase in hepatic gluconeogenesis is accomplished by induction of the following enzymes: alanine aminotransferase, pyruvate carboxylase, phosphoenolpyruvate carboxykinase, phosphoglyceraldehyde dehydrogenase, aldolase, fructose-1,6-diphosphatase, phosphohexose isomerase, glucose-6-phosphatase and glycogen synthetase.

The naturally occurring and synthetic glucocorticoids are known to have important effects on the inflammatory response and on fibroblast synthesis of collagen and glycosaminoglycans (mucopolysaccharides). The inflammatory response refers to localised reactions which occur as a result of injury to the tissues, whether by actual damage or in more general allergic conditions such as asthma and hay fever. The response is suppressed by glucocorticoids in various ways, e.g. they increase capillary resistance to the migration of leucocytes (especially neutrophils) to the site of infection. This in turn means that invading bacteria or damaged tissue cells undergo decreased phagocytosis. These effects, together with the possibility that glucocorticoids interfere with the scavenging capacity of neutrophils, help to explain the decreased resistance to infection shown by patients taking corticosteroids. Coupled with this, is the inhibition of protein synthesis in lymphatic tissues, already referred to above, so that antibody synthesis is reduced. Another effect of glucocorticoids is to counteract the increases in permeability and dilation of the capillary blood vessels caused by histamine and serotonin, both of which are known to be involved in inflammatory reactions. These properties make glucocorticoids beneficial in relieving symptoms of allergic conditions like asthma and hay fever.

However, the clinical management of such patients, and of those with rheumatoid arthritis who may require long-term steroid therapy, is made difficult because of the side effects of the glucocorticoids. Besides the effects mentioned above, they also inhibit wound healing and suppress endogenous adrenocortical function. Long-term glucocorticoid therapy may lead to severe osteoporosis and capillary friability, both due to loss of protein.

5.3.2 Mineralocorticoids

Mineralocorticoids (MC) are steroid hormones with the ability to stimulate sodium (Na^+) reabsorption in the distal nephron as well as in the large intestine and salivary glands. They play a key role in controlling salt and water balance. Acting through the MC receptor, they promote Na^+ reabsorption through the apical sodium channel in exchange for K^+ and H^+. Excess MC activity raises extracellular Na^+ levels while reducing those of K^+ and H^+ (abnormally low activity will have the opposite effect). Increased body Na^+ is associated with a parallel rise in extra-cellular fluid volume and an increase in vascular smooth muscle pressor sensitivity, both tending to raise blood pressure. Thus, the stigmata of hypermineralocorticoidism are hypertension, a high body Na^+, hypokalaemia and a metabolic alkalosis. In addition, severe hypokalaemia causes neuromuscular dysfunction.

Only two naturally occurring corticosteroids have significant MC activity. Aldosterone is much more potent than 11-deoxycorticosterone (DOC) and is probably the most important in normal subjects. Whether DOC contributes significantly in normal subjects is debatable but in a number of rare inherited diseases, it assumes a dominant role. Aldosterone is a product of the zona glomerulosa (ZG), which is under the control of the renin-angiotensin system. In contrast, the origin of almost all circulating DOC is the zona fasciculata (ZF), under ACTH control. This chapter will also deal with a group of corticosteroids in which the 18-methyl group is modified. Of these, 18-hydroxycorticosterone and 18-hydroxyDOC are related to aldosterone synthesis but, like DOC, originate mainly from the zona fasciculata. Neither compound has significant MC activity (see however Nicholls et al., 1977, 1979). In addition to this, Ulick et al. (1983, 1991) also identified and characterised 18-hydroxcortisol and 18-oxocortisol that have some diagnostic value in forms of abnormal aldosterone biosynthesis (see below). 18-Hydroxycortisol in normal subjects is again produced mainly in the zona fasciculata, and has little or no biological activity. The origin of 18-oxo-cortisol in normal subjects is debated but it may originate from zona glomerulosa action on recirculated cortisol (Freel et al., 2004). Finally, corticosterone, the principal glucocorticoid in the rat and other animals and a minor glucocorticoid in man, is also an ACTH-dependent, zona fasciculata product. However, it is also a component of the late biosynthetic pathway to aldosterone.

The glucocorticoid, cortisol, binds efficiently to the MC receptor but in normal subjects is prevented from doing so by 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD-2), which co-localises with the MR and oxidises cortisol to cortisone,

which does not bind to the MR. In the syndrome of apparent mineralocorticoid excess (AME), this enzyme is inadequate and cortisol therefore masquerades as a mineralocorticoid causing changes similar to those of aldosterone excess (Stewart et al., 1996) (see also below). Methods for assaying cortisol and cortisone are dealt with elsewhere in this book.

5.4 Biosynthesis

As a result of many (now classical) experiments that were performed during the 1960s and 1970s, it has become clear that two pathways exist in the human adrenal cortex for the formation of the corticosteroids (Fig. 5.1) (full details are given in Gower, 1984a). Histological studies combined with those from biosynthesis studies have shown that aldosterone and other mineralocorticoid production occurs in the cells of the zona glomerulosa, situated immediately under the adrenal cortical capsule. The bulk of the cortex is composed of the cells of the zona fasciculata which are laden with cholesterol and cholesterol esters, and produce cortisol. The zona reticularis (ZR), consisting of 'compact' cells, is the zone nearest the adrenal medulla. These cells, together with the cells of the ZF are responsible for the biosynthesis of the corticosteroids and adrenal androgens (see Chapter 6), with the C_{27} steroid cholesterol acting as precursor. Cholesterol uptake by mitochondria is an active process involving steroidogenic acute regulatory protein (StAR) (Miller, 2007).

Part of the side-chain of cholesterol is reduced in length in the mitochondria, losing six carbons by enzymic cleavage between C20 and C22. The enzyme involved in this 'side-chain cleavage' is cytochrome $P450_{scc}$ (CYP11A1), the reaction resulting in the formation of pregnenolone (a C_{21} steroid). This then passes into the smooth endoplasmic reticulum to act as a precursor for the first, and quantitatively more important, pathway for corticosteroid biosynthesis: pregnenolone → 17α-hydroxypregnenolone → 17-hydroxyprogesterone → 11-deoxycortisol → cortisol (Fig. 5.1). The enzymes are 17α-hydroxylase known as CYP17,[1] 3β-hydroxysteroid dehydrogenase (3β-HSD2),[1] a steroid 4,5-isomerase, which catalyses the conversion of the 3β-hydroxy-ene-5-ene steroid, 17-hydroxypregnenolone to the 4-en-3-oxosteroid, 17-hydroxyprogesterone 21-hydroxylase (CYP21A2[1])

[1]In this chapter, we have tried to use the modern terminology for the steroid hydroxylases and dehydrogenases involved in biosynthesis and catabolism of the various corticosteroids discussed here. As an example, use of the shorthand term 'CYP11A1' (the cholesterol side chain cleavage enzyme) refers to the cytochrome P450 protein, which, together with the other two or three proteins involved, constitutes the active enzyme system. *CYP11A1* refers, when in italics, to the gene which codes for this protein. The same nomenclature applies in a similar fashion to the other entire CYP-steroid enzyme (CYP11B1, CYP11B2, CYP17, CYP19 and CYP21). Full details of all CYPs can be found at: http://drnelson.utmem.edu/CytochromeP450.html.
The hydroxysteroid dehydrogenases do not seem to follow the same nomenclature in that for the 11β-hydroxysteroid dehydrogenase, the protein is 11βHSD1 and the gene is HSD11B1.

and 11β-hydroxylase (CYP11B1). It should be noted that the latter enzyme is a mitochondrial one, thus requiring the transportation of 11-deoxycortisol back to this cellular compartment for cortisol to be formed. CYP11B2 is involved in the biosynthesis of aldosterone (see below).

The second pathway proceeds along a similar route except that no 17α-hydroxylation occurs. The enzymes required for this pathway are located in the zona glomerulosa and are, first, the 3β-hydroxysteroid dehydrogenase (3βHSD2), which catalyses the conversion of the 3β-hydroxy-ene-5-ene steroid, pregnenolone to the 4-en-3-oxosteroid, progesterone; second, the CYP21A2, which produces DOC (11-deoxycorticosterone); and finally, the CYP11B2 in the mitochondria, which catalyses the formation of corticosterone as well as the 18-oxidation system producing aldosterone. CYP11B exists in two forms (reviewed by Lisurek and Bernhardt, 2004): CYP11B1, in the zona fasciculata, catalysing the production of cortisol, and CYP11B2, in the zona glomerulosa, catalysing the formation of corticosterone as well as the 18-oxidation system producing aldosterone. These two isoforms of CYP11B are intra-mitochondrial (see Fig. 5.2). Synthesis of mineralocorticoids from cholesterol involves a series of enzyme-catalysed reactions in dynamic equilibrium with each other. Disturbance of one reaction results in a readjustment in the activities of all the others. Analysis of biological fluids for groups of compounds representing these reactions thus provides crucial information on relationships and a rough index of enzyme kinetics. The above examples of 11β- and 17α-hydroxylase activity illustrate this. The synthesis of aldosterone from DOC is catalysed by aldosterone synthase (CYP11B2) and involves sequential 11β-hydroxylation, 18-hydroxylation and 18-oxidation (see Fig. 5.2).

Separate syndromes of impairment of 18-hydroxylation and 18-oxidation (corticosterone methyl oxidase deficiencies types I and II) suggest separate kinetics (Ulick, 1976; Ulick et al., 1991). A combination of 18-hydroxycorticosterone and aldosterone assays has been employed in diagnosis. More recently, a series of aldosterone synthase gene (*CYP11B2*) polymorphisms has been identified which may be related to *in vivo* enzyme efficiency (Connell et al., 2003). The combination of DOC, corticosterone, 18-hydroxycorticosterone and aldosterone assays to test these polymorphisms *in vitro* is essential to a precise localisation of this variability.

The sequence of precursors and intermediates leading to the biosynthesis of the corticosteroids has been generally accepted for many decades as, at least, a reasonable approximation to the truth. In addition, during the past 15 years or so, studies of the properties of the cytochromes involved (as noted above) have added greatly to our knowledge and have tended to add credence to the overall pattern of biosynthesis (Ortiz de Montellano, 1995; Lewis, 1996). The cytochrome P450 receives electrons from NADH and NADPH. The importance of this has been realised clinically in patients with apparent defects of both CYP21 and CYP17 because of mutations in cytochrome P450 oxidoreductase gene (*POR*) (Fluck and Miller, 2006; Fluck et al., 2008).

A number of investigators have questioned this accepted ‘dogma’ of steroid biosynthesis (as it has been called) on a number of grounds, two of which will be discussed here. First, it has been pointed out that many experiments have been performed with adrenal cortical preparations *in vitro* so that the results

may not necessarily match exactly what may be taking place *in vivo*. In fact, Lieberman et al. (2005) have stated that the many single bits of evidence gained have been 'pieced or stitched together' to provide sequences such as pregnenolone → 17α-hydroxypregnenolone → 17α-hydroxyprogesterone → 11-deoxycortisol → cortisol (see above). Second, this generally accepted scheme requires that at least some of the cytochromes P-450 are multifunctional enzymes. For example, before side-chain cleavage can occur, cytochrome P-450$_{scc}$ must use O_2 to catalyse two hydroxylations in the side-chain, one at C-20, the other at C-22. Similarly, in cortisol biosynthesis, hydroxylations are required at C-21, C-17, C-11, and in aldosterone, C-11 and C-18 hydroxylations are necessary (see later sections), the hydroxylations being catalysed by a cytochrome named cyt P-450 in each case. In a series of papers Lieberman and his colleagues (Lieberman and Warne, 2001; Lieberman et al., 2005; Lieberman and Kaushik, 2006) have put forward suggestions to answer these two possible problems, as well as others (not given here for the sake of brevity), that they find with the generally accepted sequences in Fig. 5.1. These authors admit that their suggestions are speculative, based on computer-generated mechanisms. One suggestion is that the various intermediates may not necessarily be stable and soluble compounds but, rather, may be entities that are short-lived and reactive. Lieberman et al. (2005) have therefore proposed that, in the presence of O_2, cyts P-450 could catalyse the formation of peroxides or 1,2-cyclic peroxides, resulting in both atoms of activated O_2 being introduced into an appropriate precursor, such as the cholesterol hydrocarbon side-chain (Lieberman and Lin, 2001) via the 20α-peroxy radical, which could then be converted to the 21-alkoxy radical and then to DOC. Further catalytic action of CYP11B1 would lead to cortisol, while the actions of CYP11B2 on DOC would lead to aldosterone (see Fig. 5.2).

Corticosteroids are also synthesised outside the adrenal cortex, for example in the brain and the vascular smooth muscle. This is at a very low level and its contribution to plasma concentrations or to urinary excretion rates is likely to be small. However, assessing local tissue activity is important in current research (Connell and Davies, 2005).

5.5 Metabolism

The first stage in the metabolism (catabolism) of the corticosteroids (leading to inactivation) is the reduction of the A-ring catalysed by two enzymes, the 4-ene-5β-reductase (SRD5B1) and the 4-ene-5α-reductase (SRD5A2). The second stage utilises the enzymes 3α- and to a much lesser extent 3β-hydroxysteroid dehydrogenases (3-HSDs); thus, for all corticosteroids the major products are A-ring tetrahydro-reduced catabolites. From cortisol and cortisone, tetrahydrocortisol and tetrahydrocortisone, sometimes called THF and THE respectively, are produced (illustrated in Fig. 5.3). Smaller amounts of the 5α-metabolites (referred to as allo-THF and allo-THE, for example, for cortisol (F) and cortisone (E) respectively,) are also found. The 5β/5α ratio for these corticosteroid metabolites in human urine is usually about 3:1.

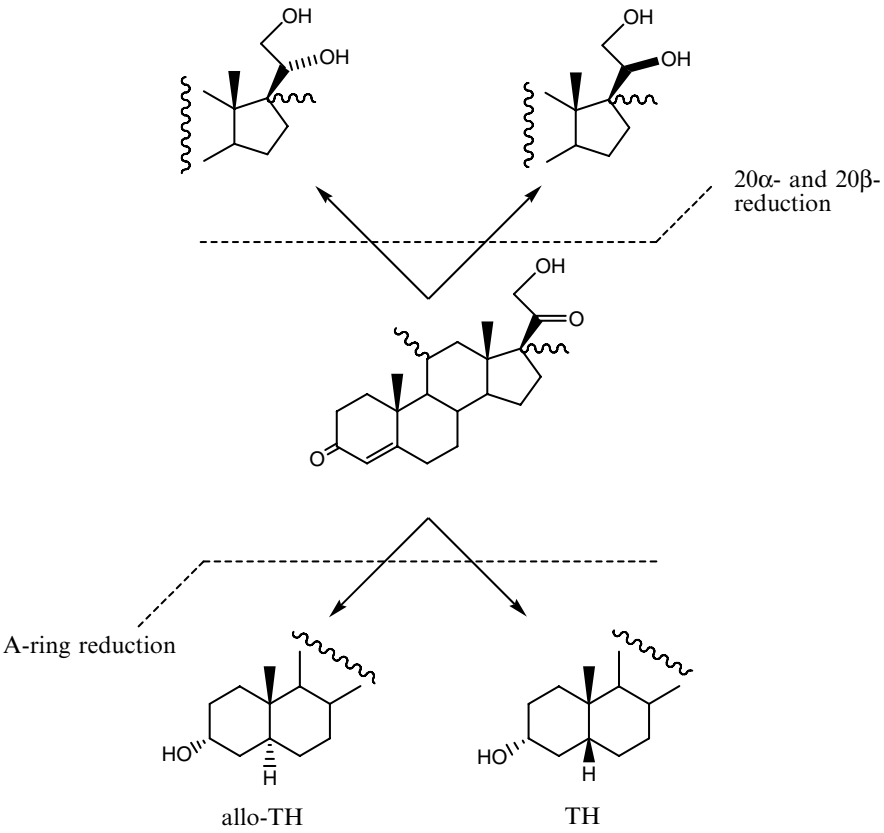

Fig. 5.3 Major catabolic products of corticosteroids (A-ring tetrahydro-reduction and 20-oxo reduction – both can occur separately and together)

Further reduction of the 20-oxo groups in the side-chain of the corticosteroids occurs, catalysed by the two enzymes 20α- and 20β-hydroxysteroid dehydrogenases (20-HSDs), producing two isomeric hydroxyl groups at C-20. In general, the 20α-isomer marginally predominates over the 20β-isomer in humans. The fully reduced derivatives of cortisol and cortisone are referred to as cortols and cortolones, respectively. The catabolism of most other corticosteroids including aldosterone occurs by the reductive methods described above, giving rise to the tetrahydro-derivatives. Corticosterone is partially converted to the 11-dehydro-derivative through the action of the 11β-hydroxysteroid dehydrogenase type 2, as described above for cortisol (see Section 5.3.3). Further hydroxylation of cortisol may occur at C-1, C-6β or, possibly, at C-16α, to give out extremely polar metabolites that are sufficiently water-soluble to be excreted in the urine unchanged, without the need for prior conjugation. 6-Hydroxylation through CYP3A4 is inducible by a number of drugs. When the

hepatic capacity for deactivation or conjugation becomes overloaded or impaired, the formation of these polar metabolites may assume more quantitative importance.

In addition to the reductive reactions described above, oxidative side-chain cleavage of 17-hydroxylated C_{21} steroids also occurs, resulting in the formation of 11-oxygenated C_{19} 17-oxosteroids. Approximately 10% of the secreted cortisol, for example, is metabolised by this method to produce four major products: 11β-hydroxyandrosterone (5α-reduced) and 11β-hydroxyaetiocholanolone (5β-reduced) and the two corresponding 11-oxo compounds. The two 11β-hydroxy compounds can also be formed via minor pathways which involve side-chain removal prior to reduction.

As many of the corticosteroid metabolites were being isolated from human urine and characterised, it was realised that these only accounted for some 80% of the total excreted steroids. In the case of the catabolism of cortisol, the unidentified material was found to be very polar in character and it was not possible to extract it with organic solvents from neutral or alkaline solution. After extensive investigations, the possibility that it might consist of conjugated material was ruled out. Instead, it was thought more probable that the polar compounds were carboxylic acids (Monder and Bradlow, 1980), formed by oxidation of the C_{21} hydroxyl group, followed by an intermolecular process. Lending credence to this mechanism is the finding of 21-hydroxysteroid dehydrogenases in the livers of humans and many other species.

Further details and references related to the topics discussed in the above sections are given by Gower (1984b) and by Andrew (2001) (in which corticosteroid metabolism is discussed in a clinical context).

Steroid metabolites are conjugated with glucuronic acid and sulphuric acid prior to excretion in the urine. Steroid analytical procedures usually involve conjugate hydrolysis using appropriate enzymes but modern mass spectrometric methods offer the opportunity of analysing intact steroid conjugates (Gomes et al., 2009). The major metabolites of corticosteroids are summarised in Table 5.1.

Table 5.1 Urinary excretion of corticosteroids and some of their principal metabolites (these data were published by Gower, 1984a, from Blackwell, with permission)

Secreted steroid	Principal urinary steroids	Principal conjugate present
Cortisol and cortisone	Cortisol	Unconjugated
	Tetrahydrocortisol and cortisone; cortols and cortolones; 11-oxy-17-oxo steroids, e.g. 11-OH-androsterone	G G,S
Corticosterone	Corticosterone	Unconjugated
	Tetrhydro derivative[a]	G
DOC	TetrahydroDOC[a]	G
11-Deoxycortisol	Tetrahydrodeoxycortisol	G
21-Deoxycortisol	3α-,17-Dihydroxy-5β-pregnan-20-one[a]	G
Aldosterone	Tetrahydroderivative	G
6β-Hydroxycortisol	6β-hydroxycortisol[a]	Unconjugated

Abbreviations: OS, oxosteroid; G, glucuronide; S, sulphate.
[a] Unique metabolite.

A major ‘catabolic’ process occurs through the interconversion of cortisol and 11-dehydrocortisol (cortisone) through the action of two 11β-hydroxysteroid dehydrogenase isoforms (Tomlinson 2005). 11β-HSD1 is expressed in numerous glucocorticoid target tissues, especially in the liver and in fat and mainly acts as a reductase generating active cortisol from inactive cortisone. In contrast, 11β-HSD2, is expressed in mineralocorticoid tissues (kidney, colon) and through rapid inactivation of cortisol to cortisone, serves to protect the occupancy of the MR by glucocorticoid as detailed above. This is reviewed by Draper and Stewart (2005) and is illustrated in Fig. 5.4. Deficiency states associated with 11β-HSD1, e.g. cortisone reductase deficiency, and with 11β-HSD2, e.g. the syndrome of apparent mineralocorticoid excess, are diagnosed from characteristic urinary steroid profiles (see Section 5.9.1) (Tomlinson et al., 2004).

In a series of detailed experiments, Diederich et al. (2002) have studied oxido-reduction reactions, catalysed by the two isoenzymes, with a large variety of synthetic corticosteroids as substrates, showing that the structural modifications of such steroids can have profound effects. For example, the oxidative action of 11β-HSD2 is diminished if the steroid substrate used is fluorinated at C-6, or C-9 (as in dexamethasone), or methylated at C-2, or C-6 (as in methylprednisolone). In the case of 11β-HSD1, the reductive action is diminished by the steric effects of methylation at C-2, 16α and 16β and by chlorination and fluorination at C-2 or C9. This could explain why 9α-fluorocortisol has mineralocorticoid activity. Further studies show that, in contrast, reductive effects of the 11β-HSD1 isoenzyme are increased in steroids that have a C1–2 double bond.

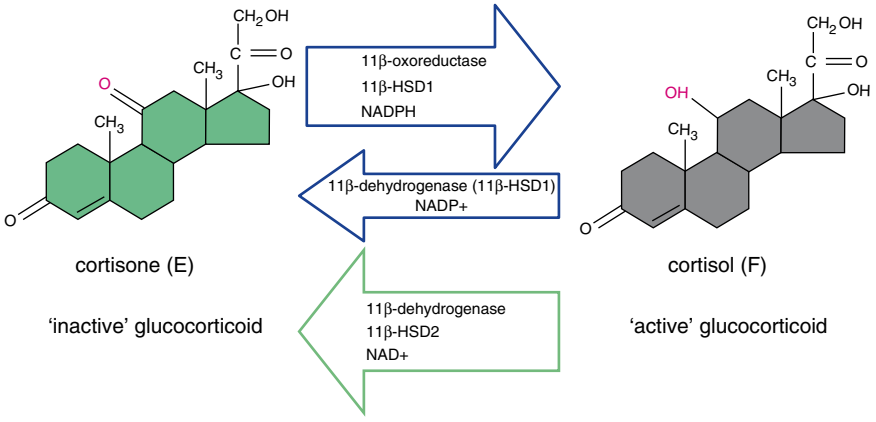

Fig. 5.4 The enzymatic actions of 11β-hydroxysteroid dehydrogenase on its substrates – interconverting inactive and active glucocorticoid (from Draper and Stewart, 2005, with permission; copyright Society for Endocrinology)

5.6 Analysis

5.6.1 Introduction

The close structural similarity of the corticosteroids produces particular analytical problems, and to devise a satisfactory analytical method it is necessary to employ a selective and specific end-point measurement, to incorporate an effective separation technique, or both. Since concentrations of steroids in biological fluids tend to be nanomolar (see Table 5.2), sensitivity is another important consideration. The measurement of steroids in body tissues is complicated by the problems of relatively lower concentrations than in blood, and also because of the large amount of lipid material present which interferes with the assay procedure. Prior separation by chromatography is therefore an essential step. Cortisol and other steroids have been assayed in human breast tumour tissue, for example, by GC-MS (Millington, 1975). Further difficulties must be surmounted in creating reliable individual assays for corticosteroids in body fluids and tissues because many steroids with similar physical and chemical properties are present and individual assays require a high degree of specificity.

In clinical practice it is necessary to determine the plasma concentration of intermediates in corticosteroid biosynthesis when there is suspicion of a metabolic disorder. The substrate for the defective enzyme will accumulate and plasma concentrations will be grossly elevated. For many of the analytes to be described, there may be relatively few laboratories performing the assays; therefore, comparative data (quality assurance schemes) using different methodologies will not be available, and any one method will have to be judged from the validation data provided. Important criteria will therefore be cross-reactivity information (note being taken of how this has been determined), recovery and dilution tests for specificity and accuracy. Even for the most commonly determined steroids there will be quite high interassay variations (>10%), but this performance should be judged along with the variation within the assay, precision, working range and sensitivity (the method of calculation again being noted).

A common adrenocortical disorder is congenital adrenal hyperplasia due to steroid 21-hydroxylase deficiency, the ‘classical’ carrier frequency in Western countries being 1 in 50–60 individuals (reviewed by Riepe and Sippell, 2007). There are about 1 in 10,000 affected live births in Western societies, many of which are detected by national screening programmes. Diagnostic strategies for the investigation of steroid metabolic defects will depend on the clinical problem being presented and the likelihood of addressing the problem by measurements of single hormones or some global approach based on non-selective determination of steroids, which will provide evidence for the site of enzyme deficiency (such as urine steroid profile analysis). Certain assays that will benefit the investigation of the rarer metabolic defects may only be practical in special centres. In many cases an early diagnosis is required, but the blood of newborn infants contains many steroids from the fetal adrenal zone that interfere in steroid assays, so caution is needed to interpret results at this time of life.

Table 5.2 Corticosteroid concentrations (nmol/L unless otherwise indicated) in various body fluids/tissues. These are merely published values and their inclusion in this table does not indicate that the values quoted are 'correct'

Steroid	Serum/plasma blood	Saliva	Urine (nmol/24 h)	References
Cortisol			28–117	Schoneshofer et al. (1980)
			63–297	Wiegand et al. (2007)
	138–331			Kasuya et al. (2002)
	58–621		7–127	Huang et al. (2007)
	143–423			Janzen et al. (2008)
	139–500	2.5–18		Cardoso et al. (2007)
		11–19.5		Nater et al. (2008)
		3–55 (2–15 years)		Groschl et al. (2003)
		4–14		Jerjes et al. (2005)
	(18–153 ng/g in hair)			Sauve et al. (2007)
			1–117	Hu et al. (2005)
			116 (mean)	Remer et al. (2007)
			130–194 (mean)	De Jongh et al. (2007)
Cortisone	27–97			Kasuya et al. (2002)
		10–40		Jerjes et al. (2005)
			225 (mean)	Remer et al. (2007)
Aldosterone	0.138–0.500	0.02–0.07		Cardoso et al. (2007)
Corticosterone	1.9–8.1			Janzen et al. (2008)
Deoxycorticosterone	0.7–1.9			Janzen et al. (2008)
17-OHprogesterone		0.006–0.225 (2–15y)		Groschl et al. (2003)
	3.6–6.5			Janzen et al. (2008)
	0–11.5	0–0.471		Mylonas et al. (2006)
	0.76–4.20			Kushnir et al. (2006)
17-OHpregnenolone	1.08–12.3			Kushnir et al. (2006)
11-deoxycortisol	2.5–8.5			Janzen et al. (2008)
	<1.5			Kushnir et al. (2006)
21-deoxycortisol	0–0.7			Janzen et al. (2008)
6β-OHcortisol			106–343	Tushar et al. (2007)
			55–267	Hu et al. (2005)

Apart from the individual analytical problems that will be discussed later, it is essential for any interpretation of the results obtained from steroid investigations to bear in mind that the production of many of the adrenal steroids is an episodic process, determined by circadian rhythms – steroid concentrations in plasma will vary depending upon the time of day. In addition, since many of the steroids to be discussed are sensitive to control by corticotrophin (ACTH), factors that cause the release of this hormone, notably stress, will also markedly influence the level of circulating steroids. In some circumstances, it may therefore be preferable for the analyst to employ a 24 h urine collection, which will better reflect the overall steroid production through this period. For the same reason, many of the clinically important tests of adrenal function depend upon a demonstration of a reserve capacity, as shown by the effect of stimulation (stress tests, ACTH tests) or suppression (dexamethasone suppression tests). These, and other aspects of adrenocortical physiology and pathology, have been reviewed extensively elsewhere (James, 1992). Aldosterone production is also episodic, but it is regulated mainly through a separate control mechanism (the renin angiotensin system) that is sensitive to the blood and kidney concentrations of electrolytes.

5.6.2 Extraction

Corticosteroids, like all other steroids, are relatively hydrophobic and circulate bound to specific binding globulins (cortisol binding globulin, transcortin) or albumin. Corticosteroids however usually contain more hydroxyl groups and are thus more hydrophilic than other steroids. In order to extract them from plasma more polar solvents are usually required. Solvent extraction has been widely used in the past for both plasma and urine and for the extraction of corticosteroids from *in vitro* incubation media. Increasingly, because of the problems with flammability of some extraction solvents, the health hazards of others and the need to evaporate relatively large solvent volumes, solid-phase extraction (SPE) methods are now widely used. This SPE methodology can also be incorporated into automated analytical sytems involving LC- and GC-MS (e.g. Rauh et al., 2006). Simple silica-based RP18 cartridges (Sep-Pak) were initially introduced for this purpose by Shackleton and Whitney (1980) but, while still widely used, are being replaced by 'Clean-screen DAU' co-polymeric SPE columns (e.g. Etter et al., 2006) or Oasis-HLB cartridges that can be incorporated as part of online analysis (e.g. Rauh et al., 2006).

Although SPE is widely used for preliminary steroid extraction, solvent extraction may on occasions have advantages. 17-OH-progesterone and cortisol can be effectively extracted from plasma using 10% hexane in diethylether. The neutral lipids, which reduce ionisation in ESI-MS/MS, are removed in an analysis as the Girard derivatives, simply by washing the derivative dissolved in the infusion solvent (MeCN/water) with hexane (Johnson, 2005). A liquid extraction with cyclohexane followed by an aqueous wash (Leunissen and Thjissen, 1978)

can, for example, be very useful for separation/purification of trimethysilyl ether derivatives of corticosteroids which have been formed to protect the steroid side-chain prior to GC, which are usually purified using Lipidex minicolumns (Shackleton, 1985).

Table 5.3 lists some common methods of corticosteroid extraction since 2000. While solvent extraction is still used today, increasing numbers of published methods use solid phase extraction. Sample preparation prior to analysis by LC/MS/MS can be simplified, removing proteins (e.g. by precipitation) which can block LC columns, and phospholipids that cause ion suppression in the LC-MS. Tissues require homogenisation and further clean-up of extracts to remove lipids-solvents and cartridge technology have been employed. Further purification (see below) can often be incorporated online into the LC system.

5.6.3 Purification

A reasonable degree of specificity can be achieved by preliminary steroid extraction, followed by chromatographic separation of the steroid by a thin-layer (TLC), paper (PC), Celite, or high-performance liquid (LC) chromatography. The end-point measurement is usually by ultraviolet (UV) absorption or by radioimmunoassay (RIA). TLC, PC, and Celite chromatography are not methods that lend themselves easily to repetitive assay. All these separatory procedures have been used in order to improve specificity of subsequent immunoassay methods. Morineau et al. (1997) undertook an investigation of various chromatographic pre-immunoassay procedures to improve specificity – using in-house packed Celite and Sephadex LH-20 mini-column chromatography as well as pre-packed silica or silica coated with a variety of functional groups obtained from commercial suppliers (Amersham, Waters-Millipore & Baker Corp). Six commercial RIA kits for serum and urine cortisol assay were examined. GC-MS and LC methods were used for reference purposes. None of the six immunoassays used without extraction/separation procedures gave values comparable to those obtained by LC or GC-MS. However, although several methods of employing LC had been reported, they were not simple or entirely technically satisfactory, and extraction problems and poor separation commonly occured. However the ability to couple LC and mass spectrometry has revolutionised the use of this methodology which is now the method of choice for many applications, where robust methods of immunoassay are not available.

Caution in the use of simple kit immunoassays for plasma corticosteroids is essential when applied to patients, or indeed to species other than human. Interference is still an unsolved problem and it is not justifiable to depend totally upon the specificity of the antiserum employed. Special care is needed in interpretation of results when patients are receiving medication (e.g. prednisolone), in cases with possible adrenal enzyme disorders, or in neonates.

Table 5.3 Some corticosteroid extraction methods used in period 2000–2007

Steroid(s)	Matrix	Extraction	References
F	Urine	Protein pption with TCA – centrifuge and use supernatant	Wear et al. (2007)
F	Saliva	Online SPME using Supel-Q PLOT column	Kataoka et al. (2007)
B	Rat plasma	Protein pption with phosphoric acid; centrifuge; supernatant on to OASIS-HLB, wash elute with MeOH	Samtani and Jusko (2007)
E, F and 17 OHprog + other steroids	Fetal cord blood	SPE after de-proteinisation with MeOH	Clifton et al. (2007)
F	Plasma and urine	Ethyl acetate	Huang et al. (2007)
F	Urine	CH_2Cl_2 before IA	De Jongh et al. (2007)
E and F	Urine	Sep-Pak Plus C8 eluted with CH_2Cl_2/EtAc (4:1)	Sharef et al. (2007)
THB	Rat brain	Homogenise tissue in MeOH/Ac acid (100:1) ×2 on to Strata-X cartridges – wash and elute with EtAc/Hx (7:3)	Higashi et al. (2007a)
17-OH-prog.	Plasma, serum, saliva	Protein ppted with MeOH/$ZnSO_4$	Rauh et al. (2006)
		Centrifuge; OASIS-HLB online	Groschl and Rauh (2006)
12 steroids including Aldosterone, F, B, DOC, S, 17-OHprog	Serum	Acetonitrile; centrifuge and use supernatant	Guo et al. (2006)
			Holst et al. (2007)
Free THE and THF	Urine	SPE – Bakerbond C18; elute with ethyl acetate	Turpeinen et al. (2006)
Pregn., 17-OHpregn., 17-OHprog and S	Serum/plasma	Diluted with H_2O and added to STRAT-X columns and eluted with methyl-*t*-butyl ether	Kushnir et al. (2006)
THE, THF and allo-THF	Urine	β-Glucuronidase, dilute with H_2O	Raffaeli et al. (2006)
F and 6β-OH-F	Urine and plasma	OASIS-HLB eluting with ethyl acetate/diethyl ether (4:1); wash with NaOH and acid	Hu et al. (2005)
17-OHprog.	Serum	Diethyl ether/ethyl acetate (7:3)	Turpeinen et al. (2005)
F and 6β-OH-F	Urine	Online SPE, using Prospect-2TM apparatus using Hysphere C18-HD 7 μm	Barrett et al. (2005)
F	Plasma	Extract with diethyl ether/Hx (9:1) – form Girard T derivatives	Johnson (2005)
Aldosterone, B, DOC and F	Adrenal ZG cells	Ethyl acetate ×2	Nithipatikom et al. (2005)
F	Urine	C8 isolute SPE, washed with borate buffer and 20% acetone in H_2O; elute with EtAc	McCann et al. (2005)

E and F	Human adipose tissue; mouse liver and adipose tissue	Homogenise in MeOH/H_2O (7:2); centrifuge; use supernatant	Ronquist-Nii and Edlund (2005)
B and 11-DH-B			
F	Urine	Methyl-*t*-butyl ether (+3% phosphoric acid)	Kushnir et al. (2004)
17-OHprog and F	Blood spots	3/16″ disk extracted with diethyl ether ×2	Minutti et al. (2004) Lacey et al. (2004)
F	Serum	Acidify to pH 1.5 with phosphoric acid; Sep-Pak C18 – wash with water, MeCN/water (2:8); elute with MeOH; evap. to dryness, dissolve in EtAc	Tai and Welch (2004)
F	Serum	CH_2Cl_2 extraction + centrifugation	Jung et al. (2004)
18-OH-E, 18-oxo-F, E and F	Urine	Sep-Pak C18 Shackleton (1983)	Freel et al. (2004)
E and F	Hair	Wash with CH_2Cl_2; 2 cm from root; pulverise in ball mill; powder incubated in Soerensen buffer for 16 h at 40°C; isolute C18 SPE; washed, eluted with MeOH; evaporated to dryness and re-dissolved in 0.2 N NaOH; extracted with EtAc	Raul et al. (2004)
F and 6β-OH-F	Urine	Sep-Pak C18 – elute with EtAc/diethyl ether (4:1); wash with NaOH, HCl	Furuta et al. (2004)
B and F	Plasma	Ethyl acetate	Chen et al. (2004b)
THE, THF, E and F	Urine	SPE on Bakerbond C18; elute with MeOH	Pujos et al. (2004)
11-deoxy-F and 21-deoxy-F	Blood	Extract (×2) with iso-octane/EtAc (1:1)	Cristoni et al. (2004a, b)
B	Plasma	Ethyl acetate	Shu et al. (2003)
F and B	Parrotfish feces	Methylene chloride	Turner et al. (2003)
F	Urine	Methylene chloride; wash with NaOH, HCl, H_2O. Evaporated to dryness; take up in MeOH/H_2O (4:6)	Turpeinen and Stenman (2003)
F and 6β-OH-F	Urine	Ethyl acetate/IPA (85:5) ×2; wash with NaOH	Rouits et al. (2003)
18-OH-5β-THDOC	Urine	Sep-Pak C18 Shackleton (1985)	Shakerdi et al. (2003)
E and F	Urine	Methylene dichloride	Taylor et al. (2002)
E and DOC	Equine urine	β-Glucuronidase hydrolysis and extraction with ethyl acetate	Tang et al. (2001)
F and 6β-OH-F	Urine	Ethyl acetate	Tang et al. (2000)

Abbreviations: B, corticosterone; E, cortisone; F, cortisol; S, 11-deoxycortisol; 11-DH-B, 11-dehydro-corticosterone; TH and allo-TH, tetrahydro (5α [allo-] and 5β A ring reduction; TCA, trichloracetic acid; MeOH, methanol; IA, immunoassay; Hx, n-hexane; MeCN, methyl cyanide (acetonitrile); SP(M)E, solid-phase (micro) extraction.

5.6.4 Quantitation

A variety of different methods is available which can be used to determine the concentration of the particular corticosteroid of interest. The method chosen for a particular application must have regard to its specificity and for the degree of purification of the analyte prior to quantitative assay. The overall degree of specificity required must also take into account the use to which the assay will be put and the need for rapid results in diagnostic situations.

5.6.4.1 Immunoassay

Immunoassay of steroids has been reviewed earlier in this book (see Chapter 4) and the application of this methodology to corticosteroids poses the same problems as its application to any other steroid. Immunoassays have for many years replaced the early methods for measuring steroids in plasma based upon the use of fluorimetric or colorimetric end points; for cortisol these were extensively and critically reviewed by Braunsberg and James (1961). The chemical assays had considerable practical value, but they had several shortcomings, lacking specificity and requiring large volumes of plasma. In the case of fluorimetry, they required the use of sulphuric acid, an unpleasant and potentially dangerous reagent. While immunoassays have largely replaced these early but occasionally still useful methodologies, the clinical biochemist must not uncritically rely on the output from commercial or other IA kits, automated or not and must remain vigilant for unusual results (Jones and Honour 2006). The recognition of the use of antibodies for the analysis of steroids and the availability of tracer-labelled steroids made possible the development of protein-binding or immunoassay methods, with their greatly enhanced sensitivity. Immunoassays are now extensively used, both for research and clinical purposes. In general polyclonal antibodies are used. Few monoclonal antibodies for steroids have been reported, though one recent such antibody was used for cortisol assay in blood and urine (Kobayashi et al., 2002). One general difficulty with RIA today is the decreasing availability of radioactive-isotope labelled standards as more and more compounds are deleted from commercial catalogues. The investment of time required to synthesise high specific activity labelled ^{3}H- or ^{14}C-corticosteroids or the cost of custom synthesis may be a major deterrent in setting up these methods on a small laboratory scale. For many assays, automated techniques dominate the methods used today. Time-resolved fluorescence in the Delfia sysytem has been adapted for a number of steroids, e.g. 18-hydroxycortisol (Reynolds et al., 2005), 17-hydroxyprogesterone (Boudi et al., 2000) and 21-deoxycortisol (Fiet et al., 2000).

A number of procedures have been used to separate the bound and free components at the end of an immunoassay (El-Gamal and Landon, 1988). The assays that use tritiated steroid have used dextran-coated charcoal (DCC) to adsorb the free fraction (Abraham et al., 1971; Janne et al., 1974; Youssefnejadian and David, 1975; Walker et al., 1979) but other separation techniques have been employed, including ammonium sulphate and second antibody precipitation (Wicking, 1975; Butkus et al., 1985),

and extraction of the free steroid into scintillant (Tea et al., 1975). DCC separation is simple and cheap but may be time and temperature-dependent and requires centrifugation, although this can be avoided by coupling the antibodies to a magnetisable solid phase or to the walls of the assay tubes. Antibodies have been immobilised into microcapsules (Wallace and Wood, 1984; Kobayashi et al., 2003) or directly coupled to magnetisable particles (Dyas et al., 1984). In some assays, antibodies are adsorbed indirectly via a second antibody to the assay tubes (Hofman et al., 1985) and many antibody-coated tube assays are available. The liquid is removed from the tubes by aspiration or decantation making sure that all the liquid is removed.

There is a general tendency to switch to the use of ^{125}I-labelled steroids. This is not without problems, particularly when measuring low levels of steroid, because in many cases the antisera are raised against a conjugate of the steroid coupled via a bridge to a protein such as bovine serum albumin (steroid-3-carboxymethyloxime 3-CMO). In order to iodinate most steroids, a derivative of the steroid, e.g. histamine, is used as the site for iodination. In many cases this derivative is homologous with the bridge used in the hapten, and the antiserum shows higher affinity for the label than for the natural steroid that is to be measured in both the standard (standard curve) and the samples. The timing of the incubation between antiserum, label and steroid has therefore to be carefully studied if bias at low level is to be avoided (Honour and Rumsby, 1993). The label has a short half-life and is easier to dispose of than the beta radiation emitting isotopes. Although it is still possible to obtain suitable reagents and set up in-house methods, and in some cases it may pay to do so, commercial kits, which are highly convenient to use, are widely available, provide all the necessary reagents together with a recommended methodology and have therefore largely replaced other techniques.

The chemistry behind manufacture of antibodies and labels continues to evolve (Kohl and Lejeune, 2002). Histamine was a common bridge for steroid to albumin in haptens and for radiolabelling. In enzyme immunoassay adipic acid dihydrazide has been tested as a spacer with reduction in cross reactivities (Basu et al., 2003, 2006; Shrivastav, 2003, 2004) although this information is not disclosed in commercial kit inserts. Reactions in solution have usually been used but solid-phase labeling of 17-hydroxprogesterone was used for a fluorescent label (Peuralahti et al., 2004).

Automated immunoassay analysers are now widely used in clinical laboratories. Enzyme, chemiluminescent and fluorescent labels have replaced radioactivity for common automated platforms that have cortisol assays. These systems have more sensitivity than is required for analysis of corticosteroids. Reagents are kept cold on the analyser and are stable on the instrument (or in the refrigerator) for long periods of time thus maintaining a level of stability not possible with radioactive reagents. Some systems are compatible with general laboratory automation so the workload can permit the processing of 200 samples in 1 h. Assays benefit from short incubation time (10 min typically), smaller sample volumes and a high level of precision. A rapid cortisol assay has been used to monitor adrenal venous sampling during the catheterisation procedure to locate an aldosterone secreting tumour

(Mengozzi et al., 2007). A dipstick test for cortisol gives results from whole blood in 15 min (Leung et al., 2003). Cortisol assays on many systems have been compared by Roberts and Roberts (2004) and Pujos et al. (2005). The workloads for other corticosteroid assays are much less than for cortisol. 17-Hydroxprogesterone has been automated on the Delfia system because of its application for the screening of all newborn babies in some countries. A Nicholls Advantage system was designed for low volume esoteric analyses (including aldosterone) but this system has been withdrawn as the company no longer exists. Other instruments in development may be able to replace that analyser. Some companies produce large and small instruments to meet different user requirements but results are not always comparable for practical and technical reasons (volumes of bulk reagents, time of storage on the instrument, stability and mixing of ingredients, etc.). Validity of measurements is becoming more of an issue for the industry. Current EQA schemes demonstrate comparability of results rather than accuracy and this approach may need to change.

Automated methods for blood samples can be adapted for different biological fluids like urine (Gray et al., 2003; McCann et al., 2005; Ching et al., 2006) and saliva (Chiu et al., 2003; Vogeser et al., 2006). In some animal studies steroids have been extracted from faeces (Mateo and Cavigelli, 2005) prior to automated analysis. Such adaptations need to be carefully validated.

All immunoassays can be affected by non-specific interference. This includes haemolysis (Snyder et al., 2004), sample collection in vacuum tubes (Morovat et al., 2006), heterophilic antibodies (Bolland et al., 2005) and albumin concentration in the plasma (Barnes and Swaminathan, 2007). Interference can be documented by nonlinear responses with serial dilution, pre-treatment of samples with heterophile blocking reagent and positive reaction on a mouse-antibody negative control reaction. This is not warranted for all samples but can be implemented for cases in which clinical history or suspicious results indicate a need (Emerson et al., 2003). In early immunoassays polyclonal antisera were raised in rabbits or sheep, second antibodies for precipitation of the bound component of the assay were often goat or donkey sera. Monoclonal antibodies are usually murine and people in contact with mice can have significant antibody titre to mouse proteins. Blood samples from such patients can react unexpectedly in immunoassays.

The use of automated assays is not without its dangers. Although they are often well-designed and standardised and give good results (see Chapter 13), if they are used uncritically, especially when employed without appropriate evaluation for research or with plasma from patients with certain types of disease, they can produce misleading results. For example, problems may arise when methods that have been designed and evaluated for use with human plasma are employed to determine steroid concentration directly in animal plasma or in tissue extracts. In both cases, extraneous materials in the matrix may cross-react and cause interference. Similarly, in some adrenocortical disorders, unusual steroids are produced in large amounts and these compounds may cause interference in immunological methods by cross-reacting. In all these cases, it is advisable first to consult the manufacturer for advice, to discover whether any relevant information is already available and then, if necessary, to introduce into the analytical method an extraction and purification procedure.

Experience may subsequently show that such additional steps are superfluous but this assumption should not be made without the relevant data to support it. Corticosteroids are widely used as therapeutic agents and can affect assay performance of patients' samples. Variablity in results from different assay systems can hinder clinical decisions (Cohen et al., 2006).

Some recent immunoassays used for corticosteroid assay are summarised in Table 5.4, which also includes contact data for firms supplying commercial simple IAs and high-throughput automated systems.

5.6.4.2 LC- and GC-MS

There are a number of publications that describe routine chromatography (high performance liquid chromatography (LC), Celite and Sephadex LH-20) (Anderson et al., 1976; Sippell et al., 1978; Schoneshofer et al., 1981; Makin and Heftmann, 1988; Szecsi et al., 2006) of steroids extracted from serum samples prior to individual quantification by RIA performed on eluted fractions. This has some benefits in reducing sample size but is very tedious. Profiling of plasma steroids by chromatography coupled with radioimmunoassay (Wei et al., 1990) or mass spectrometry (Wudy et al., 1992; Guo et al., 2006) has diagnostic advantages. The collection of many fractions of the eluates after LC separation and testing for 'immunoactivity' is a useful validation of the quality of antisera (Schoneshofer et al., 1981; Fisher et al., 1986). When immunoreaction is detected in fractions outside the elution position of the required analyte, there is evidence for lack of specificity (Fig. 5.5). In some cases the 'immunoreactive' material can be isolated for further analysis to confirm the nature of the interfering substance(s) (Wong et al., 1992; Horie et al., 2007).

If absolute specificity is required (e.g. for a reference method), then chromatography combined with mass spectrometry is probably the best method currently available, and several techniques have been described for cortisol using both radiolabelled and stable-isotope dilution methods in GC-MS (Siekmann, 1979; Gaskell et al., 1983; Patterson et al., 1984; Esteban et al., 1988) amd LC-MS-MS (Jung et al., 2004). The technique can also be used for the determination of cortisone alone in human plasma (Shibasaki et al., 1992) or in combination with cortisol (Makin et al., 2002; Taylor et al., 2002; Kushnir et al., 2004). These methods require expensive equipment but the specific technical skills and experience required of operators is now less than used to be the case. Use of MS is no longer limited to research investigations and to calibrating samples for use as target values in quality control applications. The equipment can be leased and since most methods need low expenditure on reagents, the overall cost per sample can be less than using IA, making the technique more widely applicable. The general principles of mass spectrometry when coupled to GC or LC separation systems are considered in Chapters 2 and 3. Soft ionisation systems employed in LC-MS systems necessarily rely to a far greater extent on the pre-MS separation to obtain satisfactory specificity than do GC-MS systems, which employ electron impact (EI) ionisation, producing specific fragments of the molecule.

Table 5.4 Some immunoassays used for the measurement of corticosteroid concentration in body fluids/tissues

Analyte	Immunoassay	Source[a]	References
Plasma 11-deoxycortisol	RIA	In-house	Fiet et al. (1994b)
Plasma 21-, 11-deoxycortisols and DOC	RIA after LC		Hill et al. (1995)
17-OHprog	RIAs – comparison of three commercial kits	2 DSL kits (Dab and coated tube – Webster, Texas and COAT-A-COUNT (SIEMENS)	Castracane and Gimpel (1997)
Urine 6β-OHcortisol	ELISA	In-house monoclonal antibody	Yeung et al. (1997)
Urine 6β-OHcortisol	EIA RIA	Three of each from US commercial sources – not specified	Lee and Goeger (1998)
Blood spot cortisol	RIA	SIEMENS-Los Angeles	Mitchell and Hermos (1998)
Blood and urine aldosterone	RIA	DPC kit	Pratt et al. (1999)
Plasma DOC and cortisol	RIA	In-house	
Urine free cortisol	Chemiluminescent IA compared to RIA	ACS:180 (Siemens) In-house	Kennedy et al. (2000)
Serum 21-deoxycortisol	TR-FIA	In-house	Fiet et al. (2000) Boudi et al. (2000)
Serum cortisol	ELISA–peroxidase		Basu and Shrivastav (2000)
Serum 11-deoxycortisol	RIA and TR-FIA	In-house (Fiet et al., 1994a)	Fiet et al. (2001a)
Plasma/serum 17-OHpregnenolone	TR-FIA	In-house	Fiet et al. (2001b)
17-OHprog	FIA	DELPHIA	Nordenstrom et al. (2001)
Salivary F and 17-OHprog	RIA	DSL, Sinsheim	Groschl et al. (2001)
Serum 18-OHcortisol	ELISA	In-house	Mosso et al. (2001)
Urine and serum cortisol	ELISA after CH_2Cl_2 extraction	In-house – monoclonal antibody	Kobayashi et al. (2002)
Salivary cortisol	RIA and EIA – comparison	COAT-A-COUNT (SIEMENS) –RIA and EIA (DSL)	Raff et al. (2002)
Salivary cortisol	Bioluminescent IA	In-house	Mirasoli et al. (2002)

Salivary cortisol	EIA and RIA – comparison with in-house assay and LC-MS-MS	EIA (Orion Diagnostica) and RIA (Salimetrics) and In-house RIA	Jonsson et al. (2003); Raff et al. (2003)
Salivary cortisol	Automated electro-chemiluminescence IA compared to RIA and EIA	ELECSYS (Roche) COAT-A-COUNT (SIEMENS) Salimetrics-HS	Chiu et al. (2003); Van Aken et al. (2003)
Plasma 11-deoxycortisol	Non-competitive immunoenzymetric IA (IEMA)	In-house	Kobayashi et al. (2003, 2006)
Blood spot and venous 17-OHprog		COAT-A-COUNT Siemens AUTODELFIA Kits	Santos et al. (2003)
Plasma cortisol	Immuno-threshold based assay using dipstick	In-house	Leung et al. (2003)
Plasma 18-hydroxy-DOC and -B	RIA after extraction and LH-20 separation	In-house	Riepe et al. (2003)
Urinary TH-aldo, aldo-18-gluc and free aldosterone	RIA	In-house	Abdelhamid et al. (2003)
Urinary free cortisol	Chemiluminescent IA after extraction	ADVIA Centaur (Siemens)	Gray et al. (2003)
Saliva and bloodspot cortisol	RIA	SPECTRA CORTISOL RIA (Orion Diagnostica)	Wong et al. (2004)
Urinary and salivary cortisol	RIA	CORT-CT2 (CIS Biointernational, France	Yaneva et al. (2004)
Serum cortisol	Comparison of 5 automated competitive IAs	ACCESS (Beckman) ADVIA Centaur (Siemens) AxSYM (Abbott) ELECSYS-2010 (Roche) IMMULITE 2000 (SIEMENS)	Roberts and Roberts (2004)
Plasma aldosterone	Immunometric IA	ADVANTAGE (Nicholls)[a]	Perschel et al. (2004)
17-OHprog	RIA	Three commercial kits	Meier et al. (2004)
Urinary cortisol and 6β-OH-cortisol	RIA	DIASORIN	Chen et al. (2004b)

(continued)

Table 5.4 (continued)

Analyte	Immunoassay	Source[a]	References
	ELISA	STABILIGEN	
Urinary cortisol and 6β-OH-cortisol	RIA	DSL-2100 ACTIVE	El Desoky et al. (2005)
	ELISA	STABILIGEN	
Serum cortisol and 17-OHprog.	FPIA	ABBOTT TDX	Wichers-Rother et al. (2005)
	RIA	SIEMENS, Biermann	
Serum 17-OHprog.	RIAs (comparison with LC-MS/MS)	In-house and COAT-A-COUNT (SIEMENS)	Turpeinen et al. (2005)
Urinary free cortisol	Chemiluminescent IA –comparison with LC-MS/MS	Adapted ACS:180 (Siemens) – not widely used	McCann et al. (2005)
Plasma and urine aldosterone	RIAs	QUEST DIAGNOSTICS MAYO clinic	Nishizaka et al. (2005)
Salivary cortisol	TR-FIA	DELFIA	Hoferl et al. (2005)
Serum cortisol	ELISA	IDS, Ltd. (Bolton UK)	Butcher et al. (2005)
Fecal cortisol and corticosterone in primates	EIAs	In-house using commercial antibodies	Heistermann et al. (2006)
Serum 21- and 11-deoxycortisol and 17-OHprog.	RIA	In-house after LC	Tonetto-Fernandes et al. (2006)
Blood 17-OHprog.	ELISA		Peter et al. (2006)
Serum 17-OHprog	RIA (compare with LC-MS/MS)	COAT-A-COUNT (Siemens)	Etter et al. (2006)
Plasma, serum and salivary 17-OHprog	RIA (compare with LC-MS/MS)	RIA (DSL, Sinsheim)	Rauh et al. (2006)
Salivary 17-OHprog	RIA	IMMUNOTECH	Mylonas et al. (2006)
Serum aldosterone	Solid-phase RIA	BIODATA Diagnostics	Giacchetti et al. (2006)
Serum aldosterone	3 commercial assays (2× RIA and one chemiluminescent) and one RIA	ADALTIS RIA DSL ACTIVE Nicholls ADVANTAGE[a] In-house RIA	Schirpenbach et al. (2006)

Plasma aldosterone	Chemiluminescent IA compared to ×2 RIAs	Nicholls ADVANTAGE[a]	Pizzolo et al. (2006)
		SORIN RIA	
		MAIA ADALTIS RIA	
Urinary free cortisol	Chemiluminescent IA compared to LC	IMMULITE:2000	Ching et al. (2006)
Plasma cortisol	Comparison of 3 commercial kits with LC	IMMULITE 2000	Cohen et al. (2006)
		(SIEMENS)	
		Abbott TDX	
		ADVIA Centaur	
Serum and salivary cortisol	ELISA (saliva) Chemiluminescent IA (serum)	ACTIVE CORTISOL (DSL)	Poll et al. (2007)
		ADVIA Centaur	
Serum 17-OHpregnenolone SO_4	RIA	In-house Hill et al. (1999)	Vcelakova et al. (2007)
CSF and serum cortisol	Radio-immunometric assay	DSL-2000 kit	Holub et al. (2007)
Urine free cortisol	Comparison of four commercial kits	TFB (IMMUNOTECH)	Horie et al. (2007)
		DINAPACK (Abbott)	
		GAMMACOAT (Diasorin)	
		CHEMILUMI-ACS (Siemens)	
Serum cortisol	Comparison between two commercial kits	Roche ELECSYS	Barnes and Swaminathan (2007)
		ADVIA Centaur (Bayer)	
Plasma, bloodspot and salivary cortisol	Chemiluminescent IA and RIA (saliva and bloodspot)	IMMULITE:1000 (SIEMENS)	Maguire et al. (2007)
		SPECTRA CORTISOL RIA (Orion Diagnostica)	
Serum 11-dehydrocorticosterone	ELISA	In-house with and without extraction	Tagawa et al. (2007)
Serum and salivary cortisol	RIA	COAT-A-COUNT	Dorn et al. (2007)
	EIA	(SIEMENS)	
		Salimetrics EIA	

(continued)

Table 5.4 (continued)

Analyte	Immunoassay	Source[a]	References
Urine cortisol	RIA	COAT-A-COUNT (SIEMENS)	De Jongh et al. (2007)
Hair cortisol	ELISA	ALPCO Diagnostics	Yamada et al. (2007)
Salivary cortisol	ELISA	Salimetrics	Haussmann et al. (2007)
Rat serum Corticosterone	RIA	ImmuChem Double Antibody 125-I MP Biomedicals	Samtani and Jusko (2007)
Venous cortisol	EIA and Chemiluminescent IA	ST AIA-PACK CORT ARCHITECT (Abbott)	Mengozzi et al. (2007)
Salivary cortisol	EIA	Salimetrics	Harmon et al. (2007)
Salivary cortisol	RIA – comparison with LC-MS-MS	RIA sent to Esoterix Labs for assay no details on IA given. LC-MS/MS done at Mayo Clinic	Baid et al. (2007)
Hair cortisol			Sauve et al. (2007)
Hair cortisol	Saliva cortisol IA	Adapted for hair	Kalra et al. (2007)

Some of the IA systems referred to above can be used in commercial automated systems, the most popular of which, widely used for routine estimations of the most used assays (i.e. cortisol), are detailed below.

Instrument[a]	Principle	Label
Abbott Architect	Microparticle	Chemiluminescence
Siemens ADVIA Centaur	Paramagnetic particles	Chemiluminescence
Beckman-Coulter Access. Access 2, Dxl	Paramagnetic particles	Chemiluminescence
SIEMENS Immulite 2000/2500	Coated beads	Alkaline phosphatase
Roche Elecsys/E170 modular	Paramagnetic beads	Electrochemiluminescence
Tosoh A1A	Magnetic microbeads	Kinetic fluorescence
Wallac Delfia	Coated microtitre plates	Time resolved fluorescence

[a]Websites for the majority of commercial methods listed in this table are given below:

DSL (Diagnostic Systems Laboratories Inc)	www.dslabs.com
SIEMENS (Diagnostic Products Corp.)	http://diagnostics.siemens.com
Bayer AG	www.bayer.com
[Refer to Siemens for ex-Bayer products (Centaur)]	
DELFIA (Perkin-Elmer)	http://las.perkinelmer.com
Delphia (Wallac Oy, Finland)	See Perkin-Elmer
Orion Diagnostica	www.oriondiagnostica.com
Cisbio International	www.cisbiointernational.fr
Nicholls	In liquidation – no longer available
Abbott Diagnostics	www.abbottdiagnostics.com
Immunotech	www.immunotech.cz
Biodata Diagnostica	Not known
Diasorin Inc	www.diasorin.com
Sorin Biomedical Diagnostics	Not known
ADALTIS	www.adaltis.com
Siemens IMMULITE 2000/2500	http://diagnostics.siemens.com
ELECSYS /E170 modular (Roche)	www.roche.com
Salimetrics	www.salimetrics.com
Alpco	www.alpco.com
ST AIA-Pack	www.diagnostics.eu.tosohbioscience.com
STABILIGEN	www.stabiligen.com
MP Biomedicals	www.mpbio.com
Esoterix (contract laboratory)	www.esoterix.com
IDS, Ltd. (UK)	www.idsltd.com

Many of the commercial firms/suppliers quoted in the original publications listed above are no longer correct as many mergers and takeovers have occurred since 1994. The suppliers and their websites (where known) are correct as at January 2008. We are grateful to Dr. Jonathan Middle for updating the information in this table.

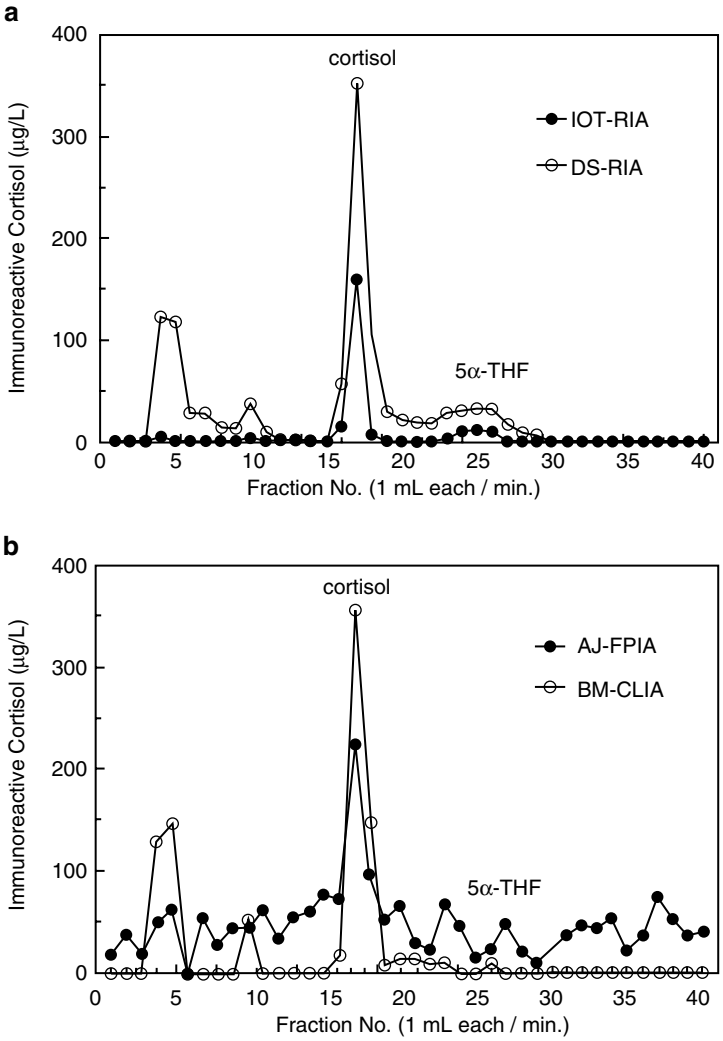

Fig. 5.5 Determination of immunoreactive cortisol concentration in each LC fraction of urine samples examined with (**a**) two commercially available RIA kits and with (**b**) FPIA kit and a CLIA kit (with permission from Horie et al., 2007; copyright Elsevier B.V)

GC-MS-calibrated samples are available both commercially and from the Commission of the European Community, Measuring and Testing Programme (M075 3/13), 200 Rue de la Loi, B-1049, Brussels, Belgium, formerly BCR (Bureau Communite de Reference). In quality-control schemes, these are used to determine 'target' values against which the results of other methods can be measured (e.g. Tai and Welch, 2004). This is obviously more relevant to specificity than the 'group' or 'consensus' mean reported in quality-control schemes,

which can only indicate how well an individual analyst is performing as compared to analysts in other laboratories using the same technique. GC-MS analysis is also used to 'calibrate' the standards supplied with some commercial kits. This technique provides a means of compensating for the various factors such as matrix interference to which all samples are subject to some extent or another. It cannot be perfect, since no two analytical samples will be identical in this respect. These materials have not been widely used.

It is important to compare the performance of individual methods with that of a reference method because adequate performance with normal samples does not guarantee similarly acceptable results with pathological or unusual (e.g. neonatal) samples or in samples from subjects receiving medication (e.g. fluticasone and/or prednisolone – Honour, 2000). Methods that use extensive purification procedures such as GC-MS and LC are excellent for achieving good specificity, but require longer processing times and are not suitable for batch analyses. Thus, when large numbers of assays are required, it may be necessary to use less sophisticated methods. These can give satisfactory results but may also require a purification stage prior to assay. The extent to which this is necessary will depend upon individual circumstances and requirements, and it can be a costly mistake to adopt a quicker method without first ascertaining that it is capable of providing the necessary degree of reliability and especially specificity. Even 'high-specificity' LC-MS/MS requires caution as it is not immune from interference (Meikle et al., 2003) – in this case fenofibrate in an assay for cortisol.

LC methods using UV monitoring of the eluate have now largely been replaced by MS or MS/MS methods. GC-MS is still widely used, primarily as a method of determining urinary steroid profiles (Palermo et al., 1996); Caulfield et al., 2002). By far the majority of modern methods use LC-MS/MS. A note of caution must be sounded here as LC-MS/MS does not always provide satisfactory information about analyte structure and the value of GC-MS with electron impact ionisation in this area should not be ignored. Vogeser and Parhofer (2007) have reviewed the application of LC-MS/MS in endocrinology.

5.7 Analysis of Glucocorticoids

The principal need to measure cortisol relates to human diseases characterised by the deficiency of adrenal steroid secretion (e.g. Addison's disease) or conversely overproduction of glucocorticoids (e.g. Cushing's syndrome). In both cases, reliable measures of cortisol concentration are required. The causes of these conditions and their consequences are beyond the scope of this chapter and are adequately reviewed elsewhere (Stewart, 2008). In this chapter we have, for the purposes of the discussion of analysis, divided the corticosteroids into the two chemical groups, glucocorticoids (17-hydroxylated) and mineralocorticoids, although we recognise that the structural division does not take account of the actual physiological function of the steroids in these two groups. In this first section therefore we deal with the

major glucocorticoids in humans, cortisol and then its metabolic precursors, the analysis of which is important in the diagnosis and characterisation of congenital hyperplasia (CAH). Concentrations of cortisol and other corticosteroids reported in various body fluids are summarized in Table 5.2.

5.7.1 Cortisol

Cortisol is quantitatively the major biologically important product of the adrenal cortex and is found in virtually all body tissues. The principal analytical applications have been in the study of biosynthetic and metabolic pathways and for the purposes of diagnosis in human and veterinary medicine. A large number of methods have been described for the quantitative measurement of cortisol in body fluids and the advent of sensitive and relatively specific techniques allows a laboratory economically to carry out large numbers of these investigations. Even so, there remain a number of problems that beset cortisol assays, and the ideal objective of a simple and reliable assay has still to be achieved.

The most widely used methods use immunoassay (IA), which is the assay most susceptible to automation. Table 5.4 lists some of the IAs available for cortisol and other important corticosteroids. There are several analytical options using the available IAs. Cortisol tracer labelled with tritium or radioactive iodine is added and reacted with antiserum, followed by the separation of bound and free fractions, using any of the standard separation techniques, such as dextran-coated charcoal, polyethylene glycol, solid-phase separation, etc. In order to measure the concentration of total cortisol in plasma or serum the method must include some mechanism to displace steroid from the binding proteins. For so-called 'direct' assays (those where extraction and/or purification is not carried out prior to assay), this can be with low pH, heating the sample, addition of a synthetic steroid and other methods, often not specified for commercial reasons. Enzyme immunoassay (EIA), luminescence and fluorescence assays have all been developed, and are available in commercial kits and automated platforms. Total cortisol is measured in plasma/serum, in saliva and in urine – the latter two assays purporting to be estimates of the 'unbound' or free cortisol. Saliva cortisol is complicated by the presence in salivary glands of 11βHSD2, which reduces the concentration of cortisol.

Plasma/Serum Cortisol Tunn et al. (1990) reported the results of a multicentre evaluation of an enzyme immunoassay for cortisol in plasma. They evaluated the Enzymun-Test cortisol method, comparing results from a number of laboratories in Germany. The within- and between-batch precision was adequate (less than 8% and 12%, respectively) although less satisfactory at concentrations lower than 100 nmol/L. With the exception of prednisolone, 6β-hydroxycortisol and 21-deoxycortisol, low interference from other steroids was found. The correlations between the results of this study and those obtained with methods using LC, luminescence, fluorescence and RIA were good, but absolute values often differed markedly. The correlation with cortisol measured by isotope dilution-mass spectrometry (IDMS) was impressive,

although it should be noted that the kit is itself 'calibrated' against IDMS and, as pointed out earler, this calibration technique is not ideal.

The methods for plasma cortisol that are in current clinical use in the UK are monitored extensively for their reliability through the National External Quality Assurance Scheme (UK NEQAS - see www.birminghamquality.org.uk, click on Chemistry). A retrospective study of the results of assays using 18 different commercial kits (Middle, 1992) showed at that time acceptable precision below the 10% level (see also Chapter 13). The data reveal that different methods show variable degrees of bias from the group laboratory mean. The kits that use GC-MS to assist with standardisation show, as would be expected, a negative bias to the group mean. The recoveries are highly variable, ranging from approximately 75% to 90%. The author concluded that there remains room for improvement in this parameter.

IA can give satisfactory results but some care is required in interpreting results on pathological sera. It is often the specificity that is at fault and especially in so-called direct assays where plasma is added directly to the reagents, there is the risk of both matrix interference and cross-reactions. Antisera raised against steroid-protein immunogens are rarely, if ever, totally selective for the steroid analyte, and in the case of cortisol, chemically similar corticosteroids, e.g. prednisolone and 11-deoxy-cortisol, invariably cross-react. A further problem arises in direct assays where the endogenous plasma proteins compete with the antibody for the antigen. The problem is diminished, although not always entirely overcome, by the addition of a blocking agent such as anilinonaphthalene sulphonic acid. The nature of the agent that is employed in most kits is not always disclosed by the manufacturer and it is not clear to what extent it is successful in preventing unwanted binding to protein.

Higher specificity demands more complex methods and there have been many changes over the years starting with improving the purity of the sample by chromatography to using sophisticated detection such as tandem mass spectrometry. Caldarella et al. (1982) published a 'Proposed Selected Method' for serum cortisol that required extraction with dichloromethane, reverse-phase C-18 chromatography and UV detection. Hariharan et al. (1992) employed solid-phase extraction and LC with UV detection, and with 1 mL of plasma, achieved a detection limit for cortisol that was about 800 pmol/L. The technique can also be adapted to measure corticosterone in plasma. McBride et al. (1991) also used solid-phase extraction followed by reversed-phase liquid chromatography, claiming good peak separation and good recovery. Their method can be applied to the simultaneous measurement of the synthetic corticosteroids, prednisone and prednisolone.

Methods employing chromatographic separation are often impractical for routine use or where large numbers of assays are required, and clinical laboratories have in general adopted simple IA methods. Turnell et al. (1988) describe a method using LC, with an automated preparation stage that required specialised dialysis and enrichment equipment. Their method is also applicable to other steroids, as is the method described by Schoneshofer et al. (1981).

Swinkels et al. (1991) evaluated a scintillation proximity assay for cortisol in which only antibody-bound ligand emits energy and thus a separation of bound and free fractions is not necessary. A virtually perfect correlation was found between

the results obtained with this method and those found with RIA. The precision was in the range of other good assays (5–6%). The method is inappropriate for the measurement of aldosterone because of the problem of interference from cortisol binding globulin.

An evaluation carried out by Ayers et al. (1989) of a polarisation immunoassay (the Abbott TDx system) reported favourably on the reliability of the method for routine use. Specificity was not examined directly, but there was a lack of bias when the results were compared with those obtained with RIA. Similar results were found by Weller et al. (1992), who compared the Serono RIA with the Abbott TDx system. As with some other commercial kits, this method is 'calibrated' against GC-MS analysis of reference plasma samples, rather than employing direct standards. This is not ideal, since it clearly implies the need to compensate the method for interference. The standard in 'direct' RIAs is a major problem area and, as Lantto et al. (1980) pointed out some time ago, an intact plasma matrix in the standard seems to be a necessary factor in achieving accuracy.

None of these assays is completely satisfactory. Problems of specificity arise, particularly in patients with adrenocortical disorders. Brotherton and Rothbart (1990) used five different types of assay (in-house tritium RIA after ethanol extraction, a direct ^{125}-I RIA, fluoroimmunoassay (FIA), enzyme immunoassay (EIA) and a luminescence immunoassay (LIA)) to measure serum cortisol levels in a group of patients with hirsutism. A number of spuriously high results were attributed to cross-reactions with other precursor steroids. After suppression of adrenocortical activity with dexamethasone, only the ^{125}-I assay and LIA produced the expected low values. The authors concluded that commercially available cortisol assays available at that time appeared to be unsuitable for studies of patients with 21-hydroxylase deficiency, and also for neonates, in whose sera other cross-reacting steroids, particularly cortisone, are present. An ELISA method for cortisol has been described and used for studies in humans and domestic and farm animals (Cooper et al., 1989). Another group reported the use of a biotin conjugate in a time-resolved fluorometric assay or an ELISA which compared favourably with an RIA (Dressendorfer et al., 1992), subsequently improved using time-resolved FIA, without extraction for bovine serum (Erkens et al., 1998).

If IA methods are not satisfactory, it is logical to consider moving towards the use of chromatographic physico-chemical methodology of which LC-MS/MS is the most obvious technology. In the past this technology, like GC-MS, has been considered too technical and operator-sensitive and unlikely to be able to provide the high throughput required in routine clinical laboratories. In the last 10 years however considerable improvements in data handling and automation have taken place and we anticipate that this will continue. Two almost identical LC-MS and LC-MS/MS methods (Jung et al., 2004; Tai and Welch, 2004) have been developed using isotope dilution (d_3-cortisol) with positive electrospray ionisation (ESI) and muliple reaction monitoring (MRM). Results were compared with Certified Reference Materials (CRM) 192 and 193 (Thienpont et al., 1991). The accuracy, precision and freedom from interference suggest that the methods qualify as candidate reference methods. Tai and Welch (2004) used Sep-Pak C18 extraction followed by ethyl acetate,

whereas Jung et al. (2004) relied upon methylene chloride extraction. Sensitivity of LC-MS methodology depends on ionisation efficiency, and increased efficiency may require other forms of ionisation and/or monitoring of negative ions, and reduction of background noise (i.e. increased S/N ratio), which can usually be achieved using MRM in LC-MS/MS. It may also be possible to increase ionisation by derivatising the analytes prior to LC (e.g. Yamashita et al., 2007a and b).

An LC-MS/MS method using negative (ESI) has recently been described (Huang et al., 2007). Prior to LC-MS-MS, cortisol was extracted into ethyl acetate from urine (1 mL), and plasma (250 μL diluted to 1 mL with water). The solvent was removed by evaporation and the steroids reconstituted in MeOH/H_2O (1:1), injected onto LC column (Inertsil ODS) and eluted with gradient of 1 nM NH_4Cl (pH 9) and MeOH. The LC was connected to a quadrupole MS and ESI(−) was carried out. The chloride adduct of the molecular ion $[M + {}^{35}Cl]^-$ was selected in MS1 and after CID, the ${}^{35}Cl^-$ adduct ion was used for quantitation in G3(MS2). The lower limit of quantitation was 0.5 μg/mL, which is perfectly satisfactory for measurement of free concentration of cortisol in plasma (around 10% of total). This method also measured the 'free' (unbound) cortisol in plasma by interpolating an ultrafiltration step, using a Millipore Centifree Micropartition column with a 30,000 Da cut-off. The ultrafiltrate (250 μL diluted with water) achieved was then analysed in the same way. In this assay 6-methylprednisolone was used as an internal standard.

LC-MS/MS using atmospheric pressure photoionisation (APPI), which has been reported to improve ionisation in comparison to that achieved with APCI (Robb et al., 2000) has also been used. Cortisol and cortisone in serum and plasma (100 μL) were extracted, after addition of the internal standard d_4-cortisol, with tert-butylmethyl ether containing 3% phosphoric acid. The solvent was evaporated to dryness and the steroids were reconstituted in mobile phase (MeOH/H_2O). An LC column (Luna Phenyl-Hexyl) was eluted with MeOH/H_2O solvent – APPI(+) –MS-MS (MRM) – positive ionisation was induced by exposure of eluent to a Krypton lamp using toluene as a dopant (Kushnir et al., 2004). The MH^+ ion was selected in MS1 and the results of the transitions *m/z* 363 to *m/z*121 and to *m/z* 97 (for cortisol) and *m/z* 361 to *m/z* 163 and to *m/z* 105 (for cortisone) were monitored in Q3(MS2).

Although LC-MS/MS is likely to find its way into routine use in the next 10 years, it is always worth looking back and considering methods based on technology, which is not expensive and has been widely used in the past. One example of this is a recent report (Fenske, 2008) which uses a TLC-based fluorescence method to measure plasma cortisol. Five millilitres of plasma was extracted using Kieselguhr mini-columns and methylene chloride and separated on aluminium backed silica gel 60 TLC plates. After separation, the plates were dipped in an isonicotinic acid hydrazide reagent, forming the hydrazones of cortisol and the internal standard, 21-deoxycortisol. Quantitation was carried out using a TLC plate scanner, measuring fluorescence developed from the hydrazone. Specificity of this method clearly resides with the adequacy of the TLC separation.

Further publications describing LC- and GC-mass spectrometry applications in this field are listed in Table 5.5.

Table 5.5 Some GC- and LC-MS methods for measurement of cortisol, aldosterone, 17-hydroxyprogesterone and corticosterone (1996-). In this table, references for each steroid are placed in date order – more modern ones first

Analyte	Matrix	Purification	LC/GC	Ionisation	Comment	References
Cortisol (internal standard used) *[other corticosteroids also measured]*						
d_2-cortisol *[11-deoxy-F, 21-deoxy-F, B, DOC and 17-OHprog]*	Human serum and dried blood spots	Extracted with acetone/MeCN (1:1)	LC on Atlantis dC18 using gradient elution with water containing 32 mM HCOOH and MeOH	Waters Ultima ESI(+)	MH^+ (363.3) in MS1 and *m/z* 122 in Q3(MS2)* (cf. Wear et al., 2007; Guo et al., 2006)	Janzen et al. (2008); Janzen et al. (2007) and Peter et al. (2008)
d_4 [9,11,12,12] cortisol *[THE and allo-THF]*	Human and primate urine	β-glucuronidase followed by extraction with *t*-butyl ether at pH 9.6 ± solvolysis	LC on Gemini C18 column with acetonitrile/water gradient at 30°C	Quattro Premier XE MS/MS Z-spray ESI(+)	MH^+ in MS1 monitoring in Q3(MS2) $(MH\text{-}18)^+$; part of 23 steroid profile	Hauser et al. (2008)
See Caulfield et al. 2002	Human urine – cortisol and corticosterone metabolites	Standard steroid profile using Sep-Pak C18 (Caulfield et al., 2002)	GC as OMO-TMS derivatives	EI(+)		Dhir et al. (2007)
No details given	Human bedtime saliva		LC-MS/MS at Mayo Clinic		No details given	Baid et al. (2007)
d_4-cortisol *cortisone, 11-deoxy-F, B, DOC,11-dehydro-B*	Human saliva	Extraction with ethyl acetate, separation on Keiselgel column and formation of picolinoyl derivatives	LC on Purisil 5C18 column with acetonitrile/acetic acid at room temp	Finnegan TSQ Quantum MS/MS ESI(+)	MH^+ in MS1 monitoring loss of 18 amu in Q3(MS2)	Yamashita et al. (2007b)
6α-methyl prednisolone	Human plasma and urine	Extraction with ethyl acetate	LC on Inertsil ODS-3 using gradient elution with NH_4OH/MeOH	Micromass Quattro II MS/MS using ESI(−)	$[M + 35\ Cl]^-$ in MS1 using 35-Cl^- ion in Q3(MS2)	Huang et al. (2007)

Flumethasone *(6-methylprednisolone, prednisone, prednisolone)*	Human plasma	Acidified plasma onto an Oasis-HLB cartridge, eluted with MeOH	LC on Waters Symmetry C18 using stepwise elution using MeOH/5 mM ammonium acetate mixtures	API 3000MS/MS ESI(−)	$[M-H]^-$ in MS1 and daughter ion *m/z* 331 in Q3(MS2)	DiFrancesco et al. (2007)
d_2 [1,2] cortisol	Human urine free cortisol	Preciptation of proteins with 5% TCA and centrifugation	LC at 60°C using Waters XBridge C18 column with stepwise elution with2 mM ammonium acetate in 0.01% formic acid in water and 0.1% formic acid in MeOH	Waters Quattro Micro MS/MS using Z-spray ESI(+)	MH^+ in MS1 *m/z* monitoring *m/z* 121* in Q3(MS2)* (cf. Janzen et al., 2008)	Wear et al. (2007)
	Cattle urine	Complex procedure using Oasis/SPE C18 cartridges and subsequent oxidation with chromate oxidation	GC separation as free and acetates	EI (+) and combustion IRMS for ^{13}C /^{12}C ratio		Bichon et al. (2007)
	Human saliva	Heat at 80°C for 5 min and centrifugation. Online in-tube SPME	LC using Eclipse ZDB-C8 at 30°C eluting with 1% acetic acid/MeOH	Agilent Model 1100 LC-MS with ESI(+)	SIM for *m/z* 363 (MH^+)	Kataoka et al. (2007)
d_4 [9,11,12,12] cortisol *[Aldosterone, B, 11-deoxy-F, 17-OHprog]*	Human serum	Protein pption with MeCN. On line washing with 2% MeOH in 15 mM ammonium acetate	LC using Supelco LC-8-DB eluting with MeOH/water gradient	Sciex API-5000 with APPI operating in both + and – ionisation modes	MS/MS MH^+ in MS1, monitoring *m/z*121 in Q3(MS2)* *(cf. Janzen et al., 2008)	Guo et al. (2006)

(continued)

Table 5.5 (continued)

Analyte	Matrix	Purification	LC/GC	Ionisation	Comment	References
[1,2,4,19-$^{13}C_4$]$^-$ cortisol and -cortisone	Human plasma	Oasis-HLB after dilution with 100 mM ammonium acetate at pH 3, vortexing and centrifugation.	LC using Waters Symmetry C18 eluting with MeOH/NH_4Ac	Sciex API 4000 LC-MS/MS using turbospray (+) ionisation	MH^+ in MS1 and monitoring *m/z* 125 in Q3(MS2)	Yang et al. (2006)
[cortisone]	Human serum	Methanol added and steroid extracted using Bakerbond C18 cartridge, eluting with EtAc.	LC using Purospher Star RP-18 eluting with linear gradient of MeOH/water	Sciex API 2000 using ESI(−)	$(M-H)^-$ in MS1 and monitoring *m/z* 331 in Q3(MS2)	Tenhola et al. (2005)
[cortisone, THE, THF and 14 synthetic corticosteroids]	Human urine	SPE on C18 Bakerbond column, enzyme hydrolysis and final Oasis-HLB	GC – no derivatization LC on Omnispher C18 column	Benchtop HP5973 MSD Using EI(+)	SIM of *m/z* 302 (loss of C17 side chain) SIM of MH^+	Pujos et al. (2005)
				HP1100 MSD with ESI (+)		
6α-methyl prednisolone *[6-OH-cortisol]*	Human urine	Online SPE extraction using Prospekt-2 extraction apparatus	LC on Symmetry Shield RP18 column eluting with mixtures of water, methanol, formic acid at 60°C	API 3000 LC-MS/MS ESI(−)	Monitoring transition *m/z* 407.5 (MS1) to 331.5 (Q3-MS2)	Barrett et al. (2005)
d_2-cortisol *[Aldosterone, DOC, B]*	Adrenal zona glomerulosa cells in culture	Extraction with ethyl acetate ×2	LC on Kromasil C18 column eluting with water/MeOH	HP 1100 LC-MSD ESI(+)	Na adducts monitored $[M + Na]^+$	Nithipatikom et al. (2005)
d_3 [9,12,12] cortisol *[17-OHprog]*	Human plasma	Formation of Girard-T and extracted with EtAc; modified Girard reagent improves ionisation for 17-OHprog.	Direct injection into ion source	Sciex API365 ESI(+)	MRM of neutral loss of 59 amu – for cortisol 476–417	Johnson (2005, 2007)

d_3 [9,12,12] cortisol	Human serum	Acidification to pH 1.5 and extraction with Sep-Pak C18. Eluted with MeOH evaporated to dryness and extracted with EtAc	LC on Zorbax Eclipse XDB-C18 column, eluting with gradient of 1 mL/L acetic acid n water-MeOH	HP 1100 MSD with ESI(+) or Quattro Ultima LC-MS/MS ESI(+)	SIM of MH^+	Tai and Welch (2004)
					MRM MH^+ in MS1 and $(MH\text{-}2H_2O)^+$ in Q3(MS2)	
d_4-cortisol *[cortisone]*	Human serum or plasma	Extraction with methyl-*t*-butyl ether	LC on Lunar Phenyl-Hexyl column eluting with MeOH/water (1:1) at 45°C	Sciex API 3000 using APPI (+) and toluene as dopant	MH^+ in MS1 monitoring *m/z* 121 in Q3(MS2)	Kushnir et al. (2004)
d_3 [9,12,12] cortisol	Human serum	Extraction with CH_2Cl_2	LC on a Hypersil ODS column eluting with 20 mM NH_4Ac–MeOH	Quattro Ultima LC-MS and LC-MS/MS ESI(+)	SIM monitoring MH^+ in MS1 or MRM monitoring transition $(MH\text{-}2H_2O)^+$ in Q3(MS2)	Jung et al. (2004)
Flumethasone *[prednisone, prednisolone and dexamethasone]*	Human serum	Methanol added, centrifuged and extracted using Oasis-HLB cartridge	LC on Symmetry C18 column eluting with gradient of MeOH in 5mM acetate buffer, pH 3.25	Sciex API 3000 with 'turbo ion-spray' ESI(−)	$[M\text{-}H]^-$ *m/z* 361 in MS1, monitoring $[M\text{-}H\text{–}CHOH]^-$, *m/z* 331	Frerichs and Tornatore (2004)
d_8-17-hydroxy-progesterone *[17-OHprog]*	Dried blood spots	Extracted with diethyl ether	LC on Symmetry C18 column eluting with gradient of MeOH/water	Sciex API 3000 with ESI(+)	MH^+ selected in MS1 and product ion *m/z*121 monitored in Q3(MS2)	Minutti et al. (2004)

(continued)

Table 5.5 (continued)

Analyte	Matrix	Purification	LC/GC	Ionisation	Comment	References
d_4 [9,11,12,12] cortisol *[14 synthetic corticosteroids]*	Human serum and urine	Extraction with CH_2Cl_2.	LC on SYNERGI 4 μ MAX-RP eluting with MeCN/0.1 mM ammonium acetate	Sciex API 3000 with ESI(+)	MH^+ selected in MS1 and product ion *m/z*121 monitored in Q3(MS2)	Taylor et al. (2004)
d_3 [9,12,12] cortisol *[cortisone]*	Human hair	Powdered in ball mill. Eluted with buffer pH 7.6 for 16 h at 40°C. Isolute C18 SPE, eluted with MeOH	LC on Nucleosil C18 eluting with 2 mM ammonium formate, pH 3.0/MeCN	Sciex API-100 with ESI(+)	MID monitoring MH^+ (*m/z* 363), $[MH\text{-}2H_2O]^+$ and $[MH\text{-}3H_2O]^+$	Raul et al. (2004)
Stigmasterol *[Profile of 63 steroids]*	Newborn urine	Hydrolysis, extraction with CH_2Cl_2	GC as O-methyl oxime-TMS ether derivatives	HP5890II MSD with EI(+)	SIM of two mass ions	Homma et al. (2003)
d_4-cortisol	Human urine	Refers to Taylor et al. (2002) but description simply says centrifugation and further purification using online C18 guard column	LC on Luna C18 column eluting with gradient of MeOH/5 mM ammonium formate at 40°C	Sciex API 2000 using APCI (+)	Selecting MH^+ in MS1 and monitoring transitions 363/121 and 363/97 in Q3(MS2)	Kushnir et al. (2003)
6α-methyl-prednisolone	Human urine	Filter and extract with CH_2Cl_2	LC on Purospher Star C18 column, eluting with gradient of MeOH/water	Sciex API 2000 with ESI(−)	Selecting $[M\text{-}H]^-$ in MS1 and monitoring transition 361/331 in Q3(MS2)	Turpeinen and Stenman (2003)
d_4-cortisol	Human saliva	Precipitate protein with MeCN and centrifuge	LC on Genesis C8 column, eluting with water/methanol gradient	Sciex API 3000 with ESI(+)	Selecting MH^+ in MS1 and monitoring transition 363/309 in Q3(MS2)	Jonsson et al. (2003)
d_4 [9,11,12,12] cortisol *[cortisone]*	Human urine	Centrifuge and extraction with CH_2Cl_2	LC on Supelco LC-181 column, eluting with MeOH/water	Sciex API 2000 with ESI(+)	Selecting MH^+ in MS1 and monitoring transition 363/121 in Q3(MS2)	Taylor et al. (2002)

6α-methyl prednisolone *[6-OH-F]*	Human urine	Oasis-HLB cartridge eluted with ethyl acetate followed by diethyl ether	LC on Nova-Pak C18 column eluting with 0.06% TFA in ammonium acetate (pH 4.8) buffer/ MeCN	Hitachi M-10,000 H with APCI(+)	SIM of MH^+ ion (*m/z* 363)	Ohno et al. (2000)
Fludrocortisone acetate *[6-OH-F]*	Monkey urine	Extraction with Ethyl acetate with and without washing with M-NaOH or Bakerbond C18 SPE, eluting with MeOH or Ethyl acetate	LC on Inertsil ODS-3 column, eluting with 0.02% acetic acid-MeCN	Sciex API 3000 using APCI(+)	MH^+ selected in MS1 and *m/z* 327 $[MH\text{-}2H_2O]^+$ monitored in Q3(MS2)	Tang et al. (2000)
[1,2,4,19-^{13}C, 1,1,19,19,19-^{2}H] cortisol *[cortisone]*	Human plasma	SPE with Sep-Pak C18 cartridge, elution with ethyl acetate and derivatisation as *bis*-methylenedioxy -3-pentafluoropropionates	GC on fused silica SPB-1 columns	QP1000EX GC-MS – EI(+)	Single ion monitoring of M^+ of cortisol derivative at *m/z* 696	Furuta et al. (2000)
d_5 [1,1,19,19,19] cortisol *[cortisone, prednisone and prednisolone]*	Human plasma	SPE with Sep-Pak C18 cartridge, elution with ethyl acetate	LC on LichroCART Superspher 100 cilumn, eluting with ammonium formate-THF-MeOH/MeCN	Shimadzu LCMS-QP1000EX with thermospray (+) ionisation	Single ion monitoring of M^+ of cortisol at *m/z* 363	Shibasaki et al. (1997)
Fludrocortisone *[E and prednisolone]*	Human plasma	MeOH and centrifugation and 'restricted access' C18 pre-column. After pre-concentration, switched to main LC column	LC on LiChrospher 100 RP-18 column, eluting with MeCN/ water containing 1 g/L acetic acid	Finnegan MAT TSQ-70 triple quadrupole LC-MS with APCI(–)	SIM of $[M + CH_3COO]^-$ ion (*m/z* 421)	Van der Hoeven et al. (1997)

(continued)

Table 5.5 (continued)

Analyte	Matrix	Purification	LC/GC	Ionisation	Comment	References
6α-methyl prednisolone *[E, 20-reduced E and F, THE , THF]*	Placental perfusate	Sep-Pak C18 SPE, eluting with MeOH.	LC on a Novapak C18 column, eluting with MeOH-10 mM ammonium formate pH 4.0	Sciex API III with pneumatically assisted ESI (+)	MH^+ selected in MS1 and *m/z* 121 monitored in Q3(MS2)	Dodds et al. (1997)
d_2 [1α,2α] cortisol	Human serum	Extraction with CH2Cl2 and further purified on Sephadex LH-20 Heptafluorobutyrate esters were formed	GC on fused silica column DB-1	HP Series IIGC connected to Finnegan MAT Incos-500 SL MS EI(+)	Single ion monitoring of M^+ ion (*m/z* 489)	Thienpont et al. (1996)
d_4 [9,11,12,12] cortisol *[E, 6-OHF and 18-OHF]*	Human urine	Free steroids extracted with Sep-Pak SPE, eluting with MeOH	GC on fused silica column DB-1	HP5970-MSD using EI(+)	Single ion monitoring of $[M-31]^+$ ion (*m/z* 605)	Palermo et al. (1996)
Aldosterone						
d_8-corticosterone	Human serum	Extraction with ETAc. Xtraction through I nterSep solid-phase cartridge. Derivatised as ethyl ether-picolinyl derivative Yamashita et al., (2007b, 2008b)	LC on Cadenza CD18 column, eluting with gradient of MeOH/0.1% acetic acid at 40°C	API5000 triple quadrupole MS/MS using ESI(+)	Select MH^+ in MS1 and monitor $[MH\text{-}EtOH]^+$ in Q3(MS2)	Yamashita et al. (2008a)
d_7-aldosterone	Human serum	Extraction with CH_2Cl_2/diethyl ether (60:40)	LC on a Waters Sunfire C18 column, eluting with a MeOH/water stepwise gradient at 30°C	Sciex API 3000 using ESI(+)	$[M-H]^-$ selected in MS1 and 189.4 monitored in Q3(MS2)	Turpeinen, et al. (2008)

Flumethasone	Human serum and/or plasma	Extraction with CH_2Cl_2/ diethyl ether (40:60)	LC on Waters Novapak phenyl column, eluted with 50 mM ammonium formate/MeOH	Sciex API III LC-MS/MS – APCI(−)	$[M-H]^-$ selected in MS1 and $[M-CHO]^-$ (*m/z* 331) monitored in Q3(MS2)	Fredline et al. (1997)
Corticosterone						
Prednisolone *[dexamethasone]*	Rat biofluids and fetal tissue	Addition of 4% phosphoric acid, centrifuged and subjected to SPE using Oasis-HLB, eluting with MeOH	LC on C8 Hydrobond AQ column, eluting with 10 mM ammonium formate in 0.1 and formic acid –MeCN	Sciex API 3000 using turbo ion-spray (+)	MH^+ selected in MS1 and *m/z* 329.1 $[M-18]^+$ monitored in Q3(MS2)	Samtani and Jusko (2007)
d_8-corticosterone	Rat brain and plasma	SPE and HPLC fractionation – formation of hepta-fluorobutyrate esters	GC	Automass MS using EI(+)	M^+ and $[M-15]^+$ of Δ^{9-11} dehydroB monitored	Meffre et al. (2007)
Measuring TH-corticosterones (3α,5α) and (3α,5β)	Rat brain	Strata-X cartridge, eluting steroids with Hx-EtAc (7:3). Formation of dinitrobenzoyl and 2-nitro-4-trifluoromethylphenyl hydrazone derivtives.	LC on J'Sphere ODS-H80 column eluting with MeOH/ water (3:2) at 40°C	Thermoquest LCQ ion trap MS using APCI(−) – use of derivatives improved ionisation ×60 and ×40	Monitoring $[M-H]^-$ and $[M-18]^-$ in MS1 for NFPH derivatives. DNB gave a strong M^- ion (1.5 × more sensitive)	Higashi et al. (2007a)

(continued)

Table 5.5 (continued)

Analyte	Matrix	Purification	LC/GC	Ionisation	Comment	References
d_7 (2,2,4,4,17α,21,21) 3α,5α-THB						
d_4 (9, 11,12,12) cortisol *[11-dehydrocorticosterone, cortisol and cortisone]*	Mouse and human tissue samples	Mouse liver homogenised in EtAc. Mouse and human adipose tissue homogenised and further shaken with heptane/methanol/H_2O, heptanre phase discarded	LC on Symmetry C8 (orLuna C18) column, eluting with TFA-H_2O	Micromass Ultima Pt LC-MS/ MSusing ESI(+)	Selecting MH^+in MS1 and monitoring *m/z*121 in Q3(MS2)	Ronquist-Nii and Edlund (2005)
11-deoxycorticosterone *[11-dehydroB, 20-dihydro-11-dehydro-B, 20β-dihydroB, DOC]*	*In vitro* metabolic studies in intestinal segments of Wistar rats and oviduct of chickens	Incubation media Centrifuged and passed through Sep-Pak C18 cartridges, eluting with MeOH	LC on Zorbax Eclipse XDB-C18 column, eluting with gradient of methanol-water-acetic acid	HP 1100 LC/MSD system using API-ESI(+)	Monitoring MH^+ (*m/z* 347)	Miksik et al. (2004)
No internal standard – spiked samples used	Rat and mouse plasma	Extraction with EtAc. Formation of di-pentafluorobenzyl oxime-11-TMS	GC on HP-5MS bonded stationary phase	HP5890 GC-MSD EI(+)	Monitoring *m/z* 316, 648, 663 and 678	Shu et al. (2003)
Isotopomers synthesised in-house for all analytes: Corticosterone [F, DOC, 17-OHprog]	Bovine vitreous and aqueous humor	Addition of IS in MeCN, mix, extract with EtAc and dried with anhydrous Na_2SO_4. PFBO-TMSi derivatives	GC on Restek Rtx-1 columns	HP5890 GC-MSD NCI using methane as reagent gas	Monitoring $[M\text{-}HF]^-$ ions for all steroids. *M/z* 699 for corticosterone – base peak	Iqbal et al. (2001)

Triamcinolone	Human serum	Extraction with diethyl ether	LC on Vydac C18 microbore column, eluting with MeCN-water-formic acid	Sciex API single quadrupole LC-MS. Ionspray (nebuliser-assisted) ESI(+)	Monitoring MH^+	Ghulam et al. (1999)
17-OH-Progesterone						
[2,2,6,6,21,21,21-$^2H_7$]-17-OH-prog	Human saliva	De-proteinised with MeCN. Thru Strata-X cartridge, eluting with EtAc. Derivatised with 2-hydrazinopyridine (Higashi et al., 2007b)	LC on YMC-Pack Pro C18 RS column, eluting with MeCM/ MeOH/10 mM NH_4 formate	API 2000 triple quadrupole MS/ MS using ESI(+)	Select MH^+ in MS1 and monitor *m/z* 109 (A-ring fragmentation).	Shibayama et al. (2008)
Medroxy-progesterone acetate – assay for 17α-caproate ester	Human plasma	Diluted with water and passed through an Oasis HLB column, eluting with MeOH	LC on Symmetry C18 column, eluting with 0.1% formic acid/MeCN/water	Finnigan TSQ Quantum Ultra. ESI(+)	Select MH^+ in MS1 and monitor *m/z* 313 and 271	Zhang et al. (2007); Zhang et al. (2008)
d_8-17-OHprog *[11-deoxy-F, 17-OH-pregnenolone and pregnenolone]*	Human serum or plasma	Diluted with water and applied to Strata X SPE columns. Eluted with methyl-*t*-butyl ether. O-methyloximes formed and extracted with methyl-t-butyl ether	LC on Synergy Fusion RP18 column, eluting with MeOH/ aq HCOOH	Sciex API 4000 LC-MS/MS using V spray ESI(+)	MH^+ in MS1 monitoring transition to *m/z* 124.1	Kushnir et al. (2006)

(continued)

Table 5.5 (continued)

Analyte	Matrix	Purification	LC/GC	Ionisation	Comment	References
d_8-17-OHprog	Human serum	SPE using Clean-Screen DAU co-polymeric columns, eluting with CH_2Cl_2-IPA-NH_4OH	LC on Supelco Discovery RP-amide C16 column, eluting with gradient of 0.1% formic acid and MeOH	Sciex API 4000 using TurboIon Spray (+) ionisation	MH^+ (*m/z* 331.2) selected in MS1 and *m/z* 109 monitored in Q3(MS2)	Etter et al. (2006)
d_8 [2,2,4,6,6,21,21,21] 17-OHprog	Human plasma, serum or saliva	Protein ppted with MeOH-$ZnSO_4$. Centrifugation.	Injected onto Oasis HLB extraction column online with LC using column switching to introduce sample on to Chromolith RP-18e column, eluting with water-MeOH at 30°C	Sciex API 4000 using APCI(+)	MH^+ (*m/z* 331) selected in MS1 and *m/z* 109 and 97 monitored in Q3(MS2)	Rauh et al. (2006)
	Neonatal dried blood spots	Steroid eluted from filter paper with 0.1% HCl in MeOH; TMS ethers made using BSTFA with microwave assistance	GC using HP-5MS capillary column	HP 5973-GC-MSD EI(+)	Monitoring *m/z* 359	Deng et al. (2005)
6α-methylprednisolone or d_8 [2,2,4,6,6,21,21,21]; 17-OHprog	Human serum	Diluted with MeOH-water and extracted with diethyl ether-EtAc (7:3).	LC on Purospher Star RP-18 column, eluting with gradient of MeOH-water	Sciex API 2000 LC-MS/MS ESI(+)	Selecting MH^+ in MS1 and monitoring *m/z* 97 and 109 in Q3(MS2)	Turpeinen et al. (2005)

6-methyl-prednisolone	Dried blood spots	Extraction with MeOH and formation of Girard P derivatives on 3 and 17-oxo groups	LC on Polaris C18 column, eluting with 1% acetic acid/ MeOH (30:70); LC on C4 Vydac column, eluting with MeCN/water (1:1)	Sciex API 2000 LC-MS/MS; TurboIon; Spray (+)	Selecting MH^+ in MS1 and monitoring *m/z* 97 and 109 in Q3(MS2); selecting M^{2+} in MS1 and monitoring *m/z* 80 in Q3(MS2)	Lai et al. (2001, 2002a, b); fragmentation given in Lai et al (2001) paper
Stigmasterol and cholesteryl butyrate *[standard urinary 15-steroid profile]*	Metabolites of 17-OHprog in human urine	Bond-Elut C18-OH, elution with MeOH. Conjugate removed by enzyme hydrolysis and formation of OMO-TMS derivatives	GC on capillary DB1 column	HP 5973 GC-MSD; EI(+)	Monitoring selected ions of high intensity	Caulfield et al. (2002)
d_4 [11,11,12,12]; 17-OHprog	Human plasma	Diluted with NaCl, loaded onto Extrelut and steroids eluted with EtAc.	LC on Nucleosil RP-18 column, eluting with MeOH/ water gradient	Sciex API 3000 APCI(+)	MH^+ selected in MS1 and daughter ion *m/z* 97.2 monitored in Q3(MS2)	Wudy et al. (2000a)
d_4 [11,11,12,12]; 17-OHprog	Human plasma	Extraction with EtAc and purification on LH-20 mini columns. Formation of heptafluo-butyrate esters	GC on capillary OV1 column	GC interfaced to HP 5970B MSD	Monitoring M^+ ions	Wudy et al. (2000b)
	Mid-gestation amniotic fluid			EI(+)		Wudy et al. (1999)
d_3 [21,21,21] 17-OHprog	Human plasma	Addition of NaOH and HCl. Adjusted to pH 7.4 and extracted with SepPak cartridge, eluted with EtAc; *t*-BDMS and/or OMO-TMS ethers synthesised	GC on DB5 column	Kratos MS-5-TC; 70eV-EI(+)	Monitoring *m/z* 429.42	Masse and Wright (1996)

For explanation of some of the MS terminology used here see Chapters 2 and 3.

Salivary Cortisol The realisation that the concentration of corticosteroids in saliva is a reflection of the circulating concentration of free steroid attracted considerable interest in developing suitable assay techniques. There are clearly major advantages in the use of saliva, since it represents a non-invasive technique and one that is particularly suitable for investigations in neonates and young children. However, the concentration of cortisol in saliva is low (less than 50 nmol/L), requiring the use of an RIA or some similar sensitive competitive binding technique. Specificity is also important since saliva contains substantial amounts of cortisone. LC-MS/MS has been used to measure salivary cortisol (Jonsson et al., 2003) and demonstrated that immunoassay results were consistently higher than those achieved using MS/MS. Use of this method but using a freeze-drying pre-concentration technique in comparison to a ELISA technique showed that the LC-MS/MS method more accurately measured added cortisol (Nelson et al., 2008).

The collection of saliva presents some difficulties. Collection is however non-invasive and far less disturbing to younger patients than venepuncture. Steroid analysis in saliva has been reviewed (Lewis, 2006). It must be centrifuged before analysis to remove debris. Contamination from small amounts of blood, e.g. from the gums, which contain steroid should be avoided (Groschl et al., 2001; Gallagher et al., 2006; Mylonas et al., 2006; Whembolua et al., 2006). Breast milk or baby feeds contain interfering substances (Magnano et al., 1989) and it is therefore desirable to remove fats from neonatal saliva. Most of the original work in developing satisfactory salivary steroid assays was carried out by Riad-Fahmy and her colleagues, who described suitable methods and validated their use in clinical applications (Walker et al., 1978). Methods designed for plasma assays have been adapted for measuring salivary cortisol (Silver et al., 1983; Al-Hakiem and Abbas, 1987). Fulton et al. (1989) showed that solvent extraction not only concentrated the analyte but also improved the specificity of the assay.

Methods designed for plasma/serum assays have been adapted for measuring salivary cortisol (Silver et al., 1983; Al-Hakiem and Abbas, 1987). Some authors have employed direct RIAs for the measurement of cortisol in saliva, but Fulton et al. (1989) examined the effect of salivary proteins on the assay binding curves and considered that these could affect direct assays, and thus prior solvent extraction will not only concentrate the analyte but also improve the specificity of the assay. Automated assays for cortisol have been modified for saliva assays (Chiu et al., 2003; Westermann et al., 2004; Gozansky et al., 2005; Vogeser et al., 2006). Two saliva assays have been compared with tandem mass spectrometry (Vogeser et al., 2006; Baid et al., 2007) and produced much lower reference ranges than the IA methods. The use of bedtime salivary cortisol for the diagnosis of Cushing's syndrome as a sole test was not recommended. A sensitive assay for cortisol in serum and saliva was developed using a bioluminescence heterogeneous immunoassay based on aequorin (Mirasoli et al., 2002). This aequorin assay has now been further refined, producing a homogeneous immunoassay with increased sensitivity (by an order of magnitude) and wider linear range (spanning four orders of magnitude) (Rowe et al., 2007). Attention must also be paid to the collection method used as problems have been encountered (Strazdins et al., 2005; Groschl and Rauh, 2006;

Mörelius et al., 2006; Harmon et al., 2007; Poll et al., 2007). It is often difficult to get saliva from toddlers and use of marshmallow has been reported to be a successful non-stressful solution which does not interfere with the subsequent cortisol assay (Clements et al., 2007) – an EIA from DSL (see Table 5.4).

In a recent method, saliva collected in Salivette tubes was centrifuged to elute saliva from swabs. 0.1–0.2 mL saliva was diluted with 0.05 mL 0.2 M acetate buffer (pH 4) and the total volume made up to 0.5 mL with H_2O. The tube was heated for 5 min at 80°C and after centrifugation the liquid was used for in tube-solid-phase extraction. A Supel-Q PLOT (NB other columns were investigated) capillary column was placed between injection loop and injection needle of the autosampler. 0.5 mL samples were injected onto the PLOT column and after washing, eluted onto the analytical column (Eclipse ZDB-C8) in an eluting solvent (1% acetic acid/MeOH (1:1)). The eluent was ionised using ESI (+) and SIM of MH^+ ions (Kataoka et al., 2007). There is a recent report of an LC-MS/MS method for salivary cortisol using 0.1mL of plasma (Turpeinen et al., 2009).

Urinary Cortisol The measurement of cortisol in urine is of considerable importance in clinical laboratory medicine because, like saliva, the amount excreted in the urine is a direct reflection of the unbound, or biologically active, cortisol in plasma. It is therefore an extremely useful method of investigating patients with suspected hypercortisolaemia (e.g. Cushing's syndrome; Neiman et al., 2008; Baid et al., 2009). The particular advantage is that the amount present in urine will indicate tissue exposure to cortisol over the appropriate period of time, whereas random blood samples can obviously only indicate the level at one particular point in time. There are many published methods for the measurement of urinary free cortisol (UFC) concentration and it remains an important endocrine investigation. Because of the diurnal rhythm of cortisol excretion, it is usual to collect 24 h urine samples, which may pose difficulties in young children and in animals – as an alternative, use of cortisol/creatinine ratios and/or timed collections (e.g. 2000–0800 h – Corcuff et al., 1998) have been proposed as screening tests. The use of such ratios in untimed collections has however been criticised (Jerjes et al., 2006).

The earlier methods for urinary free cortisol were based on a fluorimetric end point or competitive protein binding, neither of which was highly specific. Increased specificity was claimed with the advent of immunoassays, but there are many warnings in the literature that these IA techniques may still suffer from lack of specificity (Murphy et al., 1981; Murphy, 1999, 2000, 2002, 2003; chöneshofer et al., 1980b). Some recent results for urinary free cortisol concentrations are given in Table 5.2 and should be compared with values quoted by choneshofer et al. (1980a). In general, it is not possible to achieve specificity without prior extraction (dichloromethane is widely used for this purpose) and/or purification, and analysts should clearly be wary of employing simple techniques that may not have been fully validated for urine and which can thus still give misleading results. It is important to examine the criteria that the authors have applied to the validation of their method, so as to ensure that it will perform reliably in the situation in which it is required. There are relatively few direct IA methods that have been properly evaluated, especially for their specificity in urine cortisol assay, and thus care is needed if erroneous

results are to be avoided. It is more difficult to achieve purification of urine extracts than of plasma. Urine contains not only steroids but also non-steroidal material, both of which may interfere with the analysis. This is very clearly illustrated in a recent paper by Ching et al. (2006), who compared urine cortisol results obtained from use of a direct immunoassay (Immulite: 2000) (DPC – see Table 5.4) with data obtained using an LC-UV system after SPE with Oasis HLB columns. The direct IA gave a normal range of <900 nmol/24 h in comparison to 19–172 nmol/24 h by LC-UV, which is very similar to the range reported by Schoneshofer et al. (1980) (see Table 5.2). A recent investigation using LC-MS/MS compared results using this methodology with results from GC-MS and from two commercial immunoassays, both of which overestimated by 1.6–1.9-fold (Wood et al., 2008).

Improved performance of 'direct' IA methods can often be achieved by solvent extraction prior to assay, usually with dichloromethane (see Table 5.3); polar solvents such as ethyl acetate are not recommended since they can extract polar steroids such as 6β-hydroxycortisol, which can interfere with such assays (Lee and Geoger, 1998). Solid-phase extractions using disposable cartridges (Extrelut, Sep-Pak or Oasis HLB) are now widely used. For satisfactory specificity using IA as the quantitation step, some form of chromatographic purification is required (Schoneshofer et al., 1980), and LC, TLC and Celite chromatography have all been employed. Canalis et al. (1982) described an LC method using a C-18 column and UV detection. An interesting and unusual approach by Nozaki et al. (1991) employed reverse-phase liquid chromatography of the acid-induced fluorescent chromogen of cortisol.

Huang and Zweig (1989) examined a direct RIA method and compared the results obtained with those following extraction and LC. The assays correlated well, but the direct assay on average showed a 33% overestimate compared with LC. Variable recoveries were also found, and considerable cross-reaction with prednisolone occurred. They concluded that the non-extraction method may give misleading results for diagnosis and management. Lewbart and Elverson (1982) combined TLC and LC to measure cortisol and cortisone in urine and demonstrated the enhanced specificity achieved as compared to an RIA method. A more extensive study by Nahoul et al. (1992) compared the accuracy of three commercial immunoassays, one enzyme immunoassay and two RIAs. These authors also used either methylene chloride or ethyl acetate extraction together with column chromatography on Sephadex LH-20. The overall results were compared with a reference method employing LC. The results confirmed that the chromatographic step greatly improved the specificity. They also found that, after chromatography, the levels obtained using the kits were similar to those of the reference technique. The clinical value of UFC measurements in the diagnosis of hypercortisolaemia can be compromised by lack of specificity – a comparison of UFC results by automated immunoassay, described as 'urinary free corticoids' and by LC-UV using samples collected from patients with surgically proven Cushings syndrome revealed a 81% successful prediction by LC, whereas by immunoassay the prediction reduced to 56% (Ching et al., 2006).

Assays for urine cortisol on automated immunoassay platforms have given variable results due to lack of specificity (Gray et al., 2003; Horie et al., 2007). When steroids are extracted from urine, the sample needs to be reconstituted into a

matrix for the immunoassay. Steroid free serum may be better than a buffer or saline (Gray et al., 2003). Cross reactions can be seen with cortisone and metabolites if the urine is assayed directly. High throughput for urine cortisol (20 samples analysed in 2 h) can be achieved using LC-MS-MS after a solid-phase extraction of steroids from urine (McCann et al., 2005) and the method is free from interference from a number of drugs. The method is sensitive to 5 nmol/L and linear to 1,000 nmol/L.

The measurement of cortisol and related compounds in urine is useful in the study of Cushing's disease, Glucocorticoid remediable aldosteronism (GRA), apparent mineralocorticoid excess (AME) syndrome, and related conditions. Taylor et al. (2002) at the Mayo Clinic published a method for the simultaneous analysis of urinary cortisol and cortisone. Measuring both these steroids allows the diagnosis of Cushing's disease and the AME syndrome. They used [$^2H_4$] cortisol as internal standard and took a 0.5 mL urine sample. There was an extensive extraction and washing step with 4.5 mL methylene chloride. An API 2000 tandem quadrupole (Applied Biosystems) was used in the positive-ion mode with MS/MS. Chromatography was conducted on a normal-bore C_{18} column (33 mm × 4.6 mm I.D.) with C_{18} pre-column (4 mm × 2 mm). An isocratic methanol/water solvent system was used at a flow rate of 1 mL/min. The source was of the Turbo-ionspray type, and using a column splitter 200 μL/min was introduced to the source. MRM was conducted in the positive-ion mode monitoring *m/z* 363 → 121 for cortisol, 367 → 121 for [$^2H_4$] cortisol and 361 → 163 for cortisone. Cortisol and cortisone were separated and both were eluted within 2 min. Inter- and intra-assay variation for both compounds was <9% for amounts above 2 μg/dL.

The results of all of these investigators emphasise the need to ensure that the method chosen for urinary cortisol assay should have undergone careful evaluation before being selected for use. It is still highly desirable, if not essential, to carry out extraction and prior purification to ensure that adequate specificity is achieved. Interference can be encountered from drugs (e.g. Fenofibrate) taken by patients even when using LC or LC- MS/MS (Meikle et al., 2003). LC with diode array detection (DAD) has been used for the analysis of cortisol in both urine and serum (Zhang et al., 2006) in the presence of prednisolone. These two steroids, not surprisingly because of the close structural similarity, were not resolved in the LC system used but use of the DAD data and second order calibration based on an alternating trilinear decomposition algorithm, allowed each steroid to be estimated in the presence of the other.

In a urine profile analysis by GC or GC-MS, a number of aspects of cortisol production and metabolism can be examined in a single analysis. This topic is considered in more detail later in this chapter (see Section 5.9). The total daily output of metabolites approximates to cortisol production rates as determined by new stable-isotope procedures (Esteban et al., 1988; Honour et al., 1991). The ratios of metabolites are useful in a number of clinical situations. A high ratio of cortisol to cortisone metabolites is a feature of 11β-hydroxysteroid dehydrogenase type 2 (11β-2HSD2) deficiency. This is associated with an apparent mineralocorticoid excess syndrome (Shackleton et al., 1985), and in a milder form is seen with high intake of liquorice and related drugs, e.g. carbenoxolone and glycerrhitinic acid

(Stewart et al., 1987). Evidence for 5α-reductase deficiency can be found in the ratio of 5α- to 5β-reduced metabolites of cortisol (allo-THF to THF). This allows detection of this disorder of the male sex differentiation from about 6 months of age (Imperato-McGinley et al., 1985).

Cortisol in Other Body Fluids There are few reports of cortisol assays in other body fluids but the specificity of the assay used is generally uncertain and usually has not been properly evaluated for the specific application described. Brotherton (1990) reported the measurement of cortisol in seminal plasma and amniotic fluid, although specificity was not investigated. An LC method for cortisol in amniotic fluid has also been described (Noma et al., 1991). Use of GC-MS for the determination of cortisol in bovine vitreous and aqueous humor has been described (Iqbal et al., 2001). Steroids have been isolated from an ACTH-producing pancreatic tumour and purified by LC before immunoassay of eluted components (Szecsi et al., 2006). Cortisol is quite frequently 'measured' (e.g. Brundu et al., 2006, Holub et al., 2007, Popp et al., 2009) in CSF using commercial IA kits but in none of the papers quoted was a proper evaluation of the application of the methodology to CSF apparently carried out by the authors. This uncritical approach is potentially a very dangerous situation, which can lead to erroneous results. Cortisol and corticosterone have been 'identified' in aqueous humor after TLC separation by 'specific' RIA (Knisely et al., 1994) but the RIA used was a commercial kit designed for use in plasma after diethyl ether extraction and LH-20 fractionation (Oka et al., 1987).

5.7.2 Cortisol Precursors

5.7.2.1 17-Hydroxyprogesterone

Plasma concentrations of 17-hydroxyprogesterone (17-OHP) in normal adults are less than 5 nmol/L (but may be higher depending on the assay used), and more sensitive assays such as immunoassay are required to detect these levels than are needed in the investigation of patients with congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency, where values of hundreds of nmol/L occur. In such patients, this hormone can be detected and measured by a UV detector after LC (Wei et al., 2006). The main value of determining 17-OH-P concentrations in serum or plasma is usually for the diagnosis and, to a lesser extent, the therapeutic management of CAH due to 21-hydroxylase deficiency. Late-onset or non-classical forms are also recognised from high basal levels (above 5–200 nmol/L) with exaggerated response to adrenal stimulation from ACTH injection (White et al., 1987), and the distinction of these levels (60–600 nmol/L) from normal (2–10 nmol/L) and heterozygotes for the classical disease (5–60 nmol/L) poses different analytical demands.

The antisera commonly used in immunoassays of this hormone have usually been raised against 17-OH-P coupled through a carboxymethyloxime (CMO) of the 3-carbonyl group to protein such as bovine serum albumin (BSA) (Abraham et al., 1971).

The antisera have high affinity for 17-OH-P although few of the BSA conjugates yield antisera with affinity 'for the steroid' as high as a haemocyanin enzyme prepared from the keyhole limpet (2×10^{10} L/mol) (El-Gamal et al., 1987). The antisera can be used at very high dilution (typically 1:50,000–1:500,000). Many radioimmunoassays use tritiated tracer (specific radioactivity 20–100 Ci/mmol), and the methods may or may not include sample extraction with or without a purification step. A non-isotopic immunoassay for the measurement of 17-OH-P in saliva from children and adolescents has been described (Dressendorfer et al., 1998).

The diagnosis of 21-hydroxylase deficiency is important in confirming or refuting the nature of the metabolic defect in a virilised newborn girl or a newborn male infant presenting with an adrenal crisis (Hughes et al., 1979). An initial extraction of the free steroids into an organic solvent is a useful step, leaving the steroid sulphates derived from the large fetal adrenal gland in the aqueous phase. The fetal adrenal cortex secretes a number of related steroids (e.g. 17α-hydroxypregnenolone sulphate) that are present in high concentration in the plasma and may react with the antisera and lead to spuriously high results. A number of problems have been reported with kits for 17-OH-P due to such interference (Makella and Ellis, 1988; Wong et al., 1992). In the validation of a blood spot assay to screen all babies in Scotland for the defect, it was clearly shown that interference in the 17-OH-P assay was greatest in stressed babies and preterm infants, both of whom might be expected to have high secretion and circulating levels of fetal zone steroids (Wallace et al., 1987). When the 17-OH-P radioimmunoassays were performed on an organic solvent extract of the blood spots rather than on steroid eluted directly into assay buffer, the 17-OH-P results were much lower, and in better agreement with plasma assay data. Preterm infants can have 17-OH-P levels above those seen in term babies.

Fluorescent and chemiluminescent enzyme immunoassays have also been developed (Tsuji et al., 1987) with the objective of improved sensitivity for neonatal screening of blood levels from spot samples on filter paper. The use of 4-carboxyethylthio-17-OH-P as hapten increased the assay sensitivity and specificity.

High specificity of the method is much more important when a clinical diagnosis of a disease requiring lifelong treatment is to be made. The recognition of late-onset form of the disease (LOCAH) is based on finding raised 17-OH-P levels basally and more especially 30 or 60 min after an intravenous or intramuscular injection of 250 μg of ACTH (synthetic adrenocorticotrophic hormone 1–24). NEQAS data show wide ranges of results for samples containing 17-OH-P below 30 mmol/L, and the accuracies of methods need to be established. In many cases, there is poor endocrine discrimination of known heterozygotes for the classical disease from normal subjects and patients suspected of having LOCAH (Hague et al., 1989). Most commercial kits for 17-OHprog are calibrated for 0–60 nmol/L. GC-MS analysis of 17-OHP has been available for many years (Wudy et al., 2000) based on isotope dilution and preparation of heptafluorobuyrate derivatives. Simultaneous assay of androstenedione, testosterone, dihydrotestosterone and DHEA makes this a useful diagnostic test for congenital adrenal hyperplasias (Wudy et al., 1995). Derivative formation can now be speeded up using microwave irradiation (Deng et al., 2005). LC-MS/MS has been used for the simultaneous

determination of both 17-hydroxyprogesterone and 17-hydroxypregnenolone (Higashi et al., 2008) in dried blood spots.

17-OH-P is metabolised to 5β-pregnane-3α, 17α, 20α-triol, mainly in the liver, and to different 17α-hydroxypregnanolones (see Section 5.5). These steroids are conjugated with glucuronic acid before excretion in urine. In CAH, due to 21-hydroxylase deficiency, 21-deoxycortisol levels are also raised and this is excreted in the urine, mainly as 11-oxopregnanetriol (Homma et al., 2004). In newborn infants a number of other polar metabolites are excreted within a complex pattern of metabolites. 15β,17α-Dihydroxpregnanolone is a marker for the defect in the newborn, and is more reliable than pregnanetriol (Honour, 1986). Capillary column gas chromatography with mass spectrometry is the method of choice for definitive diagnosis of CAH. The mixture of steroids in urine from a newborn infant is very different from that at other times of life, due to the large amounts of steroids derived from the fetal adrenal cortex. Cortisol metabolites tend to be oxidised and excreted as cortisone metabolites. After initial Sep-Pak extraction of steroid conjugates, the steroids can be grouped according to their conjugation with glucuronic acid or sulphuric acid. A Sephadex LH-20 chromatography system is available to do this. The glucuronide steroids elute with a salt-saturated solvent mixture of chloroform/methanol (1:1, v/v). The sulphates are eluted later with methanol. After hydrolysis, the two steroid groups can be separately derivatised and run by GC-MS, allowing for the fact that 10–20 times more sulphated steroid will be found from the fetal zone than the free and glucuronide conjugated corticosteroid metabolites from the definitive adrenal zone. The latter fraction will contain the steroids that most reflect abnormal function in the case of congenital adrenal hyperplasia.

LC-MS/MS methods for 17-OHP have been described that include solvent extraction (Turpeinen et al., 2005) and solid phase extraction (Etter et al., 2006) and online SPE (Rauh et al., 2006). 17-OHP has been converted to derivatives in order to improve sensitivity. Whether this is needed will depend on the instrument (see Chapter 3). 17-Hydroxyprogesterone can be measured in saliva and when assays are performed on samples collected at regular intervals during a 24 h period (less often during the night-time) the results give a good picture of the success of treatment for CAH (Hughes and Read, 1982; Groschl et al., 2001; Groschl et al., 2002, 2003; Mylonas et al., 2006; Shibayama et al., 2008). Other GC- and LC-MS methods for 17-hydroxyprogesterone are listed in Table 5.5. In cases of raised 17-OH-progesterone levels, further investigation can be carried out using blood spots collected on Guthrie cards, using LC-MS/MS to determine the precise location of the defective enzyme (Peter et al., 2008) by measuring concentrations of 21-deoxycortisol, 11-deoxycortisol, 11-deoxycorticosterone, 17-hydroxyprogesterone and cortisol.

5.7.2.2 21-Deoxycortisol

This steroid is a key analyte overproduced in congenital adrenal hyperplasia (CAH). This is the precursor of the urinary metabolite pregnanetriolone, which has long been considered a hallmark analyte for confirming CAH in infancy. Early

methods for this analyte in blood (Cassorla et al., 1980; Milewicz et al., 1984; Gueux et al., 1985, 1988; Fiet et al., 1989; Nahoul et al., 1989) are based on extraction and chromatography before immunoassay using tritium-labelled steroids. RIA, after Celite mini-column chromatography (Fiet et al., 1994a, b) and LC (Hill et al., 1995; Fernandes et al., 2003; Tonetto-Fernandes et al., 2006; Claahse-van der Grinten et al., 2007) has continued to be used and an HPLC-UV method has also been published (Saisho et al., 1990). An IA method based on time resolved fluorescence improves throughput (Fiet et al., 2000). GC-MS urinary profiles (see below) have been and still are widely used for the diagnosis of CAH due to 21-hydroxylase deficiency by the methods (Caulfield et al., 2002) introduced by Shackleton and colleagues (Adachi et al., 2004; Homma et al., 2004; Mussig et al., 2006). Cristoni et al. (2004b) report the analysis of 21-deoxycortisol by ES-MS and APCI using a Thermo Finnigan LCQ ion-trap instrument operated in the positive-ion mode (Cristoni et al. 2004a). There are a number of more modern LC-MS/MS methods for 21-deoxycortisol in blood spots and serum (Janzen et al., 2007, 2008).

5.7.2.3 11-Deoxycortisol (Compound S)

This steroid is measured when disorders of the enzyme 11β-hydroxylase are suspected, and in the metyrapone test. Metyrapone (2-methyl-l,2-bis-(3-pyridyl)-1-propanone) prevents the conversion of 11-deoxycortisol to cortisol by blocking 11β-hydroxylation. In normal subjects the resultant fall in plasma cortisol concentration evokes an increased release of ACTH, which in turn augments adrenal steroidogenesis. The metyrapone test has therefore been used to evaluate pituitary reserve. The increase in adrenal activity after metyrapone administration is detected from the increase in 17-hydroxycorticosteroid excretion in the urine. Radioimmunoassay has permitted more precise quantification of the response by making it possible to measure plasma 11-deoxycortisol levels. LC has been used to measure plasma cortisol and 11-deoxycortisol concentrations simultaneously (Reardon et al., 1979).

Most assay methods for 11-deoxycortisol have used an initial solvent extraction step because of the lack of specificity of the antisera, particularly with regard to the cross-reaction from cortisol. Solvent mixtures have been utilised which fully extract 11-deoxycortisol whilst poorly recovering cortisol (e.g. chloroform/carbon tetrachloride; Cassorla et al., 1980). Perry et al. (1982) were able to displace 11-deoxycortisol from binding proteins by dilution of the sample (1 in 10) in pH 4 buffer.

11-Deoxycortisol levels in body fluids have been measured by LC with UV detection (Reardon et al., 1979) and by radioimmunoassay (Mahajan et al., 1972; Kao et al., 1975; Lee and Schiller, 1975; Perry et al., 1982). Large numbers of samples can pose problems in LC methods. For immunoassay, antisera are raised to 11-deoxycortisol-2l-hemisuccinate or 3-(O-carboxymethyl)oxime conjugated to bovine serum albumin. The latter conjugate gives antisera of greater specificity. Most assays have used ^{3}H-11-deoxycortisol as radiolabel, but Perry et al. (1982)

described an assay based on the ^{131}I-iodohistamine of 11-deoxycortisol. No assays, to the authors' knowledge, have been fully validated in newborn infants. In some patients with CAH due to 21-hydroxylase deficiency, high artefactual plasma concentrations for 11-deoxycortisol have been found which did not then correlate with the urine steroid profile data using GC-MS, which confirmed high excretion rates only for 17-hydroxyprogesterone metabolites.

Enzyme immunoassay of serum 11-deoxycortisol has been described using alkaline phosphatase as a label, which is stable for about 1 year (Kobayashi et al., 1984). The conjugate was synthesised from 11-deoxycortisol-2l-amine. In the assay, 11-deoxycortisol was purified by Sephadex LH-20 column chromatography after an initial solvent extraction because of the low specificity of an antiserum to a 21-hemisuccinate of 11-deoxycortisol coupled to BSA. An assay for 11-deoxycortisol has been developed recently based on anti-idiotype antibodies in a non-competitive ELISA with sensitivity to 2.9 fmol (Kobayashi et al., 2003). The assay has been further modified recently (Kobayashi et al., 2006) producing an immunoenzymatic assay for 11-deoxycortisol able to detect 20 amol/assay. 11-deoxycortisol can be measured by GC-MS after Sephadex LH-20 chromatography and formation of heptafluorobutyrate derivatives (Wudy et al., 2002).

A sensitive time-resolved fluoroimmunoassay for 11-deoxycortisol in human serum has been developed using 0.5–1 mL sample extracted with cyclohexane/EtAc (1:1). After evaporation and bringing it to a state of dryness the 11-deoxy cortisol is separated from other less polar steroids on a Celite column using Ethylene glycol as a stationary phase. The 11-deoxy-cortisol was eluted with iso-octane/dichloromethane (53:47) and assayed using RIA and the new TR-FIA. A biotinylated 11-deoxycortisol tracer was synthesised through C3 (Fiet et al., 2001a). Three deoxycorticoids (11-deoxycortisol, 11-deoxycorticosterone and 21-deoxycortisol) can be measured in a single extract by separate immunoassay after LC purification (Hill et al., 1995). 11-Deoxycortisol is included in the steroid panel by TMS of Guo et al. (2004) and Guo et al. (2006), reported above, monitoring the transitions *m/z* 347→97 for analyte and 349→97 for [$^{2}H_{2}$] internal standard.

Tetrahydro-11-deoxycortisol (THS) is the main metabolite of 11-deoxycortisol in the urine, although in the first week of life an infant with congenital adrenal hyperplasia due to 11β-hydroxylase deficiency may have high excretion rates for 6β-hydroxy-THS (Hughes et al., 1986) by GC-MS and this is the technique of choice for the analysis. THS has been measured in large patient groups with hypertension in conjunction with genetic studies of *CYP11B1* (Keavney et al., 2005; Barr et al., 2007).

5.7.2.4 17α-Hydroxypregnenolone

If ACTH stimulation tests are performed in patients with clinical signs of excess androgen production, increased production of 17α-hydroxypregnenolone may provide evidence of mild defects of 3β-hydroxysteroid dehydrogenase activity – (Type II deficiency) (Johannsen et al., 2005; Mermejo et al., 2005).

Baseline 17α-hydroxypregnenolone and DHA concentrations, although not as high as in patients with classical 3β-hydroxysteroid dehydrogenase deficiency, may also be above the normal levels. 17-Hydroxypregnenolone is usually extracted from plasma and purified by Celite chromatography before RIA (e.g. Brenner et al., 1973; Aedo et al., 1976; Cassorla et al., 1980; Pang et al., 1985; Hague et al., 1989) using tritiated label. An RIA using ^{125}I-label has also been described (Fiet et al., 1994b) – again after separation by Celite column chromatography. An RIA system has been described utilising RIA after automated Sephadex LH20 chromatography (Riepe et al., 2001) and has been used for a longitudinal study of the plasma concentration of 17-hydroxypregnenolone in pre- and full-term neonates and in the neonatal period (Riepe et al., 2002). Fiet et al. (2001b) have described the development of a TR-FIA, which uses Europium fluorescence and is non-isotopic. A GC-MS isotope dilution (using d_7-labelled IS) method has been developed (Wudy et al., 2001) using a simple GC-MSD system after extraction and separation on mini-LH20 columns and derivatisation as heptafluorobutyrates. Other GC-MS methods have been described, using human serum after solvent extraction and TMS derivatives (Starka et al., 2006) or extraction and purification on SPE C18 and NH2 cartridges and TMS derivatives for the analysis of fish plasma and bile (Budzinski et al., 2006). More recently an LC-MS/MS electrospray (+) method (Kushnir et al., 2006) has been described which includes 17-hydroxypregnenolone amongst a panel of four serum/plasma steroids (see Table 5.5), using d_3 internal standard. 17-hydroxypregnenolone sulfate has also been measured by RIA (Hill et al., 1999) in human serum after pre-extraction of 17-hydroxypregnenolone with ether, recovery of the aqueous phase, methanolysis and re-extraction (Vcelakova et al., 2007). Homma et al. (2003) have used GC-MS to measure 17-hydroxypregnenolone in the urine of Japanese newborn infants as an CMO-TMS ether derivative as part of a 50 steroid profile using the method of Caulfield et al. (2002).

5.7.3 6β-Hydroxycortisol

The urinary concentration of 6β-hydroxycortisol was first identified in human urine by Burstein et al. (1954) and has been used for many years as a marker of CYP3A activity. Alternatively the 6β-OHF/F ratio can be used for the same purpose (Luceri et al., 2001) but criticism has been levelled at this approach (Chen et al., 2006; Fenske, 2007 and reply by Chen et al. on following page). An excellent and comprehensive review of 277 references on this topic up to 2003 was published by Galteau and Shamsa (2003). The development of methods for the determination of this steroid is a very good illustration of the progress of corticosteroid methods in general, starting with the use of paper chromatography and colorimetric assay (Katz et al., 1962) and progressing to LC-MS/MS (e.g. Dilger et al., 2007). The methods available for 6β-OH-cortisol have been fully detailed by Galteau and Shamsa (2003) and a survey of papers on this topic published between 2003 and late 2007 identified only a few fully described methodology papers on this topic, using LC with UV detection

(Furuta et al., 2004; Hu et al., 2005; Micuda et al., 2007) and LC-MS/MS (Chen et al., 2006) without derivatisation. Micuda et al. (2007) pointed out that while LC-MS/MS may provide more definitive results, the methodology may not always be available in routine clinical laboratories. Barrett et al. (2005) describe an LC-MS/MS method with automated online SPE (Prospect-2™ extraction apparatus), separation on a RP-18 column (eluting with a formic acid/water/methanol gradient) at 60°C and measurement/detection by negative ion ESI, monitoring for 6β-OHF, the transition being 423.5:347.5. Dilger et al. (2007) also describe the use of a 'validated' LC-MS/MS method and although no full description of the methodology is given, it might be inferred that they used a modification of a method described previously for budesonide (Hempfling et al., 2003). A study of Table 5.4 identifies the recent use of at least two commercial immunoassays for urine 6β-OHF (Chen et al., 2004b; El Desoky et al., 2005). It is worth noting that 6α-OHF as well as 6β-OHF has been demonstrated to be formed *in vitro* from [^{3}H]cortisol by human liver, the steroids being extracted with ethyl acetate, and identified by co-migration with authentic standards in LC and by EI and CI mass spectrometry (Abel et al., 1992). A monoclonal immunoassay for 6β-hydroxycortisol developed by Yeung et al., (1997a, b) had a 50% cross reaction with the 6α-epimer but it was noted that this potential interference was not significant as the epimer was formed only in small amounts.

5.8 Analysis of Mineralocorticoids

5.8.1 Relevance

The clinical rationale for the measurement of mineralocorticoids relates to conditions with MC deficiency or excess. Detailed reviews are available elsewhere (Stowasser et al., 1996; Vallotton, 1996a, b; Stowasser and Gordon, 2000; Gordon and Stowasser, 2007). While we have given some historical coverage, emphasis has been on the approaches currently in use or those under development. Measurement of aldosterone concentration in plasma and urine is thus an essential aid to the diagnosis and differential diagnosis of 'primary hyperaldosteronism' (Schirpenbach and Reincke, 2007; Young, 2007). Briefly, it was originally defined as autonomous hypersecretion from a unilateral benign adrenal tumour in which high levels of aldosterone are accompanied by suppressed plasma renin activity or concentrations of plasma renin and low concentrations of plasma K^+. This condition is rare and in addition to peripheral and adrenal vein blood aldosterone, renin and cortisol, diagnosis can be confirmed by visualising the adenoma using CT or NMR scanning, in response to MC receptor antagonists and by the effects of removing the adenoma. These tests also serve to distinguish it from the related condition, bilateral nodular adrenal hyperplasia (see review: Vallotton, 1996a, b). It has been claimed that simultaneous evaluation of 18-hydroxycorticosterone (Biglieri and Schamberlan, 1979; Kem et al., 1985) or 18-hydroxycortisol and 18-oxocortisol (Ulick and Chu, 1982) aid differential diagnosis. With the exception of the inherited form of primary hyperaldosteronism,

glucocorticoid-remediable aldosteronism (GRA) (Ulick, Wang & Morton, 1992), this has not been our experience. In addition to high aldosterone:renin ratio (ARR), GRA patients have very high levels of 18-oxygenated steroids. Definitive diagnosis of GRA depends on molecular biology tests (Lifton et al., 1992a, b).

There is currently, considerable interest in the role of aldosterone excess in patients with essential hypertension. Indeed a high aldosterone/renin ratio (ARR) (Gordon et al., 1993; (Kaplan 2001) is present in up to 10% of all hypertensive patients, most of whom will have bilateral adrenal hyperplasia but may respond more optimally to targeted MC blockade. Recent studies have also shown that aldosterone is an important risk factor in heart failure (Pitt et al., 1999) and that even small increases can predispose to cardiovascular disease (Vasan et al., 2004). Furthermore, hypokalaemia is now regarded as an insensitive indicator and should no longer be a requirement for further testing and screening for primary aldosteronism (Gordon and Stowasser, 2007). Higher perceived incidence may increase the demand for simple, economic (but specific – Stowasser and Gordon, 2006) methods for aldosterone (and renin) assay.

Massive DOC excess, suppressing the production of both aldosterone and renin, is characteristic of both 11β-hydroxylase and 17α-hydroxylase deficiencies. In the former condition, 11-deoxycortisol levels are also elevated and those of cortisol lower; in the second condition, 11-deoxycortisol and cortisol levels are low but those of corticosterone are very high (see review by Fraser, 1982). Again, these genetic disorders are rare and biochemical analysis can be supplemented by molecular biology tests to reach a diagnosis. However, much greater demand for these assays may result from the discovery that 11β-hydroxylation is commonly mildly impaired in essential hypertension (Simone et al., 1985), that this variability in this deficiency is heritable (Inglis et al., 1999) and that there is a clear statistical relationship with raised ARR in these patients (Lim et al., 2002; Connell et al., 2003).

Profiling of mineralocorticoids or their metabolites in blood and urine may become a more frequent demand for the above reasons and because of the increasing need for the accurate phenotyping of the genetic variation made more obvious by improvements in molecular biology (Halushka et al., 1999). This will determine the choice between those methods that study individual steroids and those that study groups of compounds simultaneously. The options for each approach are summarised below. The two important mineralocorticoids, aldosterone and DOC are discussed in detail together with corticosterone. Other zona glomerulosa steroids are discussed as a group.

5.8.2 Aldosterone

Aldosterone production rate is the key index of normal zona glomerulosa function. Assays are therefore important in the investigation of the control of salt and water metabolism and of blood pressure in human subjects, animals such as the dog and the rat, and *in vitro* tissue-incubation systems. Production rate and plasma concentration are controlled by potassium intake, by sodium status through the action of the renin-angiotensin system, and by posture. Aldosterone is less dependent than other

corticosteroids on ACTH (Fraser, 1982). Reliable clinical interpretation requires that electrolyte status and posture are noted or preferably standardised, and that concurrent renin concentration is measured. Plasma cortisol is a valuable control assay. Principal clinical applications are in diagnosing the various forms of hyper- and hypoaldosteronism (Armanini et al., 1987; Fraser et al., 1989).

Three different indices of aldosterone production are available, plasma concentration and urinary excretion rate of either aldosterone-18-glucuronide or tetrahydro (TH)-aldosterone. Plasma analysis is appropriate in the study of short term variability and is necessary when correlation with concurrent plasma renin (i.e. ARR) is sought. Plasma aldosterone levels are posture-dependent; a short period of recumbency before venesection is desirable. Extremes of dietary salt intake are also a confounding factor.

Historically, several quantitation techniques of varying sensitivity have been used. Immunoassays, most commonly radioimmunoassays, are now the methods of choice for routine clinical investigation. Chemiluminescence and ELISA methods have also been described (Schwartz et al., 1990; Stabler and Siegel, 1991). Immunoassays for corticosteroids are summarised in Table 5.4. Antisera of high affinity and specificity are readily available commercially as are ^{125}I- and ^{3}H-aldosterone. The latter compound tends to isomerise at C17 and should be periodically re-purified. Improved antisera characteristics now allow assay in 100–200 μL of plasma without prior extraction or partial purification. Samples, labelled standard and antiserum are incubated. Separation of bound and free hormone is usually accomplished by dextran-coated charcoal or the use of antibody-coated tubes. Ideally, unlabelled authentic compound for constructing a standard curve must be dissolved in ‘aldosterone-free’ plasma. In practice, either charcoal-stripped plasma or commercially, an artificial matrix of undisclosed composition is used. These lack competing steroids and other non-specifically interfering compounds present in the sample so much so that results tend to be higher than those from methods involving pre-purification. In the majority of samples, this discrepancy is small and the simplicity of direct assays results in a high degree of precision.

Commercial aldosterone assay kits (see Table 5.4) are largely designed for use with human plasma and should not be used for other media without careful evaluation. Salivary aldosterone assays provide a valuable index of aldosterone status during pregnancy, in infants or where frequent sampling is required; the constraints of direct assays have been discussed (Few et al., 1984). Similarly, tissue and faecal analysis also need extraction and purification before assay. Species differences in steroid profile affect these assays (e.g. Abayasekara et al., 1993). Rat plasma contains high concentrations of 18-oxygenated steroids that may explain the tendency of kits, in our experience, to overestimate the aldosterone concentration. In contrast, they tend to underestimate canine aldosterone levels. Finally, human plasma samples from patients with steroid metabolic disorders which cause abnormal plasma corticosteroid patterns cannot be analysed reliably by direct assay. For example, in both chronic high ACTH stimulation and classical 17α-hydroxylation, plasma aldosterone concentration is suppressed to very low

levels; because concentrations of other corticosteroids are very high, direct assays may give spuriously high aldosterone readings.

In the past, a series of aldosterone assays involving extraction and purification prior to immunoassay have been described. It is unlikely, not least because of economic factors, that these would now be considered for routine use. Nevertheless, they retain value as means of initially evaluating simpler methods and for the confounding situations described above. Extraction by solvent partition (e.g. dichloromethane) or solid phase substrates (e.g. C-18 cartridges) predominate. Partial purification by chromatography, for example, paper (Bayard et al., 1970; Mayes et al., 1970; Fraser et al., 1973; West et al., 1973), Sephadex LH20 or Celite (e.g. Ito et al., 1972; Salmenpara and Kahri, 1976; Sippell et al., 1978), or by LC (O'Hare et al., 1976; Schoneshofer et al., 1981; Wei et al., 1987), is necessary. Farmer et al. (1972) used aldosterone-γ-lactone as the antigen to simplify initial sample purification.

Two quantitation techniques for aldosterone as alternatives to immunoassay have been used, gas-liquid chromatography (GLC) with electron capture detection (EC) (Mason and Fraser, 1975) and gas chromatography-mass spectrometry (GC-MS) (Dikkeschei et al., 1991; Stockl et al., 1991). Both these GC-MS methods have the advantage of positive analyte identification as well as quantitation and, for this reason, are valuable calibrating or confirmatory methods for immunoassay. The immense sensitivity of GLC-EC (within the femtogram range for standards) is offset by susceptibility to detector contamination by biological extracts and subsequent loss of sensitivity. Moreover, the detector contains ^{90}Sr, so precautions in handling and eventually disposing of the detector are obvious. Extracted aldosterone must be converted to the γ-lactone by periodate oxidation which is then derivatised to the heptafluorobutyrate (Mason and Fraser, 1975). Schemes for plasma aldosterone analysis by GCMS, using either methyloxime-trimethylsilyl ethers or heptafluorobutyrates, have been described; in our experience, even using specific ion monitoring (SIM) and tandem MS, sensitivity (>100 pg injected) is inadequate for plasma analysis. However, these methods find wider application in urine analysis. An early MS-MS system has been described for aldosterone assay at nanogram levels in complex biological mixtures (Prome et al., 1988).

An important development of recent years has been the greater accessibility of LC-MS/MS technology. As stated earlier, tandem MS offers an improved degree of specificity and LC maximises separation from similar compounds and from matrix-derived contaminants. Improvements in computer software greatly simplify optimisation of the ionisation/fragmentation process and subsequent quantitation. There have been recent descriptions of the application of LC-MS/MS to the measurement of aldosterone in human serum (Turpeinen et al., 2008; Yamashita et al., 2008a; Taylor et al., 2009) and Fig. 5.6 shows the LC-ESI(+)-MS/MS chromatograms produced using the method of Turpeinen et al. (2008), which did not use derivatisation and suggested a limit of quantitation (S/N = 10) of 30 pmol/L. Interestingly this LC-MS/MS method is very similar to that described by Fredline et al. (1997) – nearly 10 years ago. The method of Yamashita et al. (2008a and b) used ethyl ether-picolinyl derivatisation (Yamashita et al., 2007b) which seems to improve the sensitivity to 11 pmol/L – indeed a comparison of sensitivity between underivatised ESI(−) to derivatised ESI(+)

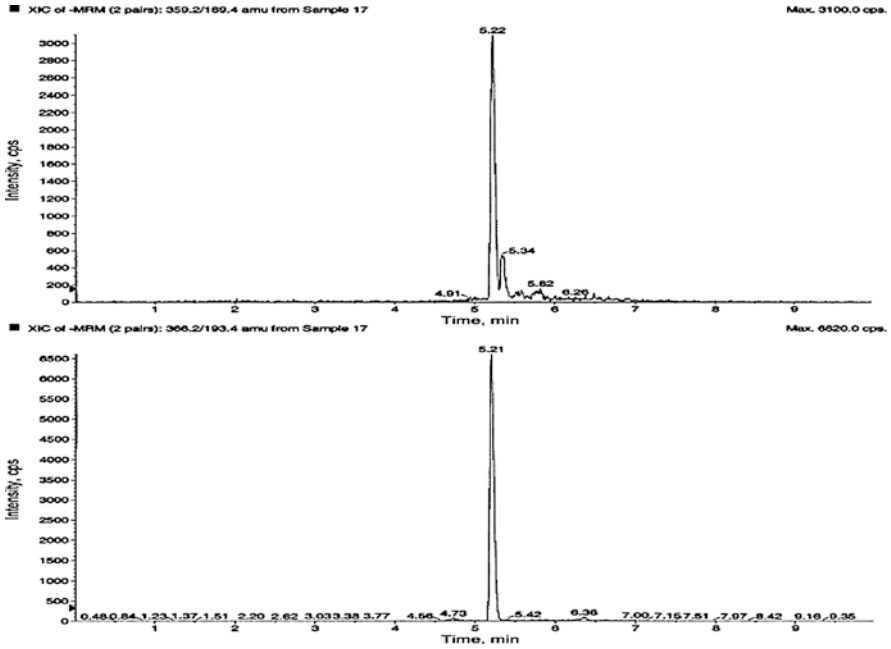

Fig. 5.6 LC–MS/MS electrospray chromatograms from a patient serum containing 704 pmol/L aldosterone. Panels: Ion chromatogram following the fragmentation 359/189 of aldosterone (*upper*) and 366/193 of IS (*lower*) (from Turpeinen et al., 2008, with permission; copyright Elsevier BV)

suggested a tenfold improvement in sensitivity but a comparison of the two methods suggests only a threefold improvement. There are three publications describing what appear to be the only LC-MS/MS methods developed over the past 10 years for aldosterone alone (Yamashita et al., 2008a & b and Taylor et al., 2009) although it is measured as part of steroid 'profiling' procedures in serum (e.g. Guo et al., 2006; Holst et al., 2007 together with *inter alia* cortisol and 17-hydroxyprogesterone) using LC-MS/MS and in urine by similar profiles using GC-MS/MS (Homma et al., 2003). The dearth of specific GC- and/or LC-MS methods for measurement of aldosterone suggests that results from immunoassay methods are regarded as satisfactory in most clinical situations, although there has been a call for urgent improvement of aldosterone assays (Stowasser and Gordon, 2006). Twenty years ago, the use of tandem MS/MS was investigated for aldosterone assay (Prome et al., 1988). A recent publication describes the development of a method for aldosterone by LC-ESI(+)-MS/MS using an ethyl ether-picolinyl derivative (Yamashita et al., 2008a and b) but it was not evaluated except briefly for use in serum/plasma.

Tissue analysis is also possible (Ohtani et al., 2007) but this paper demonstrates the weakness of LC-MS/MS relative to GC-MS in that the specificity of detection relies primarily on the retention time in the LC system as the transition monitored in the MS/MS (loss of 18 amu) is not specific for aldosterone. The technique generally is not yet devoid of potential pitfalls. Lambert (2004) points out the problems of

matrix effects and adduct formation as concerns which 'should be considered in any bioanalytical method based on LC-MS/MS'. It is not clear exactly how significant these problems are for corticosteroid analysis when an isotope labelled-analyte is used as the internal standard. An earlier general study of adduct formation in a wide spectrum of corticosteroids by Honing et al. (2000) summarises the conditions under which adducts form and stabilise. It refers mainly to acetate adducts but adducts of sodium, potassium and ammonium also occur. An example of an LC-MS/MS method involving the selection of a chloride adduct ion in LC-MS/MS and monitoring the chloride ion lost during CID to quantitate cortisol levels is described earlier in this chapter (Huang et al., 2007). Although immunoassay methods, particularly 'direct' or non-extraction methods, for aldosterone in plasma/serum are seldom specific, they are widely used for clinical diagnostic purposes. The advent of LC-MS/MS may provide not only valuable reference methodology but also the means for measuring aldosterone as part of a profile (see below).

The urinary excretion rates of two unique aldosterone metabolites, aldosterone-18-glucuronide, representing about 10% of total excretion and 3α,5β-tetrahydroaldosterone (TH-aldo) representing about 40%, have been used as indices of aldosterone secretion rate. Aldosterone-18-glucuronide is uniquely hydrolysed at pH 1. Thus, after pre-extraction of the urine sample, for example with dichloromethane (note: redistillation of this solvent is advised since it may generate destructive phosgene during storage), to remove all free steroids, acidification of the sample (HCl), incubation and re-extraction, only free aldosterone should be removed by a second extraction. Radioimmunoassay or, less commonly, GCMS have been used to quantitate aldosterone content (e.g. Vetter et al., 1973). The simplicity of this assay makes aldosterone-18-glucuronide the metabolite of choice where aldosterone excretion rate alone is sought. Excretion rates of this metabolite and those of TH-aldosterone in 155 hypertensive patients correlated closely ($r = 0.365$; $p < 0.001$) (E.M. Freel et al., unpublished observations, 2005) despite their different modes of metabolic clearance (aldosterone-18-glucuronide is a renal metabolite; TH-aldo is a hepatic metabolite). Where analysis is part of a urinary steroid profile (see below), the TH-metabolite may be a more convenient analyte (most other corticosteroids are excreted as glucuronides in man). For theoretical reasons (Tait et al., 1965), TH-aldosterone glucuronide is more appropriate for measuring aldosterone secretion rates by isotope dilution although this assay finds little use today (Ulick et al., 1958; Cope et al., 1961). After solid phase extraction and hydrolysis, TH-aldosterone is most conveniently quantitated by GLC (Wotiz et al., 1961) or GC-MS as the methyloxime-trimethyl silyl ether (Honour and Shackleton, 1977; Ulick et al., 1991). Ulick et al. (1991) combined assay of TH-aldosterone with simultaneous assay of the TH derivatives of 18-hydroxycorticosterone, 18-hydroxycortisol and 18-oxo-cortisol by GC-MS. Simplicity and sensitivity could in theory be improved by the use of LC-MS, particularly if, as has been suggested, the conjugates are extracted and analysed without prior hydrolysis (Shackleton and Straub, 1982; Shackleton, 1993). Although LC-MS-based methods have been described for synthetic and for naturally occurring C19 steroids (see later), and have the potential for aldosterone or TH-aldosterone assay, their respective

glucuronide standards are not readily available and this is currently an impediment to direct conjugate assay. In the future this could be overcome by recombinant technology or enzyme assisted synthesis used elsewhere (Kuuranne et al., 2003; Hintikka et al., 2008).

5.8.3 11-Deoxycorticosterone (DOC) and Corticosterone

Corticosterone in biological material is almost always assayed by immunoassay. Methods based on radioactively labelled or enzyme labelled standard and antisera to the 3-carboxymethyl-BSA or 21-hemisuccinate-BSA conjugate have been described. The high plasma concentrations in the rat allow direct assay, for example by a commercially available kit, but, in our experience, lower, more reproducible results are obtained if protein is first precipitated with alcohol (Gomez-Sanchez et al., 1975). Human plasma samples require prior extraction and partial purification, for example by solvent partition (Etches, 1976), paper chromatography or chromatography (Vagnucci et al., 1974) on Celite or Sephadex LH20 (Gross et al., 1972; Sulan et al., 1978). In the rat, faecal excretion rate provides a non-invasive index of production (Pihl and Hau, 2003). Solvent extraction followed by solid phase adsorption and differential elution and radioimmunoassay gives reproducible results. A method based on GLC-EC has also been published (Mason and Fraser, 1975).

Unique metabolites are TH-corticosterone, alloTH-corticosterone and TH-11-dehydrocorticosterone. After conjugate hydrolysis and solid-phase extraction, these are readily measured as their methyloxime-trimethylsilyl ethers by GLC with flame ionisation detection or by GC-MS (Shackleton 1985).

DOC is a corticosteroid with no unique chemical characteristics distinguishing it from other corticosteroids. All methods for plasma analysis, whether by double isotope derivative assay (Arnold and James, 1972; Oddie et al., 1972), GLC-EC (Wilson and Fraser, 1971) or immunoassay, require extraction and extensive purification. Antisera are too non-specific for direct immunoassay (Fraser et al., 1975; Schoneshofer et al., 1975). A commercial kit is available but sample extraction and solvent partition are necessary before assay.

THDOC is the unique urinary metabolite and can be assayed as the methyloxime-trimethylsilyl ether by GC-MS (Shackleton 1985).

5.8.4 Other 18-Hydroxy- and 18-Oxo-Steroids

5.8.4.1 18-Oxygenated Mineralocorticoids (18-Hydroxy-Corticosterone and 18-Hydroxy-Deoxycorticosterone)

There are few situations in which 18-hydroxycorticosterone (18-OHB) analysis has significant clinical value (see above) but plasma and urine analysis remain of some interest; for example, it is relevant in *in vivo* and *in vitro* research on aldosterone

synthesis. Measurement of serum 18-OHB has been used in the differential diagnosis of primary hyperaldosteronism (adrenal adenoma (APA) or bilateral hyperplasia (IHA)). However supine plasma concentrations of 18-OHB seem to parallel those of aldosterone (Phillips et al., 2000) and do not seem to provide greater discrimination than aldosterone concentration alone and is now considered to have an accuracy of less than 80% in distinguishing between APA and IHA (Young, 2007). Although high serum 18-OHB concentrations suggest APA rather than IHA, measurement of 18-OHB/aldosterone ratios during adrenal vein sampling were useful but not as reliable as aldosterone/cortisol ratios in lateralisation (Auchus et al., 2007). Both immunoassay and physicochemical quantitation have been described. Antisera have been raised to both the γ-lactone (e.g. Martin et al., 1975) and the BSA-3-CMO derivative of the unmodified steroid (Belkien et al., 1980; Watanabe et al., 1984). Pure 18-OHB is available commercially but labelled antigen must now be prepared locally. ^{3}H-steroid has been biosynthesised from labelled precursors using duck adrenal glands. Alternatively, ^{125}I-histamine or alkaline phosphatase have been used (Watanabe et al., 1984). No reliable direct immunoassay has been achieved and extraction from plasma or saliva (solvent or solid phase) and partial purification (paper or thin layer chromatography, LC) (O'Hare et al., 1976; Schoneshofer et al., 1981, 1982; Lee et al., 1988) or LH-20 (Riepe et al., 2003) is necessary. 18-Hydroxycorticosterone interacts unpredictably with alcoholic solvents and may also dimerise in solution (Fraser and Lantos 1978). Alkaline conditions reduce or prevent this degradation and thus all solutions and solvents should therefore contain triethylamine (ca1%). There may also be specific problems with 18-hydroxylated corticosteroids when attempting reverse-phase LC if the reverse-phase material is not fully end-capped (O'Hare et al., 1980).

A GC method using electron capture detection was devised before the availability of immunoassay. It required complicated preparation of the 11-oxo-γ-lactone by periodate and chromic acid oxidation (Wilson et al., 1976). Although sensitive and specific, it was subject to detector contamination, and its technical difficulties would now make it hard to recommend. Very few modern GC methods use any detector other than MS or MS/MS for steroid determination at very low concentrations (e.g. Homma et al., 2003).

18-Hydroxytetrahydocorticosterone and 18-hydroxy-11-dehydrocorticosterone are unique metabolites. A secretion rate method based on double isotope dilution analysis of the former metabolite has been published (Ulick and Vetter, 1965). However, methods involving urinary excretion rates are more accessible. The steroid, after extraction and conjugate hydrolysis and oxidation with periodate, is derivatised (methyloxime-trimethyl silyl ether) and analysed by GC-FID or GC-MS (Ulick et al., 1991; Shakerdi et al., 2003).

A frequently encountered problem in analysing biological samples for metabolites of minor corticosteroids is the difficulty in obtaining authentic standards. In some circumstances, at least during the development of the method, it may be legitimate to use a more readily available heterologous standard compound, one with a similar retention time and an ion in common with the analyte, for GC-MS analysis. For example, β-cortol has been used successfully for urinary

18-hydroxy-11-dehydrocorticosterone assay (urinary excretion rates using β-cortol standard or authentic homologous standard correlated very closely: $r = 0.998$, $p < 0.001$). Moreover, periodate oxidation of the analyte was not necessary (Shakerdi et al., 2003).

Like other 18-hydroxysteroids, 18-hydroxyDOC degrades in alcoholic solution unless the solution is made alkaline, for example with triethylamine. Radioimmunoassay reagents must also be synthesised in-house (see for example, Ulick and Vetter, 1965; Roy et al., 1976). Although one method for plasma analysis applies radioimmunoassay to a crude lipid extract, most authors recommend prior partial purification, for example by thin-layer or paper chromatography or by LC (e.g. Chandler et al., 1976; Agrin et al., 1978). A method based on GLC-EC of the heptafluorobutyrate that required a complex preparation was highly sensitive but difficult to maintain for routine use (Mason and Fraser, 1975).

Urinary TH-18-hydroxyDOC excretion rate has been measured by GC-MS. Unlabelled and labelled standard compound were synthesised by borohydride reduction of 18-hydroxyDOC (Palem-Vliers and Sulan, 1978; Ulick et al., 1991). Urine free aldosterone and 18-hydroxycorticosterone have been measured by RIA after automated LC (Schoneshofer et al., 1982).

Table 5.6 summarises details of the measurement of some 18-oxygenated mineralocorticoids.

5.8.4.2 18-Oxygenated Glucocorticoids (18-Hydroxycortisol and 18-Oxocortisol)

18-hydroxycortisol was first confirmed in urine from a patient with primary hyperaldosteronism by Chu and Ulick (1982). The measurement of 18-hydroxycortisol and 18-oxo-tetrahydrocortisol in urine has been recommended as a means of detecting the rare sub-type of primary hyperaldosteronism, glucocorticoid-remediable aldosteronism (GRA). Elevated 18-hydroxycortisol returned to normal after dexamethasone suppression (McMahon and Dluhy, 2004; Vonend et al., 2007). Schirpenbach and Reincke (2007) acknowledge the value of 18-OH-cortisol measurements in the differential diagnosis of GRA but do not include these measurements as part of their recommended protocol (diagnostic algorithm) for investigation of primary aldosteronism. GRA is an autosomal inherited disorder which arises because of the presence of a chimaeric gene originating from a cross over between the two genes coding for CYP11B1 (cortisol synthesis) and CYP11B2 (aldosterone synthesis) (Lifton et al., 1992a, b). This results in the production of these so-called hybrid steroids as a result of the ACTH stimulated production of cortisol, which can act as a substrate for aldosterone synthase (CYP11B2). The hyperaldosteronism arises because aldosterone is produced in the zona fasciculata and is under the control of ACTH.

Methods have been described for assessing both plasma concentration and urinary free steroid excretion rate of 18-hydroxycortisol. Neither standard compounds nor antisera are available commercially. Gomez-Sanchez et al.

Table 5.6 Measurement of 18-oxygenated mineralocorticoids 1993–2007

Analyte	Matrix	Method	Purification	References
18-hydroxy-B	Adrenal vein sampling			Auchus et al. (2007)
18-hydroxy-THA	Urine			Shakerdi et al. (2003)
18-hydroxy-DOC and 18-hydroxy-B	Plasma –neonates and infants	RIA	Extracted with CH_2Cl_2 and separated on LH-20	Riepe et al. (2003)
18-hydroxy-DOC and 18-hydroxy-B	Rat and mouse adrenocortical cells	LC Neri et al. (1993)		Neri et al. (2003)
18-hydroxy-B and 18-hydroxycortisol; as part of 63 steroid profile	Newborn infant urine	GC-MS as OMO-TMSi ethers Caulfield et al. (2002)	Xtraction with CH_2Cl_2 – derivitisation	Homma et al. (2003)
18-hydroxy-DOC	Serum	RIA (SRL Laboratories, Tokyo)	No details given	Kawamura et al. (2003)
18-hydroxy-DOC	Rat plasma	LC-ESI-SIM	Xtraction with diethylether	Marwah et al. (2001)
18-hydroxy-B	Transfected COS-1 cells	TLC Mulatero et al. (1998)	Xtraction from culture medium with CH_2Cl_2	Portrat et al. (2001)
18-hydroxy-DOC	Cultured human melanoma cells	Incubation with ^{3}H-Prog; TLC and LC	C18 BondElut SPE	Slominski et al. (1999)
18-hydroxy-DOC and 18-hydroxy-B		Capillary electrophoresis		Palmer et al. (1998)
18-hydroxy-DOC	Rat brain mince	Use of ^{3}H substrate	Extracted with 7% CH_2Cl_2 in Hx and separated on TLC	Gomez-Sanchez et al. (1997)
18-hydroxyB, 18,19-dihydroxyB and 18-hydroxy-19norB	Male urine after ACTH	RIA	Sep-Pak C18 extraction and RP-LC Takeda et al. (1992)	Takeda et al. (1996)
18-hydroxy-DOC	Plasma	RIA	CH_2Cl_2 xtraction and LH-20 Den et al. (1978)	Toyoda et al. (1996)
18-hydroxyB and 18-hydroxyDOC	Rat adrenal mitochondria	LC and use of ^{3}H-substrate	CH_2Cl_2 xtraction; TLC and LC	Matkovic et al. (1995)
18-hydroxy-DOC and 18-hydroxy-B	Rat adrenocortical cells	LC-UV (at 290 and 240 nm)	Xtraction with CH_2Cl_2	Neri et al. (1993)

(1985) describe a method of synthesis of the unlabelled compound; ^{3}H- and ^{125}I-iodohistamine-labelled 18-hydroxycortisol have been used for immunoassay. More recently, we have synthesised 18-hydroxycortisol standard by incubating cortisol with V79 Chinese hamster cells stably transfected with 11β-hydroxylase (prepared by Dr. Rita Bernhardt, University of Saarbrucken) Freel et al. (2004). That 18-hydroxycortisol is the sole product simplifies subsequent purification. Corrie et al., (1985) devised a direct immunoassay for diluted acidified plasma or urine using an antiserum raised to the 3-carboxymethyl oxime-BSA conjugate. However, extraction and TLC (but not LC) improve performance (Corrie et al., 1985; Gomez-Sanchez et al., 1987).

Ulick et al. (1991) measured urinary 18-hydroxycortisol excretion rate by GC-MS following solid phase extraction and derivatisation (methyloxime-trimethylsilyl ether) using ^{2}H-labelled hormone as internal standard. In a three-way comparison of radioimmunoassay, a Delfia-(time-resolved immunofluorimetric assay) based method and GC-MS in urine, Reynolds et al., (2005) found a good correlation between results from the latter two methods. Correlation of these results with those from radioimmunoassay was poorer.

Free urinary 18-oxocortisol has been measured by radioimmunoassay following solid phase extraction and LC (Ulick et al., 1983; Gomez-Sanchez, 1984) or without prior separation (dilution ×400 for urine and ×8 for serum – Corrie et al., 1985). Low pH conditions throughout enhance recovery. Like 18-hydroxycortisol, specific reagents must be prepared in house. Again, the compound can be synthesised conveniently by incubating cortisol with V79 Chinese hamster cells stably transfected with aldosterone synthase Freel et al. (2004). Alternatively, the principal urinary metabolite, TH-18-oxocortisol has been analysed by GC-MS following conjugate hydrolysis, periodate oxidation and derivatisation (Ulick et al., 1991).

Table 5.7 summarises details of the measurement of some 18-oxygenated glucocorticoids.

5.9 Steroid Profiles

As discussed earlier, in recent years, interest has moved from the physiology and pathophysiology associated with individual steroid hormones to the implications of variability in relationships between them. Very wide spectrum urinary steroid metabolite profiles have been available for several decades. The robust schemes devised originally by Shackleton and his colleagues (Shackleton et al., 1976; Shackleton, 1985, 1986) have changed only with the addition of further compounds and later developments in reagents and apparatus. Currently, GC-MS with SIM is widely accessible. Examples of the clinical conditions relevant to corticosteroid metabolism are listed in Table 5.8. The possibility of harnessing LC-MS has already been raised. Schemes for studying plasma mineralocorticoid profiles have so far been much more cumbersome, expensive and have needed considerable technical expertise. In general, they comprise either separate samples or elaborate separation

Table 5.7 Measurement of 18-oxygenated cortisol

Analyte	Extraction	Method	Details	References
Plasma and urine 18-OHF	Direct but diluted 1:400 urine and 1:8 f plasma	RIA	In-house method using 125I-ligand and rabbit antiserum	Corrie et al. (1985)
Urine 18-OHF		EIA	In-house method using rabbit antiserum	Chiba et al. (1993a, b)
		HPLC		
Urine 18-OHF		EIA	Using mouse monoclonal antibody KTM-41 (? Available from techsupport@kamiy abiomedical.com)	Kohno et al. (1994)
Urine 18-OHF	Extraction using Sep-Pak C18	GC-MS of OMO-TMSi derivatives		Palermo et al. (1996)
Formation of 18-oxo- and 18-OHF *in vitro*		TLC and auto-radiography; RIA	In COS-7 cells transfected with recombinant CYP11B. For RIA refer to Aupetit-Faisant et al. (1993) but no details given	Mulatero et al. (1998)
Urine 18-oxo-F		RIA	In-house (Stowasser et al., 1995a)	Stowasser et al. (1999)
Urine 18-OHF		GC-MS		Kikuchi et al. (2000)
Serum 18-OHF	Extraction with 10 volumes of IPA	ELISA	In-house method (modified from Gomez-Sanchez et al., 1992)	Mosso et al. (2001)
Urine 18-oxo- and 18-OHF	Direct after dilution	Time-resolved FIA	In-house method compared with ELISA (Yamakita et al., 1994a, b)	Morra Di Cella et al. (2002)
Neonate and infant urine 18-OHF	Hydrolysis and Sep-Pak C18 extraction	GC-MS of OMO-TMSi derivatives	Enzyme hydrolysis and extraction with CH_2Cl_2. GC-MS method of Palermo et al. (1996; see also Caulfield et al., 2002)	Homma et al. (2003)
Urine 18-oxo- and 18-OH-F	Sep-Pak C18 extraction	GC-MS of OMO-TMSi derivatives	Method of Shackleton (1993) and Palermo et al. (1996)	Freel et al. (2004)
Urine 18-OHF	Hydrolysis and Sep-Pak C18 extraction	GC-MS of OMO-TMSi derivatives	Shackleton (1993); Lovati et al. (1999)	Nicod et al. (2004)
Urine 18-OHF	Sep-Pak C18 for GC-MS. IAs direct	DELFIA, RIA and GC-MS	Comparison of three methods	Reynolds et al. (2005)
Urine 18-oxo- and 18-OHF	Hydrolysis and Sep-Pak C18	GC-MS of OMO-TMSi derivatives	See Palermo et al. (1996)	Freel et al. (2007)

Table 5.8 Inborn errors of steroid metabolism

1(A) Congenital adrenal hyperplasia (CAH) due to deficiency of
- Pregenenolone synthesis resulting from StAR protein defect in cholesterol uptake or, more rarely, cholesterol side chain cleavage enzyme (CYP11A1) defect (lipoid adrenal hyperplasia)
- 3β-hydroxysteroid dehydrogenase 2 (3β-HSD2)
- 17α-hydroxylase (CYP 17)
- 11β-hydroxylase (CYP 11B1)
- 21-hydroxylase (CYP 21A2)
- Cytochrome P450 oxidoreductase (POR, associated with Antley-Bixler syndrome)
- 11-hydroxysteroid dehydrogenase type1 - cortisone reductase deficiency (here the HSD11B1 is actually normal and cases occur because of mutations in an accessory redox enzyme for 11β-HSD1 - hexose-6-phosphate dehydrogense)
- 7-Dehydrocholesterol reductase (DHCR7, associated with Smith Lemli Opitz syndrome, SLOS)
- Glucocorticoid receptor action

1(B) Deficiency of
- 5α-reductase (SRD5A2)
- Steroid sulphatase (STS)
- Aldosterone synthase (CYP 11B2)
- Aromatase (CYP19)
- 11-hydroxysteroid dehydrogenase type 2 (11β-HSD2 - Apparent Mineralocorticoid Excess (AME), liquorice ingestion)

2. Steroid-secreting tumours
3. Steroid resistance
4. Adrenocortical hyper- and hypofunction
5. Changed steroid metabolism due to drug treatment (e.g. metyrapone, ketoconazole) or to clinical condition (e.g. Cushing's syndrome, anorexia nervosa)

protocols of sample extracts, followed by individual steroid quantitation. Here, LC-MS may come to the rescue (Soldin and Soldin 2009). The use of simple TLC to separate the components of the aldosterone pathway (Mulatero et al., 1998) in extracts of *in vitro* DOC incubation with aldosterone synthase transfected cells demonstrates that LC separation could easily be applied in this situation. LC-MS analysis of other steroids suggests that sensitivity may be adequate (e.g. Que et al., 2000; Fluri et al., 2001) and LC-MS/MS offers further improvement in sensitivity especially when the steroid analytes are derivatised to improve ionisation. Figure 5.7 shows the LC-UV separation of picolinoyl derivatives of six important corticosteroids but this technique has the added advantage in LC-MS/MS of increasing the sensitivity of detection by ESI(+) by five- to tenfold over that achieved using underivatised steroids during LC-MS (Yamashita et al., 2007b). It can be combined with the use of stable isotope-labelled internal standards (Dehennin et al., 1980; Wudy, 1990). Tandem mass spectrometry has recently been used to determine increasing numbers of steroids simultaneously (9, later 12 steroids) in a single serum sample with minimal sample preparation (Guo et al., 2004, 2006; Kushnir et al., 2006). The technology has already been shown to be effective in detecting adrenal

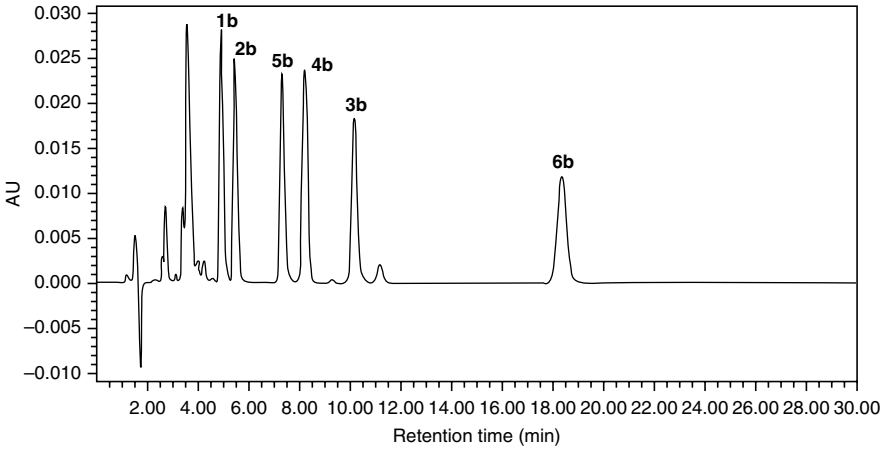

Fig. 5.7 Representative LC chromatogram of the picolinoyl derivatives of six corticosteroids (10 ng each as injected amount) with UV detection (λ: 240 nm). Column: Puresil $5C_{18}$ (150 mm × 4.6 mm I.D.), mobile phase: CH_3CN–0.25% CH_3COOH (45:55), flow rate: 1 mL/min. 1b: cortisol, 2b: cortisone, 3b: cortexolone (11-deoxycortisol), 4b: corticosterone, 5b: 11-dehydrocorticosterone, and 6b: 11-deoxycorticosterone (with permission from Yamashita et al., 2007b; copyright Elsevier B.V)

insufficiency (Holst et al., 2007), congenital adrenal hyperplasia (Turpeinen et al., 2005; Rauh et al., 2006), during and after pregnancy (Soldin et al., 2005) and in newborn screening for CAH (Minutti et al., 2004).

For clinical studies, another important decision to be made is the choice of assay medium. As with other steroid hormones, the alternatives are plasma or urine, with saliva as a possible alternative to plasma. While 24 h urine samples have the advantages of higher concentrations of hormone (or hormone metabolites), avoidance of the short-term fluctuations seen in plasma concentrations and non-traumatic collection, differential diagnosis frequently depends on concurrent estimation of other blood-borne variables such as peptide hormones or electrolytes. Moreover, accuracy of urine collections may be less easy to accomplish than is often assumed. Plasma may frequently be the medium of choice.

5.9.1 Urine Steroid Profiles

Analysis of urine steroids (Schoneshofer et al., 1986) and metabolites by IA after LC chromatography can be applied to the investigation of abnormalities of steroid production and metabolism. However gas chromatography has the added advantage that the identity of any steroid in a gas chromatographic system can usually be determined and/or confirmed by mass spectrometry (Shackleton, 1985, 1986). In cases where GC-MS does not unequivocally provide identity, LC fractionation and collection of unidentified steroids allows further study. The range of steroids in

urine can be viewed in a single gas chromatography analysis using a capillary column coupled to a non-specific flame ionisation detector or to a highly specific detector such as a mass spectrometer. Steroid conjugates usually require enzyme hydrolysis before derivatisation so as to stabilise the steroids for the high temperatures needed to elute them from the GC column (see, however, Shackleton, 1993). Carbonyl groups are protected by methyloxime formation and hydroxyl groups are converted to trimethylsilyl ether derivatives. The GC retention times are helpful in assigning possible steroids to each peak in the chromatogram, but mass spectrometry (MS) additionally produces a spectrum (a fingerprint) that is characteristic for the steroid.

Urinary steroid profiling by high-resolution gas chromatography (GC) with GC and mass spectrometry (GC-MS) provides a composite picture of the quantitatively major steroid biosynthetic and catabolic pathways (reviewed by Honour, 1997; Wudy and Hartmann, 2004). Metabolites of cortisol, progesterone, corticosterone, dehydroepiandrosterone sulphate are readily detected whereas those metabolites of testosterone, oestradiol and aldosterone in low concentration are not. A profile is more informative on adrenocortical than gonadal steroidogenesis and is especially useful for investigation of adrenal disorders in newborn infants, taking account of steroid metabolism being markedly different from that of children and adults. A profile overcomes the problems of interference in serum ligand assays from unusual steroids that are present in the neonatal period. Over more than 30 years, characteristic steroid profiles have been described in a range of clinical conditions including a number of recent findings. Specific clinical situations can be addressed effectively with urinary steroid profile analysis. The applications are listed in Table 5.8.

17-hydroxyprogesterone is the substrate for the 21-hydroxylase (CYP21B) enzyme. 17-hydroxypregnanolone, pregnanetriol and 11-oxo-pregnanetriol are the characterictic metabolites in excess in urine although 15-hydroxylated metabolites are also prominent in urine of affected newborn infants. In 11β-hydroxylase deficiency (CYP11B1), 11-deoxycortisol accumulates in blood and tetrahydro-11-deoxycortisol is high in urine whereas in 3β-hydroxysteroid dehydrogenase 2 (3β-HSD2), DHAS is raised in blood and urine. High excretion of cortisone metabolites (tetrahyrocortisone and cortolones relative to cortisol metabolites (tetrahydrocortisol and cortols) (Biason-Lauber et al., 2000) defines 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) deficiency. In cytochrome P450 oxidoreductase deficiency, metabolites of 17-hydroxyprogesterone, progesterone and corticosterone are raised (see Fig. 5.8) suggesting defects of 17-hydroxylase and 21-hydroxylase. The defect is in the electron transport system to both enzymes. CAH due to 21-hydroxylase (CYP21A2) deficiency has recently been reviewed (Riepe and Sippell, 2007).

Virilisation of a female at birth is the typical presentation of these disorders except 11β-HSD1 deficiency. Males with defects of CYP21 and CYP11B1 have normal genitalia, but a defect of 3β-HSD2 is associated with incomplete masculinisation. CYP21 defects are by far the commonest of these disorders so measurements of 17-hydroxyprogesterone are the first test for the condition but can be subject to interference (Borucka-Mankiewicz et al., 1992; Wong et al., 1992). Blood tests on samples taken in the first 3 days after birth for the above steroids are unreliable (or only available in specialist centres) unless assayed after a solvent extraction

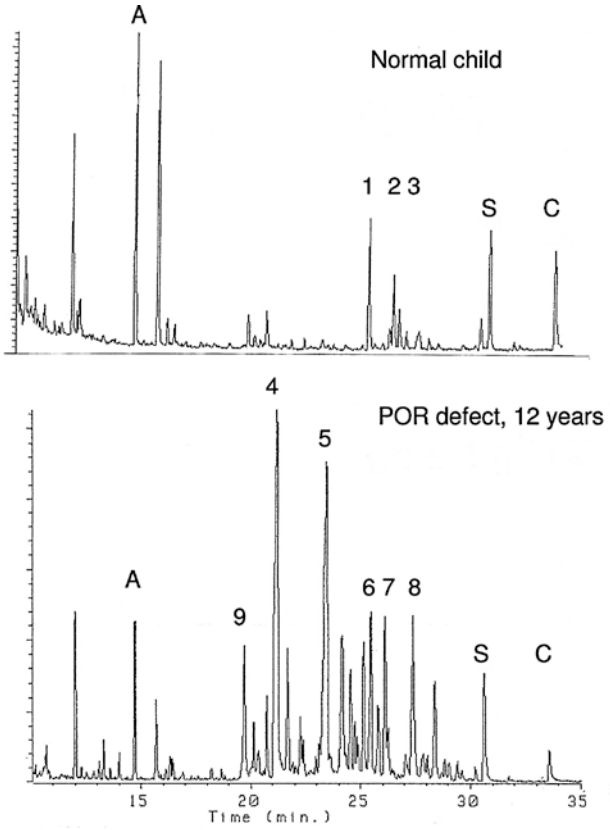

Fig. 5.8 Urine steroid profile of normal child and patient with cytochrome P450 oxidoreductase (POR) defect. Note increased outputs of metabolites of progesterone, 17-hydroxyprogesterone and corticosterone

Key to steroids: A, B, C Internal standards; 1 – tetrahydrocortisone; 2 – tetrahydrocortisol; 3 – allo-tetrahydrocortisol; 4 – pregnanediol; 5–11-oxo-pregnanediol; 6 – tetrahydro-11-dehydrocorticosterone; 7 – allo-tetrahydrocorticosterone; 8 – hexahydrocorticosterone; 9 – 17-hydroxypregnanolone

(Wallace et al., 1987). Other blood assays can be unreliable due to interference from endogenous (Ratcliffe et al., 1982) or exogenous steroids (Fink et al., 2002). Characteristic abnormal steroid profiles in adrenal disorders are found three days after birth (Caulfield et al., 2002). CAH requires life-long treatment so a urinary steroid profile is desirable on any patient with a suspected inborn error of steroid metabolism to define the nature of the steroids in excess.

CAH due to StAR or CYP11A1 defects can be inferred from a urine steroid profile devoid of steroids and those due to deficiency of 17-hydroxylase, 3β-hydroxysteroid dehydrogenase and cytochrome P450 oxidoreductase can be detected from typical patterns of steroids (Honour et al., 1978; Caulfield et al., 2002; Arlt et al., 2004). These disorders present with ambiguous genitalia. A steroid profile is of limited use

in the newborn period for the diagnosis of disorders in a 46XY male of testosterone production or metabolism. If the boy is older than 3 months a defect of 5α-reductase is revealed by the finding of a low ratio of 5α- to 5β-reduced metabolites of cortisol. In a pubertal child, the defect is also clearly reflected in a low ratio of 5α- to 5β-reduced androgen metabolites (Corrall et al., 1984). 17β-Hydroxysteroid dehydrogenase deficiency and other causes of low testosterone production are not detected by urine steroid profile analysis.

Cortisol synthesis can be impaired prior to the generation of cholesterol due to inactivity of 7-dehydrocholesterol reductase. Incomplete development of the male genitalia in combination with congenital anomalies (microcephaly, mental retardation, hypotonia, short nose with anteverted nostrils, and, in some cases pyloric stenosis) are features of the Smith Lemli Opitz syndrome The urinary steroid profile shows Δ^7 steroid metabolites of 16-hydroxy DHA and 16-hydroxypregnenolone (Shackleton et al., 1999a). During pregnancy the fetal condition can be detected by the presence of 7-dehydro-oestriol in the maternal urine (Shackleton et al., 1999b). Mass spectra of the delta-7 steroids have molecular and fragment ions 2 amu less than the saturated steroids.

A corticosteroid cause of hypertension may be indicated by a low renin level and not invariably, hypokalaemia. 17α-hydroxylase deficiency is only rarely detected in childhood and usually presents in phenotypic females with delayed puberty, primary infertility, amenorrhoea and hypertension. Progesterone and corticosterone in excess are found in this disorder (Honour et al., 1978). In 11β-hydroxylase deficiency hypertension is not obvious in young patients. Girls are virilised at birth, boys usually present with precocious puberty. 11-deoxycortisol and 11-deoxycorticosterone are elevated, as are androgens (Hague and Honour, 1983). Polymorphisms of CYP11B1 have been defined by urine steroid analysis (Keavney et al., 2005; Barr et al., 2007). 11β-hydroxysteroid dehydrogenase II deficiency (apparent mineralocorticoid excess syndrome) is associated with severe hypertension. Cortisol is not inactivated to cortisone which would normally prevent action of cortisol at the aldosterone receptor (Shackleton et al., 1980). Liquorice can inhibit the enzyme and a similar pattern of steroids is seen (Dellow et al., 1999). The genetic defect presents with severe hypertension from early childhood. Glucocorticoid remediable aldosteronism (GRA) is characterised by very high concentrations of 18-hydroxycortisol in the urine (Lifton et al., 1992a, b; Reynolds et al., 2005) but immunoassays and GC-MS assays for this steroid vary in accuracy.

In the absence of aldosterone, salt-loss will be a presenting feature. This may be due to a defect of mineralocorticoid synthesis due to lipoid adrenal hyperplasia, 3β-hydroxysteroid dehydrogenase deficiency, 21-hydroxylase deficiency, or defects of aldosterone synthase (CYP11B2). Mineralocorticoid receptor action can also be impaired (pseudohypoaldosteronism PHA) due to defects in the mineralocorticoid receptor. More often PHA is the result of defects in the epithelial sodium channel (ENaC) in the renal tubules. Production of aldosterone and its precursors are increased through profound renal salt loss leading to hyperreninaemia and stimulation of the adrenal cortex (Honour et al., 1982).

Glucocorticoid deficiency in a newborn infant can be due to lipoid adrenal hyperplasia (Bose et al., 1996), 3β-hydroxysteroid dehydrogenase deficiency or

congenital adrenal hypoplasia. Two types of congenital adrenal hypoplasia are distinguishable by the urine steroid profile. In the miniature adult type, no fetal zone adrenal steroids are found in urine from newborn infants. In the X-linked cytomegalic type (DAX-1 defect) all steroids are absent or at low levels. Absence of the major steroids may also be due to ACTH deficiency or insensitivity.

Adrenal tumours may secrete corticosteroids (e.g. cortisol, 11-deoxycorticosterone), androgens (DHA or 11-hydroxyandrostenedione) (Wolthers et al., 1999) or inactive steroids (16α-hydroxy DHA or pregnenolone) or be non-functional (no steroid production by the tumour). In the case of tumours secreting cortisol (Cushing's syndrome) urine steroid analysis of a 24 h sample will give an indication of the raised production when total cortisol metabolite excretion is compared with reference ranges. Urinary concentrations of oestrogens and androgens are increased by the presence of uterine leiomyomas (Jung et al., 2004). Many laboratories using this technology find that cortisol metabolite excretion approaches secretion rates 6–9 mg/day (Esteban et al., 1991) but there is wide variation in the published urine steroid reference ranges so local reference data must be used in interpreting results of one patient. A urine steroid profile collected before surgery may be of value in monitoring for recurrence. Gonadal tumours may result in hyperandrogenism and these changes are reflected in metabolites in the urine steroid profile.

Modest hyperandrogenism before or after puberty may be due to a mild form of CAH, most commonly the 21-hydroxylase defect, or to apparent cortisone reductase (HSD11B1) deficiency (Biason-Lauber et al., 2000). Premature adrenarche is characterised by high excretion rates of metabolites of cortisol and androgens for age and body size (Remer et al., 2006). PCOS is associated with enhanced 5-alpha reductase activity (Stewart et al., 1990; Rodin et al., 1994; Tsilchorizidou et al., 2003).

Endogenous steroid metabolites may be suppressed in subjects receiving exogenous glucocorticoids (Yiallouros et al., 1997). When synthetic glucocorticoids are suspected in herbal medicines that patients are taking, analysis of cortisol metabolites in a 24 h urine collection may show low levels indicative of suppression and this may be a more productive first approach than direct analysis for the preparation. When inhaled steroids are taken for treatment of asthma, the adrenal function can be shown to be suppressed, at sensible doses (for body size) these drugs rarely suppress the HPA axis to produce adrenal hypoplasia. Quantitation of cortisol metabolites in a 24 h urine collection is more reliable than urine free cortisol in demonstrating the extent of adrenal suppression (Fink et al., 2002). The only effective way of showing suppression is to monitor plasma cortisol concentrations at 15 min intervals throughout a 24 h period. Loss of peaks of cortisol during the night is dose dependent (Law et al., 1986).

Many synthetic steroid metabolites (e.g. from gestogens, equine oestrogens, androgenic anabolics) can be detected in a urine steroid profile. For a full screen of anabolic steroids the doping control centres within the structure of the World Ant-Doping Agency (to be found at www.wada-ama.org) have the most experience (Saugy et al., 2000; Hatton, 2007).

Urine steroid profiles need to be interpreted with caution. Excretion rates of the steroid metabolites vary throughout the day, with a peak in the late morning/early

afternoon (Jerjes et al., 2006). There are also gender differences in steroid excretion rates (Finken et al., 1999; Shamim et al., 2000). Thyroid hormones affect total activity of 11β-HSD2. THE and allo-THF are increased in hyperthyroidism. The widely used THF + allo-THF to THE ratios were lower (Hoshiro et al., 2006). Reference values have been documented in children (Honour et al., 1991; Homma et al., 2003). The activity of the fetal zone in premature babies is programmed according to post-coneption age (Midgley et al., 1996). Expressing steroid excretion as steroid/steroid or steroid/creatinine ratios is potentially misleading but some studies report excretion rates in this manner in an attempt to overcome unreliability of urine collection (Caulfield et al., 2002; Remer et al., 2002; Dimitriou et al., 2003). Specific gravity may be an alternative to creatinine when creatinine values are low or variable (Miller et al., 2004). Steroids are usually quantified through the addition of internal standards and response factors when reference steroids are available. Steroids, especially as conjugates are becoming more difficult to source. The required enzymes have however been transferred to cell systems capable of conjugate synthesis (Kuuranne et al., 2003). In GC-MS, deuterium labelled steroids are used in isotope dilution experiments. Isomeric steroids are also used. Urinary 18-hydroxy-tetrahydro-11-dehydrocorticosterone was determined in a novel manner using allo-tetrahydro-11-deoxycorticosterone as an internal standard (Shakerdi et al., 2003).

In cases of ambiguous genitalia it is important to obtain a karyotype. If the patient has hypertension, plasma renin activity and plasma aldosterone concentrations should be checked before considering steroid profile analysis. For diagnosis of the cause (other than CAH) of salt-loss in a neonate, salt intake and mineralocorticoid treatment should be reduced as much as possible. Endogenous cortisol production cannot usefully be examined if hydrocortisone (cortisol) or cortisone acetate is being given. If glucocorticoid treatment is essential, dexamethasone is preferred since dexamethasone metabolites do not interfere in the assay. A depot Synacthen test can be used to assess adrenal function during dexamethasone cover. A 24 h urine collection with no preservative is ideal for steroid profile analysis. Untimed collections may be acceptable for the identification of inborn errors of steroid metabolism. GC-MS methods continue to be described for particular steroids in urine (i.e. Shama et al., 2008 – progesterone and seven corticosteroids) and serum/plasma (i.e.Magnisali et al., 2008 – 17-hydroxyprogesterone, pregnanolone, cortisol and three androgens; Shibasaki et al., 2008 – cortisol, cortisone, prednisone and prednisolone).

New profile methods for urinary steroids include assays for free cortisol (McCann et al., 2005) and three tetrahydro metabolites (Raffaelli, 2006; Turpeinen et al., 2006). A recent LC-MS/MS analysis of steroids after hydrolysis of 200 μL of urine and subsequent extraction with *t*-butylmethyl ether and solvolysis produced a profile of 23 steroids in primate urine (Hauser et al., 2008). Synthetic corticosteroids have been detected in urine for doping control purposes (Leung et al., 2005) following systemic use and inhalation (Deventer, 2006). Steroid conjugates are usually hydrolysed before analysis but in doping control some benefit has been found by analysing anabolic steroid conjugates directly by LC-MS/MS (Antignac et al., 2005) and this may lead to analysis of corticosteroids in a similar manner.

5.9.2 Plasma Steroid Profiles

Steroid profiles for example in CAH provide more clinically useful data than is available from a single steroid measurement. LC coupled with UV detection has been useful in some cases (Wei et al., 2006). A more time-consuming process has included chromatography before immunoassay of the collected eluates in fractions (Hill et al., 1995; Peter et al., 1995; Fernandes et al., 2003; Riepe et al., 2003). This approach is still in use (Szecsi et al., 2006). Tandem mass spectrometry has recently been used to determine increasing numbers of steroids simultaneously (9, later 12) steroids in a single analysis with minimal sample preparation (Guo et al., 2004, 2006; Kushnir et al., 2006; Soldin & Soldin, 2009). The separation achieved by LC-MS/MS is illustrated in Fig. 5.9, which should be compared with similar separations for corticosteroid metabolites in primate urine (Hauser et al., 2008). The first method for multiple steroids that used atmospheric pressure photoionisation source needed 760 μL of serum and took 18 min. The second-generation assay with photospray took 11 min from 200 μL of sample. The technology has already been shown to be effective in detecting adrenal insufficiency (Holst et al., 2007), congenital adrenal hyperplasia (Turpeinen et al., 2005; Rauh et al., 2006), during and after pregnancy (Soldin et al., 2005) and in newborn screening for CAH (Minutti et al., 2004). Cortisol and cortisone are monitored in blood (Anderson et al., 2007) and in cell-assays for 11β-HSD1 inhibitors (Xu et al., 2006). Synthetic corticosteroids are monitored in a number of situations (Frerichs and Tornatore, 2004; Taylor et al., 2004).

17-Hydroxyprogesterone is the preferred analyte for diagnosis of 21-hydroxylase deficiency. A panel of three relevant steroids (17-hydroxyprogesterone, cortisol and androstenedione) in blood spots is used in a follow-up test for newborn screening (Lacey et al., 2004; Minutti et al., 2004; Janzen et al., 2007, Peter et al., 2008) (see Chapter 3 for more detail). 17-OH-progesterone, 17-OH-pregnenolone, 11-deoxycortisol and pregnenolone have been measured by LC-MS/MS in serum and plasma after SPE column extraction and elution with methyl-tert-butyl ether. Oximes were prepared with aqueous hydroxylamine (Kushnir et al., 2006). The simultaneous analysis of 17-OH-progesterone, androstenedione and testosterone has been automated with online SPE (Rauh et al., 2006).

Quest Diagnostics use an LC-MS/MS panel for diagnosing cortisol-related disorders. This panel was designed to diagnose Cushing's syndrome and the hypertensive conditions AME syndrome and GRA. The panel quantifies cortisone, cortisol, 6β-hydroxycortisol and 18-hydroxycortisol. The Quest analysis uses [$^2H_4$]cortisol as internal standard and HTLC for online extraction. Cortisol can usefully be measured along with synthetic corticosteroids (DiFrancesco et al., 2007). LC-MS/MS profiles in serum have been described for seven underivatised C21 steroids, using positive ion APCI (Cavalho et al., 2008) and six derivatised (as diethylether picolinyl esters) C21 steroids again using ESI(+) LC-MS/MS (Yamashita et al., 2007b). LC-MS/MS has also been applied to the measurement in urine of the A-ring reduced metabolites of cortisol and cortisone (Yamashita et al., 2008c).

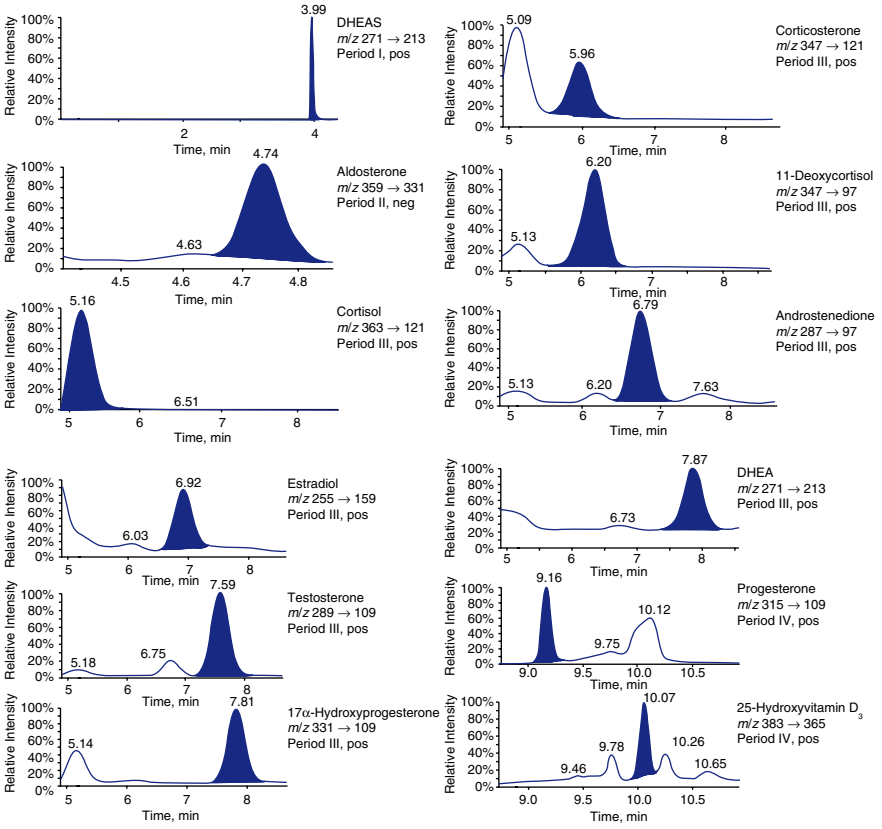

Fig. 5.9 Tandem mass spectrometric chromatograms of a steroid calibrator mixture. Chromatograms were obtained using four different periods for the whole HPLC-MS/MS MRM analysis. Period I: 0 → 4.40 min in positive ion mode; Period II: 4.40 → 4.87 min in negative ion mode; Period III: 4.87 → 8.74 min in positive ion mode; Period IV: 8.74 → 11.0 min in positive ion mode. pos: positive ion mode; neg: negative ion mode. The concentration for each steroid in the calibrator is: DHEAS 698 ng/mL; aldosterone 257 pg/mL; cortisol 143 ng/mL; corticosterone 2.86 ng/mL; 11-deoxycortisol 1.43 ng/mL; androstenedione 1.44 ng/mL; estradiol 314 pg/mL; testosterone 2.87 ng/mL; 17α-hydroxyprogesterone 2.88 ng/mL; DHEA 3.12 ng/mL; progesterone 3.8 ng/mL; 25-hydroxyvitamin D_3 20.7 ng/mL (with permission from Guo et al., 2006; copyright Elsevier B.V)

5.9.3 Tissue Steroid Profiles

Liquid chromatography with electrospray ionisation MS/MS has been used to examine steroid synthesis in zona glomerulosa cells (Nithipatikom et al., 2005), corticosteroid content of mouse adipose tisse (Ronquist-Nii and Edlund, 2005), placental perfusate (Dodds et al., 1997) and after *in vivo* microdialysis stable isotope labeled cortisol and cortisone in monkey adipose tissue as a means of assessing 11β-HSD

activity (Sun et al., 2008). Faecal steroid assays are used in animal studies as a non-invasive technique for assessing stress. Immunoassays for cortisol and corticosterone have been compared for studies in primates (Heistermann et al., 2006) and squirrels (Mateo and Cavigelli, 2005). LC measurements of cortisol and corticosterone in faeces from parrotfish were validated with mass spectrometric and radioactive tracer experiments (Turner et al., 2003).

5.10 Synthetic Corticosteroids

5.10.1 Introduction

There have, to the best of our knowledge, been no simple (sometimes referred to as 'direct') immunoassays reported over the last 10 years for most of the synthetic steroids in plasma, which do not require some extraction/purification prior to assay. Although a simple direct on-filter IA system has been described for use as a screen for dexamethasone in equine urine (Hassan et al., 1998) and a radioimmunoassay for dexamethasone in saliva and plasma after ether extraction (Thijssen et al., 1996), modern methods of assaying synthetic steroids in plasma utilise GC- or LC-MS. Many of the immunoassay reagents used for the measurement of cortisol cross-react with prednisolone and thus provide the basis for an assay method, but this emphasises the necessity for chromatographic purification if it is intended to measure cortisol in the presence of prednisolone or vice versa. In general, therefore, it is necessary to employ a separation technique followed by immunoassay or some other endpoint. Kong et al. (1988) used normal-phase LC to measure methylprednisolone hemisuccinate, cortisol and methylprednisolone in plasma, and Cheng et al. (1988) used a reverse-phase C-6 column for prednisone and prednisolone. McBride et al. (1991) also described a method for the measurement of plasma prednisolone, prednisone and cortisol by reversed-phase liquid chromatography. Recoveries and precision were claimed to be good, and reasonable separation on the column was achieved. The limit of sensitivity for each steroid is about 30 nmol/L. Dexamethasone in plasma can be assayed after purification, using an RIA procedure (e.g. Kream et al., 1983). A more recent study using an antibody generated against a new type of hapten, carboxymethylthiodexamethasone, claimed adequate precision and specificity for a direct enzyme immunoassay (Nishiguchi et al., 1992).

Synthetic steroids are sometimes referred to as xenobiotic steroids but both these terms encompass a large group which have in the main been synthesised by pharmaceutical firms in attempts to emphasise the physiological features associated with various steroid structures in order to use various formulations for clinical treatment. Clearly these interest the steroid analyst as compliance monitoring and efficacy of treatment may need estimates of active component concentrations in blood or urine and also measurements of metabolites in urine. Knowledge of pharmacokinetics is a pre-requisite of licensing by regulatory authorities. The structures of these steroids are based on cortisol with modifications:

- An extra double bond at C-1.
- Methylation at C-6, C-9, C16.
- Halogenation (chlorine of fluorine) at C6 and/or C9.
- Alkylation at C17, C21.
- Isomerisation of the side chain.
- Acetonide at C16, C17 – examples of some synthetic steroids are given in Fig. 5.10.

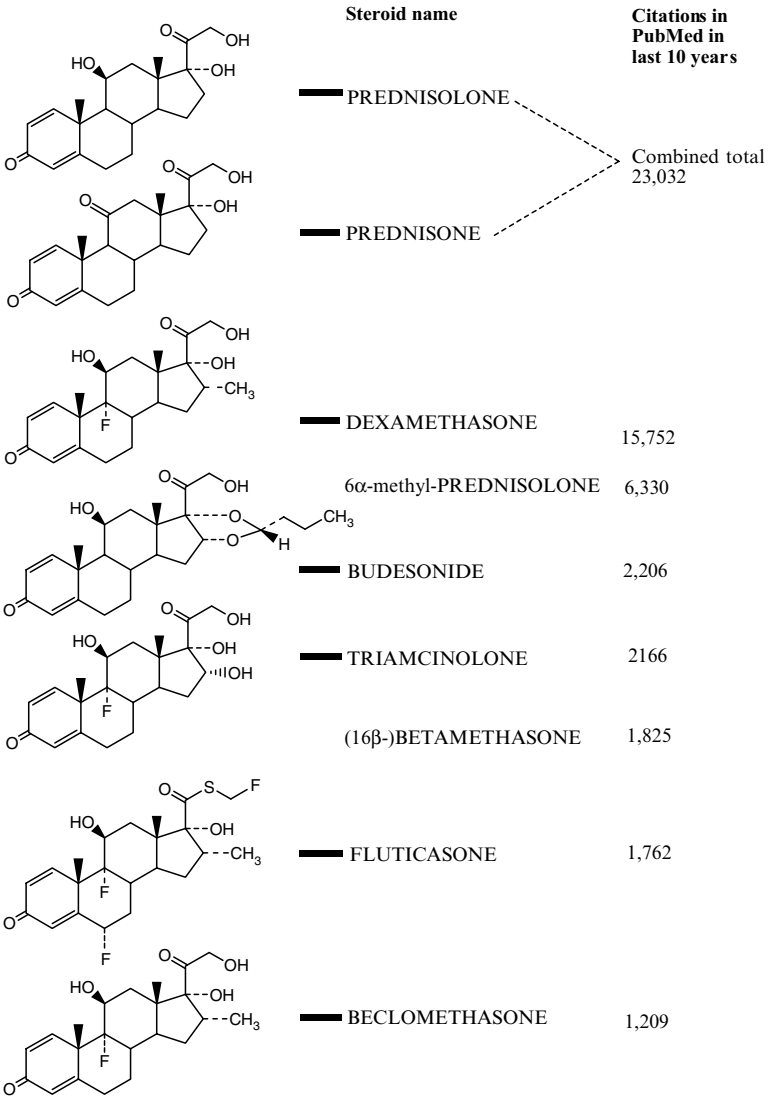

Fig. 5.10 Structures of the most cited synthetic steroids – the remaining steroids clobetasone, clobetasol, fluorocortisol, fludrocortisone, mometasone, fluocinolone are all cited <500 times

5.10.2 Metabolism of Synthetic Glucocorticoids

It is well-known that the structures of the majority of the most widely used synthetic glucocorticoids contain an A-ring containing two double bonds (at C-1,2- and C-4,5-). It is this delta-1,2-structure that renders the A-ring significantly resistant to the usual reductive metabolism which occurs in naturally-occurring glucocorticoids, such as cortisol (vide supra) This is illustrated by the fact that 9α-fluorocortisol, not a widely used steroid, is metabolised by A-ring reduction despite the presence of the 9α-fluoro moiety. Some of these synthetic steroids are formulated as esters, which require hydrolysis before catabolism can occur, further increasing the half-life (e.g. betnovate – betamethasone-17α-valerate). As an example the initial metabolism of beclomethasone-17,21-dipropionate (BDP) involves hydrolysis of the 21-ester, producing beclomethasone-17-monopropionate (Dollery, 1991), which is more active than the dipropionate. Foe et al. (1998a, b) have studied further degradation of BDP that can occur in human plasma at 37°C resulting in the formation of 17-BMP, as well as the 21-BMP, beclomethasone, and a degradation product separated by LC and characterised by UV spectroscopy and ^{1}H-NMR, as 9β,11β-epoxy-16β-methyl-1,4-pregnadiene-17, 21-diol-3,20-dione. Further metabolism (and inactivation) of budesonide occurs by enzymic formation of the 6β-hydroxy metabolite or, by loss of the C17 acetonide moiety, conversion to 16α-hydroxy-prednisolone (Phillips, 1990; Johnson, 1996).

Mometasone furoate (MF) is also converted to its major metabolite, 6β-hydroxyMF (Teng et al., 2003). In addition, there is good evidence for the formation of a degradation product, 9,11-epoxyMF, confirmed by studies by Valotis et al. (2004) and Valotis and Hogger (2004) using LC-ESI-MS-MS and NMR. The formation of this epoxide seems to be a common feature of 9α-fluoro steroids. Methyl prednisolone (MP) and its succinate ester (MPS) have been widely used for many years in the treatment of various disorders, such as asthma and other respiratory problems, in glucocorticoid replacement therapy and in the treatment of multiple sclerosis. Vree et al. (1999a, b) used LC with NMR, IR, UV and MS to isolate and identify metabolites hydroxylated at C-6 and reduced at C-20, as well as MP glucuronide in the urine of ten patients who had received MP-succinate.

In summary therefore the catabolism of the more widely used synthetic steroids involves hydrolysis of esters, 6β-hydroxylation, 20-reduction, loss of the acetonide moiety and, in 9α-fluorinated steroids, the formation of a 9α,11β-epoxy-derivative. The metabolism of a number of synthetic glucocorticoids, which is, like naturally occurring steroids, primarily hepatic, is summarised in Fig. 5.11.

5.10.3 Analysis

5.10.3.1 Introduction

Advances in analysis of xenobiotic glucocorticoids in biological matrices have been driven largely by the need to control their use in sport, and also because of the need to monitor their use as growth promoters in livestock. Although glucocorticoids are

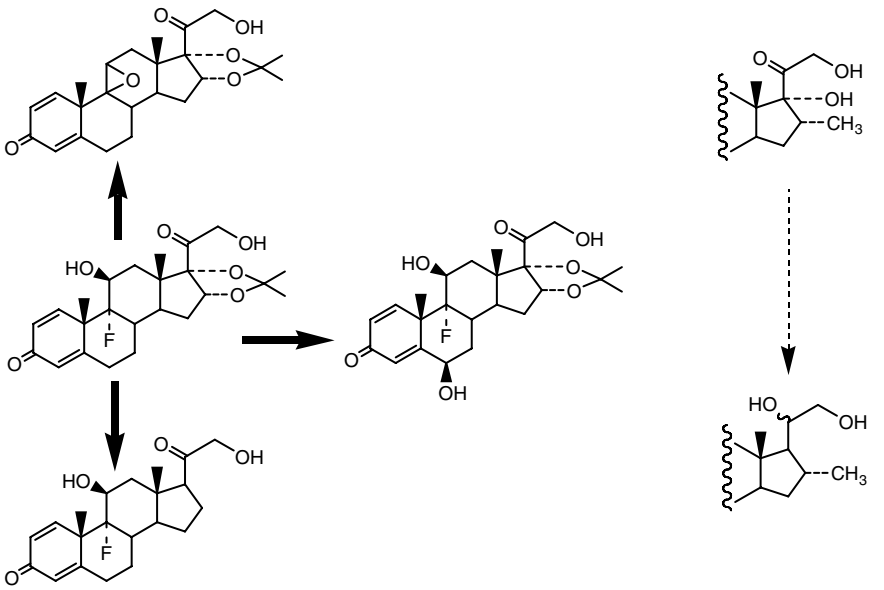

Fig. 5.11 An outline of the catabolism of synthetic corticosteroids. Triamcinolone acetonide (*middle left*) and dexamethasone (*top right*) are used as examples

generally catabolic, at low concentrations in cattle they can promote weight gain and reduce the feed conversion ratio. The use of corticosteroids as growth promoters is illegal in Europe and consequently analytical techniques have been developed to detect these steroids in biological matrices, generally in urine and meat from cattle, and more lately hair (Antignac et al., 2001). Their use in equine sports has been controlled for many years, and their potential for performance altering effects include the increase in blood glucose from non-carbohydrate sources and the stimulation of appetite, which in humans has been associated with euphoric or stimulant effects (Frisbie, 2000). A report was published as early as 1981 on the application of LC-MS using a moving belt interface to confirm their administration (Houghton et al., 1981). With respect to human sports, glucocorticoids have been considered as potential doping agents for many years due to their potentially performance enhancing effects when administered acutely, and further to their euphoric and stimulatory effects, their anti-inflammatory effects (systemically) may be particularly advantageous to those participating in extremely challenging endurance sports such as the Tour de France in cycling. The screening and confirmatory analysis for these compounds in urine has recently become part of the laboratory proficiency test programme organised by the World Anti-doping Agency (see www.wada-ama.org).

Within clinical chemistry, there is a need to extend the analysis of corticosteroids to include the mainstream exogenous ones. Such a need is to help elucidate the aetiology of disease states associated with glucocorticoid excess where the pathology shows no obvious intrinsic cause but where there is a history

of use of non-approved medications. A major concern is that these 'medications' may contain corticosteroids, particularly those purported to be natural herbal treatments. Another major concern is the use of skin bleaching (lightening) products that contain such steroids.

Herbal treatments are known to be used for dermatological conditions, such as eczema and psoriasis, and some have been shown to be adulterated with glucocorticoids (Ernst, 2000). In particular Chinese herbal creams have been shown repeatedly to contain anti-inflammatory drugs, including dexamethasone and the superpotent steroid clobetasol propionate (Ramsay et al., 2003). Moreover, herbal 'medicines' for oral administration have contained dexamethasone, prednisolone and prednisone.

Topical corticosteroids cause skin bleaching and the hypopigmentation process is completely distinct from the rapid and temporary skin blanching caused by the vasoconstriction properties of these compounds. The mechanism by which skin is lightened by these corticosteroids is not completely understood but the result is the inhibition of melanogenesis (Gupta et al., 2006). Medically, hydrocortisone, triamcinolone, betamethasone, fluocinolone, dexamethasone, and other corticosteroids are used to treat hypermelanosis (melasma) with other skin-lightening agents, such as hydroquinone, to enhance their activity or to decrease their irritating effects (Petit and Pierard, 2003). Regrettably, there is a booming market in skin bleaching products for cosmetic rather than medicinal purposes, most likely, the market being driven by the perceived success of a new generation of black celebrities in the UK and USA, many of whom have a lighter skin (see the article in The Observer of October 16, 2005, 'Toxic creams for sale as thousands seek whiter skin'). The corticosteroid that appears most often in such preparations is clobetasol propionate.

There is an increasing number of case reports and documented studies describing the complications that can arise from chronic use of these herbal creams and cosmetic skin products,with reports of skin thinning and the possibility of permanent skin damage. There is also a risk of iatrogenic Cushing's syndrome and much more so with adulterated herbal preparations which are take orally, e.g. (Goldman and Myerson, 1991; Edwards et al., 2002; McConkey, 2003), and also adrenal insufficiency when the product is suddenly stopped. Further, pregnant women of sub-Saharan Africa origin are known to use skin bleaching products, where there may be consequences not only for the mother but also for the child (Mahe et al., 2005, 2007).

For the analysis of xenobiotic corticosteroids in biological matrices, mass spectrometry is the method of choice, either coupled to a gas or liquid chromatograph, as it can offer unambiguous identification at a suitable sensitivity. In forensic analysis, a minimum required performance limit is set for the type of work being performed, i.e. the minimum content of an analyte in a sample, which has to be detected and confirmed by all laboratories. For example, in sport, the World Anti-doping Agency has set a minimum required performance limit of 30 ng/mL for the analysis of urinary glucocorticoids.

For clinical purposes, legally defensible data are not required, but good rather than watertight evidence of the presence of the drug and/or its metabolite is usually sufficient for the purpose. Often the clinical chemist is presented with a urine

specimen (and /or serum sample) and sometimes a herbal or skin care product is available for analysis. With a topical skin application that may be adulterated with corticosteroid, only a very small proportion of the dose enters the systemic circulation, the extent of transcutaneous penetration being dependent on the region of the body, the potency of the steroid and the dose. Even if the steroid is administered orally rather than topically, the short half-lives of these steroids still means that post hoc detection in serum and urine is challenging, even more so with potent and superpotent steroids where the dose will often be smaller. Method development thus can be weighted to achieving excellent sensitivity, ideally around 1 ng/mL, by standard LC-MS/MS with one transition for identification purposes being sufficient, at least, to presume that a corticosteroid is present. Xenobiotic corticosteroids can be also detected in human hair (Gaillard et al., 2000) for forensic purposes and this may offer some opportunity for a greater window for detection in clinical cases, particularly important, for example, where there is a suspicion that a child may have been exposed to adulterated products.

The recent advances in LC-MS/MS technology over the last decade is making this the analytical approach of choice for the screening for corticosteroids in biological matrices, particularly as time-consuming derivatisation usually associated with gas chromatographic analysis (Pujos et al., 2005) can be avoided, Selected reaction monitoring (multi-stage MS) offers far superior sensitivity for these compounds compared to selected ion monitoring (single stage MS). For confirmatory analysis, GC-MS usually provides better chromatographic resolution of complex extracts, better isomer differentiation and generally more structural information. The application of these hyphenated techniques in the analysis of these compounds is elegantly and concisely reviewed by Antignac et al. (2004), and even though their interest is in residue analysis, the philosophy of the approach to analysis of corticosteroids is of general relevance. The article compares chromatographic separation of corticosteroids by LC and GC, compares the merits of different ionisation sources, these being electron impact (positive mode), chemical ionisation (negative mode) and atmospheric pressure ionisation (electrospray; positive and negative mode) and finally the application to monitoring corticosteroid misuse and kinetic and metabolic investigations.

5.10.3.2 GC-MS

The moderately polar nature of corticosteroids, natural or xenobiotic, require that the hydroxyl and keto groups are derivatised to increase their volatility, to avoid column adsorption phenomena leading to peak tailing and natural water loss. An interesting exception to this approach is the method described by Pujos et al. (2005) who used no derivatisation but the limit of quantification was poor for some steroids (>500 ng/mL), e.g. budesonide, desonide. Another approach that also does not rely on derivatisation is to use chemical oxidation to remove the polar C-17 side chain and to subsequently oxidise the residual hydroxyl groups to ketone functionality, followed by negative chemical ionisation; this method,

however, appears to be limited to residue analysis of matrices from cattle (Courtheyn et al., 1998; Heutos Hidalgo et al., 2003). Usually, electron impact ionisation is employed for GC-MS, and a better chromatographic profile is achieved with silylation but sensitivity is lower because of mixed derivatives being formed (polysilylated products leading to multiple peaks of the same steroid). Multiple reactions can be avoided to a large extent by converting the keto groups to oximes, prior to silylation. The most common reagent used for oxime formation is methoxylamine, thus replacing the C=O functionality by $C{=}N{-}OCH_3$.

Analysis of corticosteroids in urine for clinical purposes by GC-MS following methyl oxime–trimethylsilyl (MO-TMS) derivatisation is well established, particularly for identification of inborn errors of metabolism (e.g. Thenot and Horning, 1972a; Yap et al., 1992; Caulfield et al., 2002; Taylor, 2006). In contrast, there are very few papers describing the urinalysis of xenobiotic glucocorticoids as MO-TMS derivatives by GC or GC-MS; a restricted number of analytes were targeted (usually betamethasone, dexamethasone and prednisolone), with very different derivatisation and analytical conditions being used. For example, with respect to betamethasone, methoximation has been carried out with conditions varying from 10 μL of a 10% (w/v) solution of methoxylamine hydrochloride in pyridine to 100 μL of an 8% (w/v) solution, with reaction times and temperatures varying from 30 min at 80°C to overnight at 57°C. The silylating conditions used have been equally varied, with 10–50 μL of trimethylsilylimidazole (TSIM) and reaction times and temperatures from 2 h at 80°C to 6 h at 110°C (Thenot and Horning, 1972b; Houghton et al., 1982; Rodchenkov et al., 1987, 1988a–c, 1991; Midgley et al., 1988). There is consensus that glucocorticoids with a methyl substituent on the C-16 position are much harder to derivatise, i.e. dexamethasone and betamethasone (16α and 16β methyl respectively) and require harsher conditions than prednisolone or 6α-methylprednisolone. With the exception of one publication (Midgley et al., 1988), details on assay sensitivity are not provided.

Investigating the application of urinary steroid profiling (vide supra) to doping control in sport, Walker et al. (2006) recently investigated the optimum conditions required for silylation of dexamethasone and found, following MO derivatisation of the oxo groups at C-3 and C-20, that 20% TSIM in MSTFA (v/v) for 6 h at 100°C were sufficient to form the complete di-MO, tri-TMS derivative with negligible artefact formation. These conditions were then applied to various corticosteroids: prednisone, 6α-methylprednisolone, prednisolone, 20-dihydroprednisolone, fludrocortisone, dexamethasone, 6-hydroxydexamethasone, flunisolide, desonide, betamethasone, budesonide, and triamcinolone. The silylating conditions that were developed appear to strike a balance between the methods described by Thenot and Horning (1972b) and Houghton et al. (1982). As an adjunct, silylation of betamethasone di-MO was completed in a shorter period (3 h) under these conditions, probably because the steric hindrance of the 17α-hydroxyl by a 16β-methyl is less than that from a 16α substituent.

The presence of pyridine hydrochloride and methoxylamine hydrochloride reagents is detrimental to GC columns and promotes degradation of the analytes (Thenot and Horning, 1972b). The GC column also can be damaged by TSIM and

because it is very involatile, it is not practical to try to remove it by evaporation. Lipidex (Axelson and Sjovall, 1974) or Sephadex LH-20 (Houghton et al., 1982) column chromatography is often employed to remove these harmful reagents prior to injection but this approach is time consuming. A simpler and much faster approach is to perform liquid–liquid extraction using dichloromethane (Leunissen and Thijssen, 1978) and washing with an aqueous solution. Walker et al. (2006) chose to use cyclohexane instead of dichloromethane, as the use of chlorinated solvents is discouraged for environmental reasons. The steroid derivatives appear to be shielded from the water due to the favourable partitioning into this immiscible and very non-polar solvent, as there was no evidence of hydrolysed products. Dodecane (2%) was added to the cyclohexane and this remained, following the evaporation of the cyclohexane. The advantage of this approach is that the derivatised steroids are kept in solution, preventing possible loss of silyl groups that can otherwise occur if the organic solvent is completely evaporated. Use of GC-MS for corticosteroid analysis, apart from its value in steroid profiling, seems to be replaced by LC-MS/MS. This increasing reliance on LC-MS/MS may be premature as these methods may not be as specific as GC-MS for some glucocorticoids.

5.10.3.3 LC-MS/MS

The fact that corticosteroids are moderately polar molecules makes them ideally suited for analysis by LC-MS/MS, thus avoiding the time consuming and rather laborious derivatisation processes necessary for analysis by GC-MS. Using positive electrospray ionisation (ESI), an intense quasi-molecular ion $[M + H]^+$ is formed under slightly acidic conditions, and in negative ESI, an adduct with the conjugate base of the organic acid used is formed $[M + base]^-$. These precursor ions are then selected for collision-induced dissociation to yield product ions. To maximise sensitivity, one transition can be monitored (selective reaction monitoring), as demonstrated by Taylor et al. (2004) for the detection of xenobiotic corticosteroids in human serum, urine and tablets for clinical chemistry purposes. The conditions used are described in full in the paper but in brief an acetonitrile/ammonium acetate gradient was used with a Synergi 4 μ MAX-RP C18 column (50 mm × 4.6 mm I.D.), the assay run time being relatively long (15 min per sample) to separate the isomers dexamethasone and betamethasone, and the isomers triamcinolone and flunisolide. The steroids were injected following a simple liquid-liquid extraction of 0.5 mL urine, serum or plasma with dichloromethane (methylene chloride). Electrospray in positive mode was used as it gave better ionisation compared with negative mode. The steroids analysed with transitions in parentheses were: beclomethasone dipropionate (521→ 337), betamethasone (393→147), budesonide (431→413), cortisol (363→121), dexamethasone (393→147), flunisolide (435→321), fluorometholone (377→279), fludrocortisone (381→239), fluticasone propionate 501→239), megestrol acetate; a progestagen with glucocorticoid activity (385→224), methylprednisolone (375→161), prednisolone (361→147), prednisone (359→147), triamcinolone (395→225), triamcinolone

acetonide (435→397). Stable isotopes of cortisol-9,11,12,12-d_4 (367→121) and triamcinolone-d_1 acetonide-d_6 (442→404) were added as internal standards. The assay sensitivity for each analyte was defined as the lowest analyte concentration with an interassay CV < 20% was 0.6–1.6 nmol/L (which is less than 1 ng/mL) for all analytes, making this approach the method of choice for clinical purposes, where a single transition may be considered acceptable (if the background interference is low).

In evidential analysis, several characteristic ions are required for confirmatory purposes. ESI in +ve mode, fragmentation of the precursor ion $[M + H]^+$ can produce numerous but not very specific fragment ions corresponding to the loss of water molecules and/or halogen atoms and other minor cleavages within the B and C rings (Antignac et al., 2004). Negative ESI of the adduct $[M + base]^-$ yields the quasi-molecular ion $[M - H]^-$ as the product and also the fragment corresponding to cleavage of the side chain with loss of formaldehyde $[M - CH_2O - H]^-$. With triple quadrupole instruments, if additional product ions are required a high cone voltage can be chosen to induce in-source fragmentation of the $[M + base]^-$ ion so that $[M - CH_2O - H]^-$ can then be selected for collision induced dissociation in the second quadrupole (Q2). Of course, with ion traps, where MS^n experiments can be performed, $[M - CH_2O - H]^-$ can be selected as the intermediate ion, as demonstrated by Deventer and Delbeke (2003) who performed MS^3 to differentiate between the epimers dexamethasone and betamethasone based on relative intensities of the diagnostic ions produced.

APCI appears to be particularly suitable for the ionisation and fragmentation of corticosteroids, when applied in positive mode, for example in the analysis of equine urine (Tang et al., 2001). Even so, for some steroids, APCI in negative ion mode can offer better sensitivity and reduced matrix interference, such as in the confirmation of the presence of triamcinolone acetonide and its hydroxylated metabolite (Tang et al., 2001). Indeed, De Brabander et al. (2007) comment that for analysis of xenobiotic corticosteroids, APCI techniques operating in negative ion mode 'represent today the technique of choice because of its better sensitivity and specificity compared to all ionization techniques'.

The application of GC- and LC-MS (and MS/MS) to the analysis of synthetic corticosteroids, primarily the most cited (see Fig. 5.10), is summarised in Table 5.9, which details, methods used since 1997.

5.10.3.4 Conclusions and Future Work

LC-MS/MS is currently the ideal analytical tool for the screening of xenobiotic corticosteroids, as only a simple sample work-up is required compared to the derivatisation necessary to perform GC-MS, and the sensitivity appears to be far superior. By contrast, LC separation is far inferior to GC, but this may be partially addressed with ultra-high pressure (UPLC) columns, although this can add to the expense due to the pumps required. The harsher ionisation conditions of electron impact, and the MO-TMS derivatives, associated with GC analysis can give better

Table 5.9 Some LC- and GC-MS methods for measurement of synthetic corticosteroids in human and animal body fluids and tissues and other matrices of interest (1997–)

Analyte	Matrix	Purification	LC/GC	Ionisation	Comment	References
Dexamethasone and Dexamethasone Palmitate IS: tramadol hydrochloride	Human plasma	Isopropanol to ppt proteins. Centrifuge and supernatant extracted with diethyl ether/CH_2Cl_2	LC on Venusil XBP-C8 column at 30°C with gradient elution using acetontrile/0.5% ammonia solution, pH 8.5	Sciex API 4000 using ESI(+)	Collecting MH^+ in MS1 and monitoring *m/z* 147 for dexamethasone and *m/z* 373 for palmitate ester in Q3(MS2)	Yang et al. (2008)
Dexamethasone; prednisone; prednisolone; triamcinolone; methyl prednisolone; budesonide beclomethasone + eight more synthetic CS + other steroids IS: methyltestosterone, d_3-trenbolone and d_6-triamcinolone acetonide	Human and calf urine	5 mL urine mixed with pH 7 buffer, enzyme de-conjugation and extracted with diethylether	UPLC ON Waters BEH-C18 column (50 mm × 2.1 mm I.D., 1.7 µm particle size) using gradient elution at 35°C with 0.1% formic acid/water and 0.1% formic acid in MeCN	ToF with ESI(+) and using in source CID	SIM of MH^+ ion to four decimal places (e.g. betamethasone MH^+ at *m/z* 393.2077). In-source CID can give fragment ions and provide elemental composition	Touber et al. (2007)
Budesonide, dexamethasone, triamcinolone acetronide and Dexamethasone acetate. IS: d_7-triamcinolone acetonide	Porcine plasma, after suprachoroidal administration	1 mL plasma mixed with 4% phosphoric acid to ppt proteins. Loaded on to OASIS-HLB cartridges and eluted with increasing concn of MeOH in 0.1% formic acid	µLC on Zorbax C18 (300 mm I.D. × 150 mm) Stablebond column eluting with stepwise solvent 2 mM NH_4acetate (at pH 3.2) in 10:90 MeCN/water and in 90:10 MeCN/water	API 3000 with turbospray ESI(+)	MRM, collecting MH^+ in MS1 and fragment ions in Q3(MS2) (e.g. for budesonide *m/z* 431 to *m/z* 413 – loss of 18 amu)	Qu et al. (2007)
Betamethasone and anabolic steroids IS: d_3-testosterone	Human urine	SPE extraction using BondElut Certify cartridges, eluting with MiOH	LC on RP Novapak C18 eluting with gradient of 10 mM ammonium acetate, pH 6.7 and MeCN/MeOH (1:1)	Agilent MSD single quadrupole with ESI(+) or Finnegan LTQ coupled to vMALDI ion source	Use of F20TPP as matrix for MALDI, monitored transition *m/z*393 to *m/z* 239 for betamethasone	Kosanam et al. (2007)

All CS in Fig. 5.10 plus others amongst panel of 66 drugs IS: d_4-cortisol	Equine plasma	Protein pption with 10% TCA onto BondElut Certify cartridge, elute (for steroids) with CH_2Cl_2/EtAc (4:1) or (for acidic drugs) 2% Conc NH_3 in EtAc/CH_2Cl_2/IPA(5:4:1)	LC on Supelcosil ABZ + PLUS column, eluting with 0.1% Acetic acid/acetonitrile	Applied Biosystems 4000 QTrap MS/MS ESI(+)	Collecting MH^+ in MS1 and monitoring fragment ions after CID in Q3(MS2) (e.g. *m/z*393-*m/z*373 for betamethasone	Yu et al. (2008)
Dexamethasone, betamethasone, prednisolone, 6α-Meprednisolone, Flumethasone IS: Deltafludrocortisone	Bovine milk	Filter after addition of 20% TCA. Onto Oasis-HLB columns. Elution with MeOH	LC on a Hypercarb column, eluting with isocratic 90:10 acetonitrile/0.1% formic acid	Micromass Quattro Ultima ESI(−)	Collecting $[M + HCOO]^-$ ion in MS1 and monitoring fragment ion (e.g. *m/z*345 or 361 for dexamethasone) in MS3	McDonald et al. (2007)
Triamcinolone acetonide IS: d_3-triamcinolone acetonide	Rabbit aqueous humor	Extract with EtAc/water (1:1); dried extract derivatised with acetic anhydride/pyridine and then with BSTFA	GC on a DB-5MS capillary column	Agilent 6890 GC system coupled to a 5973 MSD using NCI(−)	SIM of acetylated-TMS derivative M^- [*m/z* 548]	Chu et al. (2007)
Dexamethasone IS: Prednisolone	Rat biofluids and fetal tissue	Protein pption with 4% phosphoric acid. Centrifugation and Oasis-HLB cartridges. Elution with MeOH	LC on C8 Hydrobond AQ column. Stepwise elution with 10 mM NH_4formate/acetonitrile	Sciex API 3000 using Turbo-ion spray (+)	Collecting MH^+ [*m/z* 393] in MS1 and monitoring *m/z*373 in Q3(MS2) [loss of 20 amu is –HF]	Samtani and Jusko (2007)
Prednisone, prednisolone, dexamethasone, 6α-methylprednisolone IS: d_2-cortisol	Sewage plant influents	Filtration. Passed through Oasis-HLB cartridge, eluting with EtAc/MeCN (1:1). Dried extract reconstituted with EtAc/Hx (1:9) and re-applied to a SIL cartridge. Eluted with water saturated EtAc	LC on ACQUITY UPLC BEH C18 column, eluting with 0.1% formic acid/MeOH at 40°C	Micromass Quattro Ultima with Z-spray ESI(−)	Collecting $[M + HCOO]^-$ in MS1 and monitoring $[M\text{-}H\text{-}HCHO]^-$ in Q3(MS2) (e.g. *m/z*473 to *m/z*361 for dexamethasone)	Chang et al. (2007) (cf. Cui et al., 2006)

(continued)

Table 5.9 (continued)

Analyte	Matrix	Purification	LC/GC	Ionisation	Comment	References
Dexamethasone, methylprednisolone, prednisolone, prednisone IS: Flumethasone	Human plasma	Plasma (500 mL) + MeOH, mix, add 0.1 M Hcl. Mix. Centrifuge. Pass through OASIS-HLB cartridge and elute with MeOH	LC on Symmetry C18 column, eluting with 95% NH_4Ac buffer pH 3.2:5% MeOH and reverse mixture 5:95%	Sciex API 3000 using TurboIon spray (−)	Collecting $[M-H]^-$ and monitoring $[M-H-30]^-$ for all CS	DiFrancesco et al. (2007)
Dexamethasone IS: Flumethasone	Human plasma	Extraction with diethyl ether. Dry extract re-suspended in MeCN	LC on Hypersil Hypercarb, eluting with water/MeCN (20:80) plus triethylamine at a final concn of 5 mM at 50°C	HP5989 MS-Engine API-ESI(−) Single quadrupole	Monitoring $[M-H]^-$or $M-H-HF]^-$ produced by source CID	Damonte et al. (2007)
15 synthetic glucocorticoids (all those in Fig. 5.10) IS: d_3 [9,12,12]cortisol	Milk and eggs	Acetate buffer pH 5.2 added and glucuronidase/sulfatase enzyme added. MeOH added and homogenised; centrifuge and wash supernatant with Hx ×2. propanol added and sample evaporated to dryness; water/MeOH added and passed through OASIS-HLB cartridge; eluted with water-saturated EtAc; finally passed through an aminopropyl SPE cartridge	LC on an ACQUITY UPLC BEH C18 column at 40°C with MeOH/0.1% formic acid	Micromass Quattro Ultima Pt MS/MS system ESI(−)	Collecting $[M + HCOO]^-$ in MS1 and monitoring $[M-H-HCHO]^-$ in Q3(MS2) (e.g. *m/z*473 to *m/z*361 for dexamethasone)	Cui et al. (2006) (cf. Chang et al., 2007)

Budesonide and 16α-OHprednisolone IS: Betamethasone	Human urine	Adjust to pH 9.2 and extract with EtAc	LC on an Omnispher C18 column, eluting with 1% acetic acid/MeCN at 35°C	ThermoFinnigan MS/MS LCQ-Deca XP-Plus ESI(+)	Collecting MH^+ and monitoring $[MH\text{-}H_2O]^+$ (*m/z* 431 to *m/z* 413)	Deventer et al. (2006)
Betamethasone IS: Chloramphenicol	Human plasma	Extracted 0.5 mL plasma with diethyl ether/Hx (80:20)	LC on a Genesis C18 column, eluting with acetonitrile/water (80:20, 10 mM NH_4acetate)	Sciex API 3000 MS/MS APPI (−) with toluene as the dopant	Collecting $[M\text{-}H]^-$ in MS1 and monitoring $[M\text{-}H\text{-}HCHO]^-$in Q3(MS2) (*m/z* 391 to *m/z* 361)	Periera et al. (2005)
Mometasone furoate IS: $[^{13}C_3]$-fluticasone	Human plasma	30% ethanol added to ppt protein. Centrifuged and passed through end-capped C18 cartridge and eluted with EtAc/heptane (35:65)	LC on Waters 5mm Symmetry C18 column, eluting with MeOH/water (85:15)	Micromass Quattro-LC-Z APCI(−)	MRM, collecting *m/z* 484 $[M\text{-}H\text{-}HCl]^-$ in MS1 and monitoring loss of furoate ester (*m/z* 389 in Q3(MS2)	Sahasranaman et al. (2005)
Betamethasone, dexamethasone IS: Fluoxymesterone	Equine plasma	Extracted 1 mL plasma with methyl- *t*-butyl ether	LC on a Hypercarb (2.1 × 30 mm, 5 μm particle size), isocratic elution with MeCN/water/formic acid (95:5:0.5) with flow rate changes. 16α and β epimers completely resolved	TSQ Quantum MS/MS system ESI(+)	Collecting MH^+ in MS1 and monitoring fragment ions *m/z* 237 for dexamethasone and *m/z* 279 for betamethasone in Q3(MS2)	Luo et al. (2005a)
17 synthetic corticosteroids	Equine plasma	Extracted 1 mL plasma with methyl- *t*-butyl ether	LC on an Ace C8 column, eluting with MeOH/ NH_4formate buffer, pH 3.4, 2 mM -16α/β epimers NOT resolved	TSQ Quantum MS/MS system	MRM. Collecting MH^+ and monitoring up to three fragment ions in Q3(MS2)	Luo et al. (2005b)

(continued)

Table 5.9 (continued)

Analyte	Matrix	Purification	LC/GC	Ionisation	Comment	References
IS: Desoxymetasone or Dichlorisone acetate 14 synthetic steroids including most popular 7 in Fig. 5.10 IS: There appear to be no internal standards identified	Human urine	SPE on C18 column, elution with MeOH. β-Glucuronidase hydrolysis. Products re-purified on Oasis-HLB cartridge – water–acetonitrile (80:20 to 50:50)	GC on HP-5MS capillary column – no derivatisation	ESI(+) Agilent GC-MSD (5973)	SIM choosing different ions for each CS – 2 major fragments for prednisolone (*m/z*300 – loss of side chain and *m/z* 122 (cleavage in B ring)	Pujos et al. (2005)
			LC on Varian Omnispher C18 column, eluting with 10 mMNH_4formate pH 3.9/acetonitrile/IPA	HP1100MSD using ESI(+)	SIM using MH^+ ion	
Budesonide, prednisone, prednisolone, methylprednisolone, triamcinolone, fluocinolone	Study on LC and MS properties	Not applicable	LC on Hypersil C18 column, eluting with EtOH/MeCN/formic acid (2:30:68)	Micromass ZMD4000 single qudrupole MS with ESI(+)	MH^+, $[MH\text{-}nH_2O]^+$ and various adduct ions noted – useful fragmentation given	Hou et al. (2005); Hou et al. (2001) for LC-UV assay
14 synthetic CS IS: d_4[9,11,12,12] cortisol and d_7-triamcinolone acetonide	Human urine, serum/plasma	Protein pption with MeCN. Centrifugation. Extraction with CH2Cl2 and acid/alkali wash	LC on SYNERGI 4 μ MAX-RP column, eluting with stepwise increased concentration of MeCN in 0.1 mMNH_4acetate	Sciex API 3000 MS/MS with TurboIon spray (+)	MH^+ in MS1, monitoring fragments in Q3(MS2)	Taylor et al. (2004)

Prednisone, prednisolone, dexamethasone IS: Flumethasone	Human serum	SPE on Oasis-HLB after centrifugation. Steroids eluted with MeOH	LC on Symmetry C18 column, eluting with MeOH/5 mM acetate buffer, pH 3.25	Sciex API 3000 MS/MS TurboIon spray ESI(−) gave better S/N ratios than ESI(+)	Collecting $[M-H]^-$ in MS1 and monitoring $[M-H-HCHO]^-$ for each steroid	Frerichs and Tornatore (2004)
Dexamethasone IS: Desoxymetasone (17-deoxy-dexamethasone)	Bovine milk	10 mL milk – protein ppted by addition of 20% TCA. Homogenised on vortex mixer and centrifuged. Filter and apply to C18 cartridge. Elute with MeOH	LC on PLRP-S polymeric reverse-phase column, eluting with MeCN/0.1% acetic acid at 30°C	Finnigan MAT LCQ ion-trap MS^n with APCI(+)	Collecting MH^+ in MS1 and monitoring $[MH-HF]^+$ in Q3(MS2)	Cherlet et al. (2004) [NB Reviews other LC methods for dexa. 1990–2002]
Dexamethasone and 6β-hydroxy-dexamethasone IS: 6α-methyl-prednisolone	Human urine	SPE on Oasis-HLB cartridge, eluted with ethyl acetate followed by diethyl ether. Filtered be4 LC	LC on Nova-Pak C18 column, eluting with 0.06% TFA in NH_4Ac buffer, pH 4.8/MeCN	HP/Agilent 1100 LC-MS using ESI(+)	SIM monitoring MH^+ ions – $[MH-HF]^+$ ions also noted	Zurbonsen et al. (2004)
Beclomethasone di- and mono-propionate IS: Fluticasone propionate	Rat and human plasma and rat tissue samples	Plasma extracted using Supelco C18 cartridges after protein pption with 33% EtOH. Steroids eluted with EtAc/n-heptane (2:98) Tissue samples extracted with organic solvent – not specified	LC on Waters Symmetry C18 column eluting with 33 mM NH_4actetate/0.33%% formic acid buffer, pH 3.4/methanol	Micromass Quattro-LC-Z MS/MS using ESI(+)	MRM slecting MH^+ in MS1 and daughter ions (*m/z* 319 and 279) in Q3(MS2)	Wang and Hochhaus (2004)

(continued)

Table 5.9 (continued)

Analyte	Matrix	Purification	LC/GC	Ionisation	Comment	References
Dexamethasone IS: Methylprednisolone	Bovine urine	SPE on C18 or Oasis cartridge eluting with Hx/EtAc (1:1) for C18 or MeOH for Oasis. MeCN added to dried extract and treated with pyridinium chlorochromate	GC on capillary SPB-5 column	GC-MSD (HP5888A0 in NCI(−) mode	SIM of oxidised dexamethasone – most abundant ion of dexamethasone was *m/z* 310	Huetos Hidalgo et al. (2003)
Budesonide, 6β-hydroxy-budesonide, 16α-hydroxy-prednisolone IS: 9-fluorocortisol	Human plasma	Protein ppted with 30% EtOH. Centrifuged. SPE on Supelco C18 columns, eluting steroids with 80% MeOH ×2	LC on Waters Symmetry C18 column, eluting with 10 mM NH_4acetate buffer pH 3.5 – MeOH	Micromass Quattro-LC-Z using ESI(−)	$[M-H]^-$ selected for 6-OHPred and $[M + AcO]^-$ for others in MS1 and fragment ions monitored in Q3(MS2)	Wang et al. (2003)
Beclomethasone dipropionate and metabolites IS: Desoxymetasone (17-deoxy-dexamethasone)	Equine plasma and urine	Plasma and urine extracted with methyl-*t*-butyl ether. Urine may be treated with β-glucuronidase	LC on Ace C8 column, eluting with gradient of 2 mM NH_4formate buffer pH 3.4/MeOH	Thermo Finnigan TSQ Quantum triple quadrupole MS/MS using ESI(+) and ESI(−) for beclomethasone	MH^+ in MS1 and $[MH-H_2O]^+$ in MS3 for all CS except Beclomethasone when $[M-H]^-$ was collected in MS1 and $[M-H-HCHO]^-$ in Q3(MS2)	Guan et al. (2003)
Budesonide IS: Triamcinolone acetonide	Human plasma	Protein ppted with 30% EtOH. Centrifuged and applied to SPE C18 cartridge. Eluted with 30% EtAc in heptane. Acetates formed with acetic anhydride/Triethylamine	LC on on Waters Symmetry C18 column, eluting with methanol/water (80:20)	Micromass Quattro-LC-Z MS/MS system using APCI – presumably + ionisation, though not specified	SIM. M^+ of acetate ester *m/z* 472 monitored in MS1 – no MS/MS done despite having facility	Dimova et al. (2003); Comparison with SPE and in-house RIA

Dexamethasone, betamethasone, triamcinolone acetonide, Beclomethasone, methylprednisolone, 16a-hydroxy prednisolone Budesonide IS: Desoxymetasone (illustrated as 21-deoxy but in fact is 17-deoxy)	Male volunteer urine sample after i.m. injection of betamethasone and spiked urine samples	Urine extracted with diethyl ether after addition of potassium carbonate	LC on Nucleosil C18 column, eluted with 1% acetic acid/MeCN	Finnigan MAT LCQ Deca ion-trap MS^n used – highest sensitivity obtained using ESI(−)		Deventer and Delbeke (2003)
Dexamethasone, betamethasone IS: Fluorometholone	Bovine liver	Homogenate.Helix pomatia enzyme and buffer pH 4.6 added. Extracted ×2 with MeCN/Hx and CH_2Cl_2 added, mix and centrifuge Middle MeCN layer removed. Added to C18 SPE cartridges and eluted with EtAc	LC on Hypercarb column, eluting with MeCN/water (90:10 + 3% formic acid)	Quattro LC-Z ESI(+)	Collecting MH^+ in MS1 and monitoring $[MH-HF]^+$ in Q3(MS2)	Van den Hauwe et al. (2001)
Budesonide, fluticasone propionate IS: d_3-budesonide-21-acetate	Human plasma	Protein ppted by 30% EtOH. Centrifuge. Supernatant applied to SPE C18 cartridges, eluting with EtAc. Acetates made using acetic andhydride/ Triethylamine	LC on 5 µm ODS Hypersil column, eluting with EtOH-water (43:57)	Finnigan MAT TSQ 7000 MS/MS using APCI(+)	Collecting MH^+ in MS1 and monitoring $[MH-18]^+$	Li et al. (2001)

(continued)

Table 5.9 (continued)

Analyte	Matrix	Purification	LC/GC	Ionisation	Comment	References
Dexamethasone, betamethasone IS: Flumethasone	Bovine liver	Extraction using ASE 200 system (Dionex, CA, USA). Liver mixed with diatomaceous earth-Hydromatrix and extracted with Hx. CS eluted with Hx/EtAc:: (91:10)	LC on Phenomenex Kingsorb C18 column, elutong with MeCN/5mM NH_4Ac/MeOH (35:60:5)	PE-SCIEX API III triple quadrupole MS/MS using APCI(−)	$[M\text{-}H\text{–}HCHO]^-$ collected in MS1 and daughter ions *m/z* 307 and 292 monitored in Q3(MS2)	Draisci et al. (2001)
Triamcinolone acetonide IS: d_7-Triamcinolone acetonide	Human bronchoalveolar lavage fluid	Extraction with diethyl ether ×2; acetylation with acetic anhydride/pyridine; purification on small Pasteur pipettes filled with Porasil silica and elution with diethyl ether	GC on capillary methylsilicone column with helium as carrier gas	GC-Finnigan MAT SSQ710 negative ion CI(−)	SIM of M^- (*m/z* 476)	Hubbard et al. (2001)
Triamcinolone, prednisolone, prednisone, methylprednisolone, betamethasone, dexamethasone, flumethasone, beclomethasone IS: d_3 cortisol	Human hair	Hair samples washed with CH_2Cl_2. Pulverised in ball mill and incubated in Sorensen buffer pH 7.6 for 16 h at 40°C. Buffer added to Isolute C18 cartridges and CS eluted with MeOH	LC on Novapak C18 column, eluting with MeCN/NH_4formate buffer pH 3	Perkin-Elmer Sciex API-100 MS using both + and – ionisation	SIM of M^+ for quantitation	Cirimele et al. (2000)
Fluticasone propionate IS: $[^{13}C_3]$fluticasone propionate	Human plasma	Protein ppted with 30% EtOH. Centrifuge. Supernatant added to C18 SPE cartridges. CS eluted with EtAc/heptane (80:20)	LC on a Waters ODS2 column, eluting with MeOH/water (80:20)	Micromass Quattro-LC-Z using APCI (+ and -). For quantitation APCI(−) was chosen as S/N ratio was higher	Collecting *m/z* 500 in MS1 and monitoring $[M\text{-}120]^-$ (*m/z* 380 in Q3(MS2)	Krishnaswami et al. (2000)

Fluticasone propionate IS: [$^{13}C_3$]fluticasone propionate	Human plasma	Plasma centrifuged and tricine buffered saline pH 7 added and mixed. Applied to SPE C18 cartridge and eluted with MeOH	LC on a Hypersil BDS C8 column eluting with NH_4acetate/MeOH with formic acid	Perkin-Elmer Sciex APIII + tandem MS using ESI(+)	Collecting MH^+ ions in MS1 and monitoring *m/z* 293 in Q3(MS2)	Laugher et al. (1999)
Fluticasone propionate IS: [$^{13}C_3$]fluticasone propionate	Human plasma	Automated SPE	LC on ResElut C8 BD 5 μm column, eluting with MeOH/25 mM NH_4formate (80:20)	Perkin-Elmer Sciex APIII + triple quadrupole withTurboIon spray using ESI(+)	Collecting MH^+ ions in MS1 and monitoring *m/z* 313 in Q3(MS2)	Callejas et al. (1998)
Prednisone, prednisolone IS d_4 [1,19,19,19] analogues of both analytes	Human plasma	Sep-Pak C18, washed and eluted with ethyl acetate	LC on LiChroCART Supersphere 100 column, eluting with 0.05 M NH_4formate/THF/MeOH (180:53:17) and 0.05 M NH_4formate/MeCN (65:35)	Vestec Model 750B MS with thermospray (+)	Monitoring MH^+ ions	Shibasaki et al. (1997)

For explanation of some of the MS terminology used here see Chapters 2 and 3.

characteristic spectra for identification purposes, which is important in evidential analysis but even here LC-MS/MS more often than not can provide sufficient data to be fit for the purpose.

It is of particular interest to include clobetasol propionate as a target analyte for screening purposes, given that it is a common adulterant in herbal preparations and ingredient in skin lightening products; currently there is very little information in the published literature concerning the analysis of this steroid or its metabolites in biological matrices using hyphenated mass spectrometric techniques.

Finally, fused silica capillary LC interfaced with MS/MS offers tremendous increases in sensitivity, as demonstrated where a limit of quantification of 5 pg/mL in plasma was achieved for budesonide, dexamethasone, triamcinolone acetonide and dexamethasone acetate (Qu et al., 2007). This strategy extends the limit of quantification to areas of pharmacokinetic analysis of low-dose corticosteroids where their systemic concentrations were previously too small to be detected by current methods.

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Chapter 6
Analysis of Androgens and Their Derivatives

D.B. Gower

Abbreviations for Steroid Names

Testosterone	17β-hydroxy-4-androstene-3-one
Epitestosterone	17α-hydroxy-4-androsten-3-one
5α(β)-DHT	17β-hydroxy-5α(β)-androstan-3-one
DHEA	3β-hydroxy-5-androsten-17-one
4-Androstenedione	4-androstene-3,17-dione
5α(β)-Androstanedione	5α(β)-androstane-3,17-dione
Androsterone	3α-hydroxy-5α-androstan-17-one
Aetiocholanolone	3α-hydroxy-5β-androstan-17-one
Epiandrosterone	3β-hydroxy-5α-androstan-17-one
5-androstenediol	5-androstene-3α17β-diol

6.1 Introduction

In view of the enormous increase in the field of androgens during the past decade, an important decision had to be made concerning this present revised chapter. Some of the older material, prior to 1990, discussed in the first edition has been either omitted or, at least, summarised and presented in tabular form. This decision is not meant to detract from the importance of earlier studies. Interested readers can refer to earlier work in the first edition of 'Steroid Analysis' which is still available.

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6.2 Overview of the Molecular Biology of Enzymes Involved in Androgen Biosynthesis

The C_{19} steroids that possess androgenic activity are testosterone (T), its A-ring saturated analogue, 5α-DHT, 5-androstene-3α,17β-diol, and at least, one of the isomeric 5α-androstanediols, formed by the reduction of 5α-DHT. It is now generally accepted that T exerts its androgenic effects as a result of rapid conversion to 5α-DHT in androgen target tissues, such as the prostate and the skin, by means of the catalytic activity of 4-ene-5α-reductase (commonly known as 5α-R, two forms of which are known (Andersson and Russell, 1990; Andersson et al., 1991; Labrie, 1991).

Skalba (2006) have demonstrated the presence of 5α-R (types 1 and 2) mRNA in dermal papillae from the lower abdominal region of women with hirsutism. Twenty-four subjects had polycystic ovarian syndrome (POC) and 18 had idiopathic hirsutism (IH). The authors reported a positive correlation between the number of mRNA copies/mμg of total mRNA for 5α-R Type 2 and the serum concentrations of free T. For 5α-R Type 1, there was only a tendency towards a correlation. The authors have suggested that T increases the expression of both Types 1 and 2 of 5α-R in dermal papillae taken from the female patients mentioned above.

5α-R1 and -R2 are also known to be present in rat brain, Type 1 being involved in catabolic actions and Type 2 being involved with sexually dimorphic functions of the male. In the adult female rat brain (pre-frontal cortex), 5α-R2 mRNA is known to be only slightly regulated by T and 5α-DHT, whereas 5α-R1 mRNA is not regulated by T but very positively by 5α-DHT. The authors (Torres and Ortega, 2006) conclude that there is great sexual dimorphism in the central nervous system (CNS) with respect to 5α-R Types 1 and 2, and that there may be a crucial role for 5α-DHT in the sexual dimorphism in the CNS of female rats (see also Section 6.7.5.1 for further discussion on steroid metabolism in brain tissues).

In addition to T and 5α-DHT, 4-androstenedione (4-A) and DHEA are also discussed in this chapter, although 4-A is a weak androgen, having only 10–15% activity relative to that of 5α-DHT, while DHEA is even weaker. These results were obtained *in vivo* by the measurement of growth of, for example, rat seminal vesicles and cockscombs. Neither 4-A nor DHEA bind to the androgen receptor but exert what little activity they possess by possible metabolism to active androgens, e.g. T and 5α-DHT. 5-Androstenediol can also be converted into these active androgens, thus explaining at least some of its androgenic activity. However, Miyamoto et al. (1998) have published evidence indicating that 5-androstenediol is able to activate androgen receptor target genes in the presence of androgen receptors.

The biosynthesis of T, which occurs especially in the Leydig cells of the interstitial tissue of the testes of numerous species, has been studied extensively for over 50 years and many reviews are available (e.g. Payne and O'shaunessy, 1996). During the past 15 years, much information has been published concerning the molecular biology of various enzymes involved in the conversion of two precursors, pregnenolone and progesterone, into T. Two biosynthetic pathways are known, the Δ^5 and Δ^4 pathways, depending on whether 5-ene-3β-hydroxy or 4-en-3-oxo metabolites, respectively, are involved (see, e.g. Gower, 1984a; Lewis, 1996; for review). Figure 6.1 summarises the

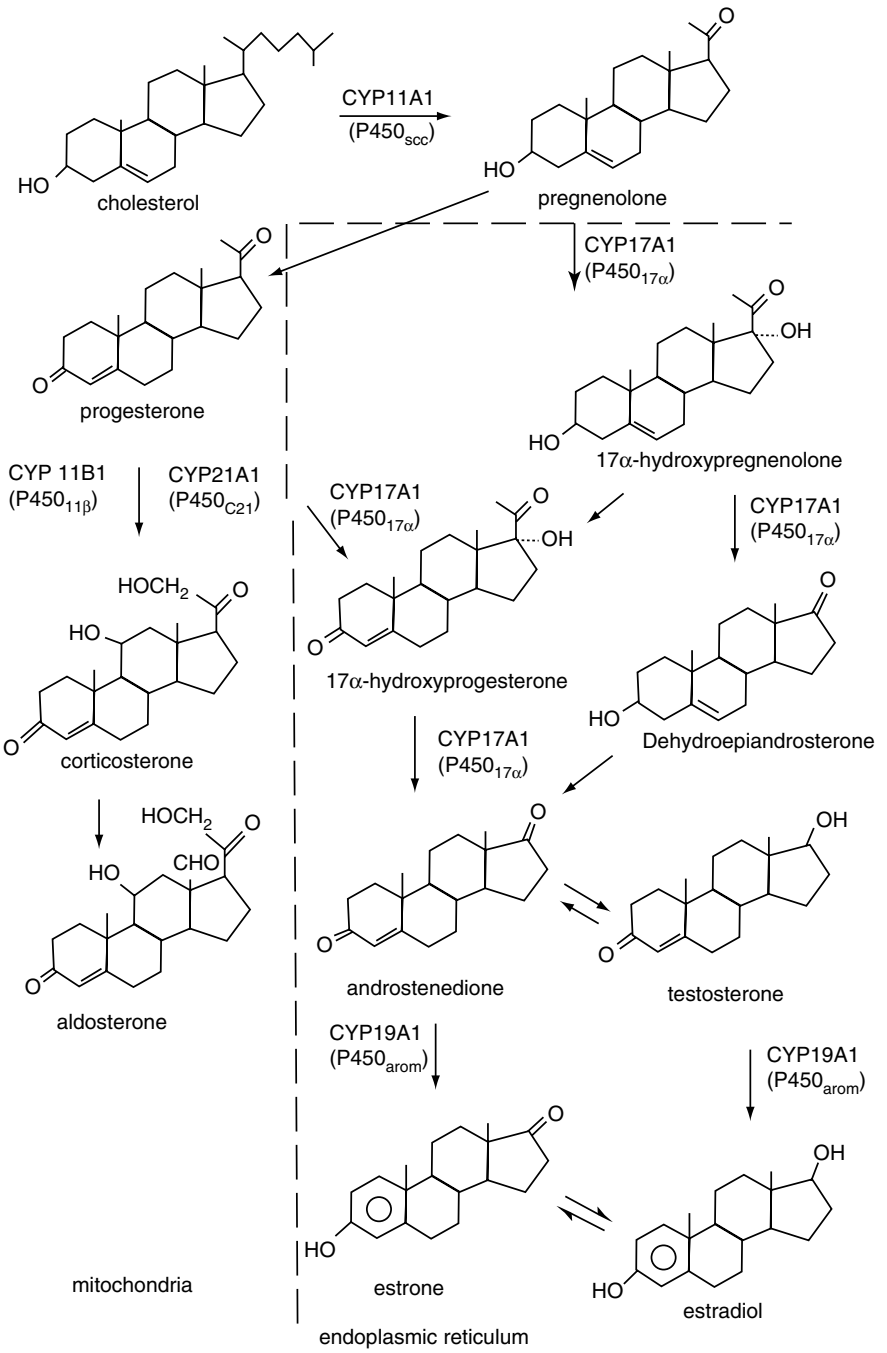

Fig. 6.1 Steroid hormone biosynthetic pathways showing roles of certain cytochromes P-450. For such enzymes, both new and old nomenclatures (in parentheses) are given (from Lewis, 1996, with permission of the author and CRC Press, Boca Raton, New York, London and Tokyo)

pathways and the cytochromes (old and new nomenclature) involved, while Table 6.1 shows the characteristics of the important enzymes (Makin and Gower, 1996; Payne and O'shaunessy, 1996).

6.3 Overview of Catabolic Reactions for C19 Steroids

As noted above, the reduction of T to 5α-DHT occurs rapidly in androgen target tissues, the reaction being catalysed by 4-ene-5α-reductase (now often known as 5α-reductase or 5α-R, as above). In rat prostatic tissue, at least, enzyme activity can be modulated by its phospholipid environment (Cooke and Robaire, 1988; Cooke, 1989). Many other characteristics of the enzyme and its inhibitors are provided in a detailed review by Li et al. (1995). Further metabolism of 5α-DHT at C-3 (Gower, 1984b) yields isomeric androstanediols, one of which, the 3α,17β-diol, possesses around 50% of the androgenic potency of 5α-DHT. This catabolic reductive reaction is thought to be a means of modulating the androgenic effects of 5α-DHT (Liao and Hippaka, 1984; Leake, 1996; Makin and Gower, 1996). Figure 6.2 summarises these reactions as well as the reversible reduction of T at C-17 leading to 4-A and its further reduction in the A-ring to yield the isomeric 17-oxosteroids (17-OS), notably androsterone (3α,5α-), aetiocholanolone (3α,5β-) and epi-androsterone (3β,5α-), excreted in urine as mixtures of glucuronides and sulphates (Gower, 1984b). Andrew (2001) has also summarised these catabolic reactions and has reviewed the use of estimating some of the metabolites in the clinical context.

The formation of 5α-androstane-3α,17β-diol from 5α-DHT has been known for many years, but evidence for the existence of an alternative pathway from the reduction of progesterone has been published by Wilson et al. (2003). These workers used testes of Tammar wallaby (*Macrobus eugenii*) pouch young, and incubated the tissues with ^{3}H-progesterone and ^{3}H-T, separately. The extracted metabolites were separated initially by thin layer chromatography (TLC) (see Section 6.4.2 and Table 6.2). The data showed that, after the reduction of progesterone, the product (3α, 17α-dihydroxy-5α-pregnan-20-one) (Gower, 1984b) acts as a key intermediate because side-chain cleavage, catalysed by CYP17 (see Fig. 6.1) then yields androsterone (inferred) with 5α-androst-3α,17β-diol as final product. Mahendroo et al. (2004) have shown that 5α-androstanediol is also formed by similar pathways in immature mouse testes and, further, that 5α-reductase Type I is involved.

Conjugation of non-polar steroids with free hydroxyl groups (at C-3 or C-17) is a metabolic reaction that yields the more polar glucuronides or sulphates, thus facilitating renal excretion. These important Phase II conjugation reactions, involving glucuronosyl tranferases and appropriate sulphotransferases, have been studied for several decades in detail and reviews are available (e.g. Gower, 1984c; Kicman et al., this volume Chapter 9). In addition to the three 17-oxosteroids, androsterone, aetiocholanolone and epi-androsterone, mentioned above, DHEA is included in this group, but is excreted in urine almost exclusively as the sulphate (Gower, 1984b).

Table 6.1 Characteristics of the genes and mRNAs of cytochromes P450 involved in steroid biosynthesis in man

Enzyme[a]	Gene	Reaction	Number of genes[b]	Chromosome	Gene length (kb)	mRNA length (kb)	Protein length[c]
$P450_{scc}$	*CYPIIA*	$Cholesterol_{scc}$ (includes C-22 and C-20 hydroxylations)	1	15	>20	2	482 (521)
$P450_{c11}$	$CYPIIB_1$	C-11 and C-18 hydroxylations	1	8			479 (503)
$P450_{c18}$	$CYPIIB_2$	C-18 hydroxylation and oxidation	1	8			479 (503)
$P450_{c17}$	*CYP17*[d]	17α-Hydroxylation; 20 bond cleavage, C-17, C-20	1	10	6.6	1.9	508
$P450_{c21}$	*CYP21*	C-21 hydroxylation	1 (1)	6	3.3	2	494–495
$P450_{arom}$	*CYP19*	C-10 demethylation and A-ring aromatisation	1 (1)	15	>70	2.9, 3.4	

[a]$P450_{scc}$, $P450_{c11}$, and $P450_{c18}$, mitochondrial enzymes; $P450_{c17}$, $P450_{c21}$, and $P450_{arom}$, microsomal enzymes.
[b]Number of pseudogenes given in parentheses.
[c]In amino acids, the presequence, given in parentheses, includes the amino-terminal extension signal peptide.
Reproduced from Makin and (Gower, 1996) with permission of VCH publishers, Weinheim, Germany.
[d]The enzyme *CYP17A1*, coded for by the gene *CYP17*, is inhibited by Abiraterone (a 17-pridinyl) derivative of pregnenolone (Attard et al., 2009), which is undergoing clinical trials for use as a potent bioavailable inhibitor of androgen production, reducing post-operative recurrence of prostate cancer.

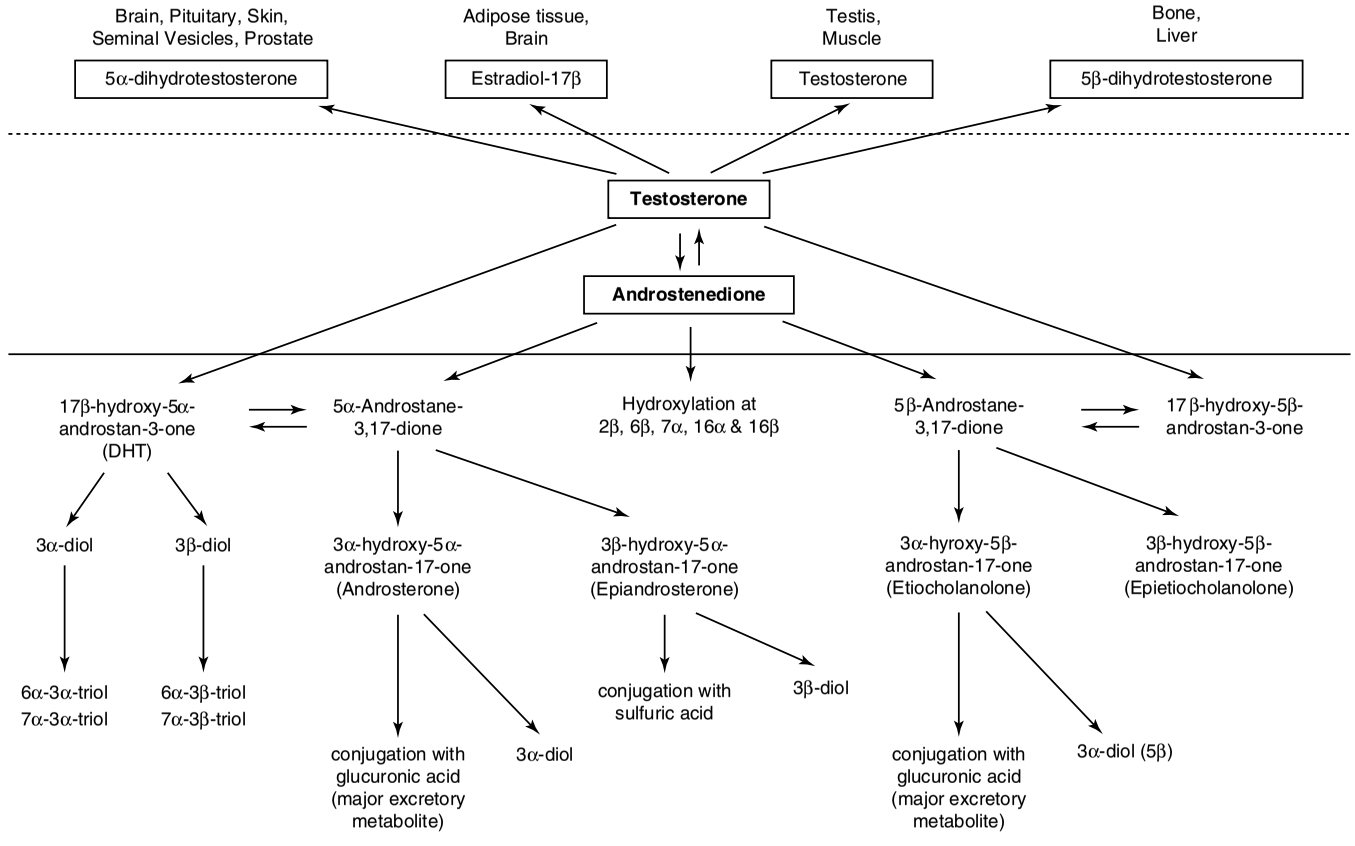

Fig. 6.2 Activation and inactivation pathways of androgen metabolism. Pathways above the dotted line show activation pathways and products responding to the products. Pathways below the solid line generally involve inactivation, leading to a decrease in activity and promoting the excretion of the products (from Sundaram and Kuman, 1996, with permission of authors and Cache River Press, Vienna, IL., USA)

Table 6.2 Selected references for the use of TLC/HPLC in purification and separation of C_{19} steroids

Application	Steroids purified/separated	Methods used	Methods used in further quantification/purification	References
Plasma steroids (free and conjugated)	T, DHA, DHAS, 4-A	SPE extraction; isocratic HPLC	RIA	Boschi et al. (1994)
T metabolism in rat liver microsomes	T metabolites	HPLC (isocratic, reversed-phase, Ultrasphere IP column; elution with methanol/water/tetrahydrofuran (35:35:10, by vol., pH 4.0)	GC-MS; metabolites Derivatised with N_2O-bis(trimethylsilyl) tri fluoroacetamide-trimethyl chlorosilane	Sanwald et al. (1995)
Effect of plasma steroid-binding proteins on androgen metabolism in human leucocytes *in vitro*	Metabolites of T, 5α-DHT, 4-A	Initial separation and quantification by HPLC	Specific RIAs	Dechaud et al. (1995)
Formation of pregnenolone and DHA fatty acid esters in human high-density lipoproteins	Fatty acid esters of pregnenolone and DHA	HPLC	None	Lavallee et al. (1996a)
3β-HSD/isomerase activity in zebra finch telencephalon in culture	DHA, 4-A	HPLC	Recrystallisations of products to constant specific radioactivity	Vanson et al. (1996)
Effect of gamma-hexachlorocyclohexane on biosynthesis of steroids in goldfish testis and ovary *in vitro*	4-A, T, 11-oxo T	TLC then HPLC	None	Kime and Singh (1996)

(continued)

Table 6.2 (continued)

Application	Steroids purified/separated	Methods used	Methods used in further quantification/purification	References
Steroid metabolism in embryonic and newly-hatched steelhead trout	4-A, T, and T glucuronide	TLC then HPLC	None	Yeoh et al. (1996b)
Steroid biosynthesis of androgens; identification of T in male frog brain	DHA, T, 4-A, 5α-DHT	HPLC	GC-MS; RIA	Mensah-Nyagan et al. (1996b)
Postnatal decline in DHA and 7-dehydro DHA in newborn foals	DHA, 7-dehydro DHA	SPE; HPLC	RIA	Raeside et al. (1997b)
T metabolism in rat brain microsomes; effects of phenytoin	6β (6α) DHT and OH-4-A; plus other isomers	HPLC, equipped with photodiode array detector	Inhibition experiments with antibodies against specific cytochromes P-450, e.g. CYP2B, 2C and 3A	Rosenbrock et al. (1999)
DHA metabolism in monocyte-derived macrophages	16-OH DHA, 5-androstenediol; 5-androstentriol; lower amounts of 4-A, T and 16-OH T	TLC	None	Schmidt et al. (2000)
Properties of 17β-HSD from *Cochliobolus lunatus*; preferred substrates and inhibition	T metabolites (oxido-reduction at C-17 and C-3)	TLC	Kinetic studies	Rizner et al. (2000)
Pregnenolone metabolism in mouse uterus; effects of LH	DHA, 4-A, T	TLC, HPLC	None	Debnath et al. (2000)
Hydroxysteroid sulphotransferase in frog brain	DHAS	HPLC	RIA	Mensah-Nyagan et al. (2000)

Blood steroid profiles and steroidogenesis in follicles and testis fragments of sea lamprey	15-OH 4-A, and 15-OH T; small quantities of T	HPLC	None	Lowartz et al. (2003)
Faecal androgens in spotted hyenas	Total androgen metabolites; plasma T (wild hyenas)	HPLC	Enzyme immunoassay	Dloniak et al. (2004)
Metabolism ^{13}C-T and ^{2}H-Oe-17β in reef-building coal homogenates	T, 4-A, 5αAdione, Oe-17βOestrone	TLC	None	Tarrant et al. (2003)
Androgen metabolism in human skin cells *in vitro*	T, 5αDHT, Adiols	TLC	None	Munster et al. (2003)
Neurosteroids in rat brain (cortex and sub-cortex)	DHA and its 7-hydroxy derivatives; also their sulphates	Ether extraction of homogenates, solvent partition	Specific RIAs; sulphates required solvolysis	Kazihnitkova et al. (2004)
Prototypic examples for substrates for CYP3A in liver microsomes (human and animal)	T, 6β -OH-T	Incubation of [^{14}C] T with cDNA-expressed CYP3A enzyme in liver microsomes TLC separations with (sequentially) CH_2Cl_2/acetone (80:20, v/v), $CHCl_3$/ethyl acetate/ethanol (80:20:14)	Radioautography and scintillation counting	Waxman and Chang (2006)

OH, hydroxy; LH, luteinising hormone; Adione, androstanedione; Adiols, androstanediols; Oe-17β, oestradiol-17β.

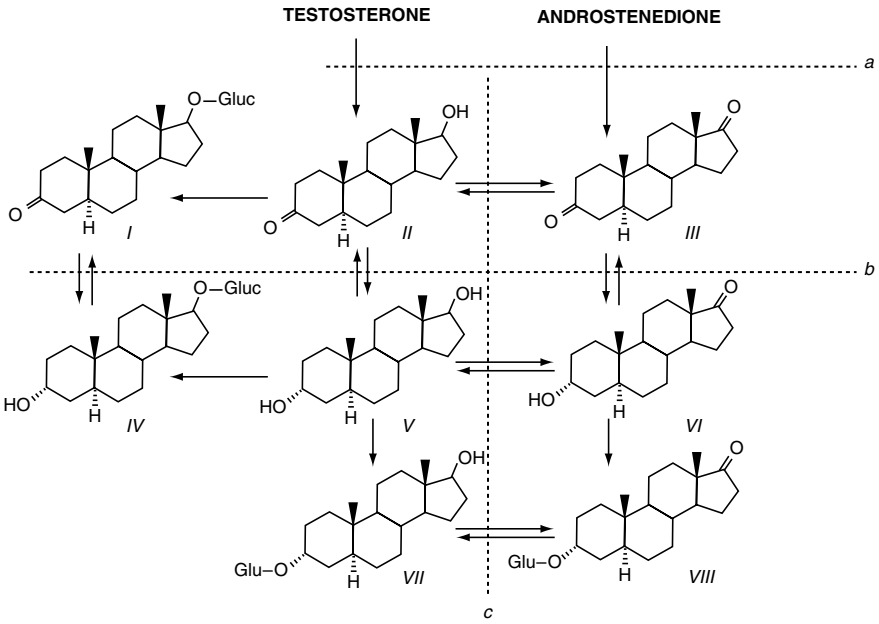

Fig. 6.3 Pathways of 5α-DHT metabolism. Gluc, glucuronide; I, 5α-DHT gluc; II, 5α-DHT; III, 5α-androstanedione; IV, 5α-androstane-3α,17β-diol 17-gluc; V, 5α-androstane-3α,17β-diol; VI, androsterone, VII, 5α-androstane-3α,17β-diol 3-gluc; VIII, androsterone gluc; *a*, 4-ene-5α-reductase; *b*, 3α-hydroxysteroid dehydrogenase; *c*, 17β-hydroxysteroid dehydroxygenase (after Rittmaster et al., 1988, with permission of authors and publishers, The Endocrine Society, USA)

Another important urinary metabolite is T, which is excreted largely, but not exclusively, as the 17-glucuronide; 11-oxygenated-17-oxosteroids (11-oxy-17-OS) are also present in urine (excreted as mixtures of glucuronides and sulphates) and occur as a result of oxidative side-chain removal of corticosteroids, such as cortisol and cortisone (see Chapter 5). Measurement of total urinary 17-OS was performed for many decades to provide a rapid indication of androgenic status, especially for patients in whom excessive androgen production occurs.

In the context of glucuronidation, the detailed study of 5α-DHT metabolism by Rittmaster et al. (1988) has revealed something of the complexity of steroid glucuronide metabolism. In the case of 5α-DHT, three major pathways have been identified (Fig. 6.3).

Glucuronyl transferases (GTs), now called uridine phosphoGTs (UGTs), have been studied, often at the molecular level (Kicman et al., this volume Chapter 9) by several research groups in recent years. UGTs occur as a super family of enzymes that catalyse the glucuronidation of a wide variety of xenobiotics, bile acids and steroids (Gall et al., 1999). Narayanan et al. (2000) confirmed the presence of four major forms of UGTs in rat liver and intestine and, in attempts to study the kinetics of the glucuronidation reaction, the authors have devised a quantitative method for various intact steroid glucuronide conjugates (see Section 6.6.4). The UGTs are also found

in mammary glands, as well as in breast and prostate tumour cell lines, MCF-7 and LNCaP, respectively (Hum et al., 1999). Far from the concept that glucuronidation of non-polar steroids is merely concerned with decreasing their hydrophobicity and, hence, increasing their renal excretion (as noted above), the process appears to play several important regulatory roles. For example, the same group (Hum et al., 1999) characterised two highly related isoforms, UGT2B15 and UGT2B17, in human LNCaP cells. Only the expression of the latter isoform was affected by treatment with androgen, growth factor or cytokines. Considering this finding with other results, the authors concluded that steroid glucuronidation by UGTs could be an important control mechanism in steroid inactivation in target tissues (Fig. 6.4).

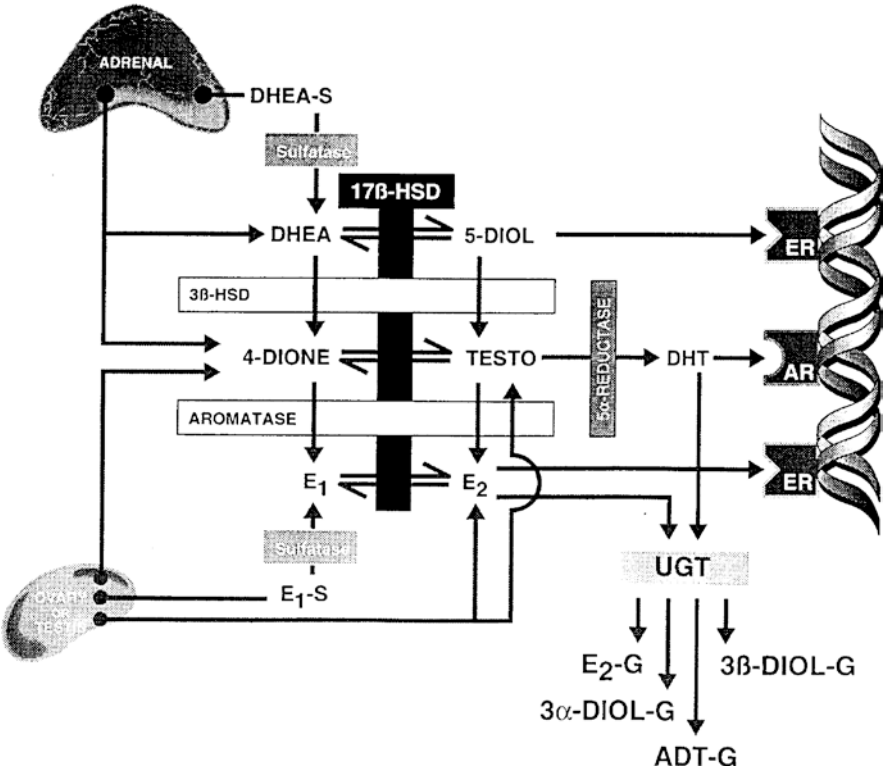

Fig. 6.4 Production and elimination of dihydrotesterone (DHT) in steroid target cells. The concentration of DHT in the cell is in equilibrium between production and elimination. The enzymes involved in steroid synthesis and catabolism are as indicated: UGT, uridine diphosphoglucuronosyl transferase; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; ADT, androsterone; 5-diol, androst-5-ene-3β,17β-diol; 3α-diol, androstane-3α,17β-diol; DHT, dihydrotestosterone; Testo, testosterone; 4-DIONE, androstenedione; DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulphate; E_1, estrone; E_2, estradiol; E_1-S, estrone sulphate; AR, androgen receptor; ER, estrogen receptor; DHT-G, DHT-glucuronide; 3α-diol-G, 3α-diol-glucuronide; ADT-G, ADT-glucuronide; 3β-diol-G, 3β-diol-glucuronide (from Hum et al., 1999, with permission of authors and publishers, Pergamon Press)

Furthermore, Belanger et al. (1998) have shown that glucuronidation of androgens abolishes their receptor interaction and facilitates their clearance from the androgen target cells. The microsomal transport of glucuronides has been determined by 'rapid filtration' transport assays. For this purpose, liquid chromatography, coupled with electrospray ionisation tandem mass spectrometry (LC-ESI-MS/MS, see Section 6.7.8), was utilised (Staines et al., 2005).

In addition to the methods detailed above, T and 4-A can also be metabolised by means of cytochrome P-450-dependent hydroxylations. For oestrogen-producing tissues, hydroxylations occur at C-1 and C-2, while in other tissues, particularly in the liver, hydroxylations occur at a variety of sites including C-6, 7, 11, 14, 15, 16 and 18. In addition, the regio- and stereospecific hydroxylation capabilities of bacteria and fungi are well known (Brzezowska et al., 1996), leading to the formation of various mono- and di-hydroxylated products, with little or no androgenic potency. Hu et al. (1995) separated and characterised some 20 metabolites of T, 4-A, progesterone and other progestagens after incubation with several fungal strains. Mono-hydroxylations occurred at C-6β, 7α, 9α, 14α and 15β, with di-hydroxylations at 7α, 14α-; 9α, 14α-; 14α, 15α-; 14α,15β-; and 14α, 16β. Such microbiogical hydroxylations can produce higher yields at lower cost than the corresponding chemical syntheses (Cotillon et al., 1997). The hydroxylated products have often been used as models in studying mechanisms involved in hydroxylation systems. Methods for estimating T and 4-A hydroxylation products are described in later sections (Sections 6.6.2, 6.7.5.1 and 6.9).

Many of the cytochromes P-450 involved have been studied intensively at the molecular level during the past 2 decades (Ortiz de Montellano, 1995; Lewis, 1996; Makin and Gower, 1996; Payne and O'shaunessy, 1996). Two important enzymes may be referred to here: the 7-hydroxylase, involved in the hydroxylation of DHEA and other neurosteroids (see Sections 6.6.2, 6.6.4.1, 6.7.5.1, 6.7.5.2, 6.7.7, 6.10.10 and Table 6.4); and 6β-hydroxylase, important in T hydroxylation (see Sections 6.7.5.1 and 6.9). This 7-hydroxylation reaction has generated considerable interest during the past few years (Cotillon et al., 1997) because of an earlier suggested key role for, especially, 7α-OH-DHEA, in regulating the immune response. 7-Hydroxylation has already been reported in tissues of various species, such as human liver, adrenals and adipose tissue, rat liver, brain and central nervous system, in mouse tissues and many microorganisms. Cotillon et al. (1997) have studied the 7α-hydroxylase of mycelia of the fungus *Fusarium moniliforme* and showed that the microsomal enzyme system contained cytochrome P-450, and was inducible by DHEA (see also Section 6.7.5.1).

Clearly, there is a plethora of metabolites of T and 5α-DHT and their conjugates. Measurement of plasma levels of many of these is important in clinical and biochemical research. Two examples of many that may be cited are measurements of the glucuronides of androsterone and 5α-androstane-3α, 17β-diol which were earlier thought to be markers of peripheral tissue androgen metabolism (Rittmaster, 1993; Rittner et al., 1997). In keeping with these suggestions, Labrie et al. (2006) have shown that there was no correlation between the T concentrations in sera of pre- and post-menopausal women and those of androsterone G or 5α-androstane-3α,

17β-diol G. Indeed, measurement of the pool of total androgens, reflected by serum concentrations of the latter two glucuronides, cannot be replaced by other steroids, such as DHEA or DHEAS (see Section 6.8.1 for further explanation, clinical and physiological significance and methodology).

It is obvious that effective chromatographic separations and reliable quantification are mandatory.

6.4 Extraction Methods for Androgens from Biological Matrices

It is true to say that each laboratory has its own favoured and well-tested protocol for androgen estimations, which includes some kind of extraction and clean-up procedure prior to separation and quantification. All these stages, the necessity for which is dependent upon the specificity of the methodology used for final quantitation, have been described in detail earlier (Gower, 1995). The methods involved specifically for T estimation in human urine have been discussed by Venturelli et al. (1995). In the following sections, various well-established methods for extraction are reviewed, together with those published within the past few years. Several specific examples of the use of each method are described, with indications of other references summarised in Table 6.2.

6.4.1 Liquid–Liquid Extraction

Most methods of steroid analysis used today rely upon an initial extraction of the steroid(s) of interest from the medium in which they are present. The principle of this well-used organic solvent extraction method relies on the partition of the C_{19} (or other) steroid between its usual aqueous environment (e.g. urine, blood plasma, tissue homogenate) and a partially miscible solvent. The aqueous and organic solvent layers can then be separated by centrifugation and the latter layer obtained by aspiration. To obtain good recoveries, two or three extractions are often performed (Baltes et al., 1998; Section 6.6.2). Ethyl acetate, dichloromethane and chloroform/methanol (2:1 v/v) have been commonly used for many years for extraction of free (unconjugated) C_{19} steroids. The organic solvent extracts can then be dried by standing over anhydrous sodium sulphate, prior to being subjected to clean-up procedures, if these are thought to be necessary (Section 6.5). For example, Fernandez et al. (1996) studied the hydroxylation of T in rat liver microsomal preparations; the hydroxylated derivatives were extracted with dichloromethane. Pertiwi et al. (2002) used ethyl acetate to extract pregnenolone metabolites from whole rat testes in studies of the movement of such metabolites through the various subcellular components during incubation.

Thienpont et al. (1994) have explored the use of cyclodextrins for the purification of steroid extracts prior to isotope dilution gas chromatography-mass spectrometry

(GC-MS) (see Section 6.7). After liquid–liquid extraction of human serum with dichloromethane, back-extraction was performed into a cyclodextrin–-water solution. After washing the aqueous phase with *n*-hexane steroid (T) was finally extracted into dichloromethane, ready for quantitative analysis. The authors studied the use of various concentrations of a number of cyclodextrins and their derivatives, finally choosing an aqueous solution of 150 mmol/L hydroxypropyl cyclodextrin, thereby achieving an overall extraction efficiency of 80% for plasma T. The authors claimed that their method was equivalent to gel chromatography purification in regard to effectiveness, accuracy and precision.

6.4.2 Solid-Phase Extraction

This type of extraction involves the use of a solid support, which can adsorb steroids from a solution when passed over it; the steroids can be recovered subsequently by washing the solid support with solvents of varying polarity. In 1968, Bradlow proposed the use of the resin, XAD-2 (a neutral cross-linked polystyrene polymer), and later modified the procedure (1977). Although this procedure was used successfully for many years, particularly for assays of urinary steroids, the throughput time was low and large volumes of polar solvents were required to displace the adsorbed steroids, thus resulting in extended time periods being needed for solvent evaporation to small bulk, prior to further procedures. The introduction by several groups (e.g., Shackleton and Whitney, 1980) of octadecyl-substituted cartridges has made the use of Amberlite resin virtually obsolete. These cartridges can adsorb mixtures of steroids (both free and conjugated) with high capacity, the different groups being recoverable by elution with appropriate solvents. Details of the use of solid-phase extraction (SPE) in analysis of C_{19} steroid conjugates are given in Section 6.5 but the extreme usefulness of this method can be noted from the following examples. Foster et al. (2002) used methanol in the initial liquid–liquid extraction of C_{18} and unconjugated C_{19} steroid metabolites from equine placental tissue preparations. Further extraction and clean-up were achieved using C_{18} Sep-Pak cartridges and elution with diethyl ether, prior to characterisation by GC-MS with selection ion monitoring (SIM). Kintz et al. (1999a, b) measured concentrations of DHEA and T (plus T esters) in human hair, first by its decontamination using dichloromethane, then by washing samples with 1 M NaOH solution. Following neutralisation and addition of [$^2H_3$]-T as internal standard, SPE was performed with solute C_{18} columns. A final wash of the columns with *n*-pentane yielded extracts that were sufficiently clean for derivatisation and GC-MS (Section 6.7.5).

6.4.3 Extraction of C_{19} Steroid Conjugates

Several methods are available for the extraction and subsequent quantification of C_{19} steroid conjugates. One method, sometimes referred to as the ‘combinatorial approach’ (e.g. Chatman et al., 1999), involves extraction of both free steroid(s) and

their conjugates using, for example, liquid–liquid extraction with, first, a non-polar solvent (for free steroids) followed by a polar solvent, e.g. chloroform/2-butanol (50:50, v/v) to extract conjugates. In earlier studies, the conjugate fraction had to be hydrolysed with preparations of β-glucuronidase for glucuronides and/or sulphatase for the steroid sulphates. Details of these procedures have been discussed earlier by Gower (1995), Venturelli et al. (1995) and Kicman and Gower (2003). In the past, many workers used the solvolysis procedure of Burstein and Lieberman (1958), involving overnight refluxing of the acidified urine extract (pH 1) with diethyl ether. Although this procedure gives excellent recoveries for 5-androstene-3-sulphates from urine and serum (98% and 96%, respectively), as reviewed by Gower (1995), there are potential hazards with this method because of the flammability and risk of explosion with large volumes of ether.

Enzymic hydrolysis is commonly used now but one has to be aware of potential problems. For example, when β-glucuronidase, prepared from *Helix pomatia*, is used for the hydrolysis of glucuronides, some workers believe that the presence of 5-ene-3β-HSD/isomerase as an impurity (Gower et al., 1995 for references) can give rise to artifactual conversion of, for example, 5-androstenediol to T. However, it should be noted that Venturelli et al. (1995) in studies on urinary T glucuronide, were unable to detect any 5-ene-3β-HSD/isomerase activity in β-glucuronidase preparations from *Escherichia coli*, *Patella vulgata* or *H. pomatia*, Type H–1 (Sigma). In 1989, Payne et al. published detailed experiments that resulted in a unified scheme for the differential extraction of conjugated and unconjugated C_{19} steroids. Using added radio-labelled steroids, it was shown that steroid conjugates were quantitatively recovered in the 47% aqueous ethanol fraction and unconjugated steroids in the 100% ethanol fraction. When the scheme was applied to human male sera, C_{19} steroids, such as T which were normally protein-bound, could only be recovered quantitatively if the serum samples were first heated at 60°C in the presence of urea. Separation of glucuronides and sulphates, eluted together in the 47% aqueous ethanol fraction, was achieved by sequential hydrolysis with β-glucuronidase (*E. coli*) to hydrolyse glucuronides, followed by organic solvolysis for sulphates. The liberated unconjugated steroids could then be eluted separately by 100% ethanol.

Tohyama et al. (2004) have studied the properties and funcion of Klotho, a novel β-glucuronidase. The hydrolysis of the glucuronides of oestradiol-17β, oestrone and oestriol could all be hydrolysed in the presence of Klotho, which was shown to be a type I membrane protein that shows sequence similarity with members of the glycosidase family 1.

Tagawa et al. (2000) extracted steroids and steroid sulphates by treating plasma samples from patients suffering from hypo- or hyperthyroidism, with methanol. The resulting extract (after the removal of precipitated protein) was subjected to SPE plus an ion-exchange column. Elution resulted in three fractions: free steroids, mono- and di-sulphates. DHEA and pregnenolone were separated by high performance liquid chromatography (HPLC) (Section 6.6) and quantified by radioimmunoassay (RIA) (Section 6.10.10). After hydrolysis of the mono-sulphate fraction with aryl sulphatase, the DHEA and pregnenolone produced were separated by HPLC (Section 6.6), prior to final quantification by GC-MS (Section 6.7).

6.4.4 *Supercritical Fluid Extraction*

Supercritical fluid extraction (SFE) is a means of extraction of, commonly, pharmaceuticals from water and serum, but is also of use for anabolic and androgenic steroids (Simmons and Stewart, 1997). In brief, water or serum samples containing the compounds of interest are added to Celite wet support contained in evaporation dishes and SFE is achieved with supercritical CO_2 in a supercritical fluid extractor under pressure at strictly controlled temperature. The mobile phase used for subsequent HPLC for each sample serves as extraction trap. After SFE, each sample is transferred to a volumetric flask and the volumes made up with mobile phase, after which HPLC is carried out. Quantification is achieved by comparison with standard calibration curves. Extraction efficiencies found in the work of Simmons and Stewart (1997) for T were 86–90% and 86–92% for water and serum, respectively.

6.5 Purification Procedures Prior to Quantification

The examples of the work of Kintz et al. (1999a, b) make it clear that, although a single extraction step may also provide a degree of purification, this may still not result in sufficient purity for final quantification, depending of course, upon the quantification step chosen. This obvious difficulty has been appreciated for many years and has been discussed by Gower (1995) and by Venturelli et al. (1995). Schmidt et al. (1985) recommended placing an amino (NH_2)-column in series after the C_{18} cartridge to eliminate impurities from hydrolysed urine samples. In their studies with urinary T estimations, Venturelli et al. (1995) confirmed improvements in clean-up when an additional NH_2 column was utilised.

Other post-extraction purification procedures include the use of TLC of steroid extracts. For example, this has been sufficient, not only to separate T from its metabolites, such as 5α-DHT, but also to result in samples which were sufficiently pure for quantification. Such has been the case with studies of T metabolism in human skin cells *in vitro* (Munster et al., 2003). This is not always the case, however, and much depends on the complexity of the biological matrix and the relative quantity of a steroid being measured and other substances also present in high abundance. If this is the situation, and the quantitation methodology is not sufficiently robust to cope, then the number of purification steps is inevitably greater.

Column chromatography has been used by some groups for the purification of extracts prior to RIA (Section 6.10.10). For example, Monti et al. (1997) quantified T and 5α-DHT in tissue samples taken from different regions of the prostate of patients with benign prostatic hyperplasia (BPH). The same methodology was used by Di Silverio et al. (1998) to measure plasma T and 5α-DHT concentrations in BPH patients who were being treated with lipido-sterol extract of *Sereno repens* (LSESr), a phyto-therapeutic drug, whose actions include inhibition of types 1 and 2 of 5α-reductase and competitive binding to androgen receptors in prostatic cells. In addition

to Celite, Sephadex LH-20 has been used in small columns by many workers, not only in purification of final extracts for quantification but also in separating free and conjugated steroids. Our own group has found this method extremely useful in preparing testicular extracts suitable for analysis by GC-MS (Pertiwi et al., 2002).

6.6 Use of High Performance Liquid Chromatography in the Separation and Quantification of C_{19} Steroids and Their Derivatives

6.6.1 Introduction

As an analytical technique, HPLC has a number of advantages, not least that it can play a dual role–first, as a rapid means of purification of steroid extracts and, secondly, in separation and quantification. Initial purification of extracts has been alluded to in Section 6.5, and we (Kwan et al., 1992) have found HPLC to be extremely useful in purifying C_{19} steroid-containing extracts so that they are suitable for GC-MS analysis, and for immunoassay (IA), particularly in situations where high-specificity antisera are not available (see Section 6.10). Makin and Heftmann (1988) have provided details of the primary role of HPLC in the separation and quantification of C_{21}, C_{19} and C_{18} steroids. Some of the relevant information specifically for C_{19} steroids is included in Table 6.2. It should be noted that liquid chromatography–mass spectrometry–mass spectrometry (LC-MS-MS) methods certainly claim greatly enhanced sensitivity; these methods are discussed in Sections 6.7.7 and 6.7.8 (Cawood et al., 2005) in which it is shown that LC-MS-MS can now be used for measurement of T down to 0.3 nmol/L, approx 90 ng/L or 90 pg/mL). To overcome the problem of the relative insensitivity (and of course specificity) of UV detection at 240 nm, Payne et al. (1989) were able to use HPLC in the separation of serum steroids by detection/quantification using specific bacterial hydroxysteroid dehydrogenases (HSDs) off-line. Extraction efficiency from human serum 93.9 ± 6.4% (mean ±SD, n = 12) was achieved for 4-A, T, 5α-DHT and 5α-androstane-3β,17β-diol. For serum DHEAS, 93% was recovered; a LiChrosorb Diol column was used, with a concave gradient of 2-propanol–*n*-hexane mixtures as eluting solvent. More sensitive detection methods are discussed below.

6.6.2 Use of HPLC in Metabolic Studies

Hydroxylation of T and 4-A as a catabolic reaction has been discussed earlier (Section 6.3) and, in order to study this reaction in detail, some hydroxy derivatives of 4-A (2β-, 6β-, 7α- and 16α-), formed in rat liver microsomes *in vitro*, have been separated by Valdes et al. (1993). Reversed-phase HPLC was used on an RP-100

column, with a linear gradient of acetonitrile-water (30:70, v/v, to 60:40, v/v) over 30 min. Detection was achieved with UV light (254 nm wavelength). Draper et al. (1998) have also studied the hydroxylation of T by devising an assay for CYP3A4/5, which catalyses the major hydroxylation of T (6β-, 75–80%). Human liver microsomes were incubated with [1,2,6,7-^{3}H]T in the presence of NADPH, and the quantity of $^{3}H_2O$ released was shown to correlate closely with the extent of 6β-hydroxylation and with the formation of terfenadine, both commonly-used markers for CYP3A4/5. The T metabolites were separated by HPLC.

Svechnikov et al. (1994) have exploited the use of gradient HPLC in their studies of the 4-en-3-oxo steroid pathway (Section 6.2) occurring in murine Leydig cells. After incubation with C_{21} steroid precursors, e.g. progesterone, and extraction, the metabolites were separated using a five-step gradient, with methanol, acetonitrile and water as mobile phase and 5 μ Lychrosorb RP-18 as sorbent. Activities of 3β-HSD, 17α-hydroxylase, C-17,20-lyase and 17β-HSD were derived from the relative quantities of purified metabolites formed, corresponding to these enzymes either as substrate or product.

Raeside et al. (1999) published results of metabolic studies of both androgens and oestrogens in the reproductive tract and accessory sex glands of the domestic pig (*Sus scrofa*). Samples of seminal vesicles and other accessory organs were incubated in suitable medium, together with ^{3}H-T, ^{3}H-oestradiol-17β or ^{3}H-oestrone. After 2 h, portions of the spent medium were removed for ^{3}H estimations, prior to SPE using C_{18} Sep-Pak cartridges (see Section 6.4.2). The steroid sulphates and glucuronides were hydrolysed and the resultant unconjugated fractions, together with the free steroid fraction were submitted to gradient HPLC (acetonitrile-water) on a reversed-phase C_{18} column. The results showed that 5α-DHT was the major metabolite of T (yields in vas deferens and prostate were >25% and >30%, respectively), but very little was formed in other accessory organs. No clear evidence for C_{19} steroid conjugation was obtained, even though conjugation of oestrogens was quantitatively very important. The authors concluded that their overall results highlight the need to consider the local metabolism of steroids in the porcine reproductive tract.

Another example of the use of HPLC in separating T metabolites formed in metabolic studies is provided by Baltes et al. (1998) who developed a method for liquid–liquid extraction of the metabolites after incubation. It was also shown that two extractions of microsomes with ethyl acetate gave improved specificity and overall recoveries being around 90% (see also Section 6.4.1). T and its major metabolites were separated by isocratic HPLC (reversed-phase) with a mobile phase consisting of phosphate buffer/methanol/acetonitrile (50:38.5:11.5, by vol.). The authors experimented with numerous chromatographic parameters, the ratio of acetonitrile to methanol being the most important.

DHEA, pregnenolone, their sulphates and other derivatives have been studied in brain tissues and CNS during the past 15 years and are now considered to be important neurosteroids. Their significance and quantification by GC-MS are described in detail in later sections (Sections 6.7.5.1, 6.7.5.2, 6.7.6–6.7.8). In this context, Jellinck et al. (2005) have utilised TLC and HPLC in studies on the relationship in

rodent brain cells of 7-hydroxylation of DHEA and aromatisation. Different neuronal cell lines of mouse and rat brain were compared with embryonic rat hippocampal cells in primary culture. Incubations were performed with ^{3}H-labelled 4-androstenedione (4-A) and DHEA. After incubation, extracted metabolites (oestrogens from 4-A and 7-hydroxylated derivatives from DHEA) were separated by TLC and then by HPLC for further purification and identification. It was found that all brain types produced oestrogens from 4-A (although to different extents). However, although most mouse hippocampal cells were able to 7-hydroxylate DHEA, mouse brain BV2 microglia were virtually unable to do this but, instead, converted DHEA via 4-A to T (see Fig. 6.1). The authors believe that these results highlight the pivotal role of 3β-hydroxysteroid dehydrogenase/4-en-3-oxosteroid isomerase in regulating the conversion of DHEA to 4-A and thence to oestrogens via T, and it is suggested that differences in the metabolism of DHEA by brain cells may account for differences in their immunomodulatory and neuroprotective functions. Further studies on DHEA metabolism, involving identification of metabolites by LC-GC-MS are described in Section 6.7.

6.6.3 Use of HPLC in Analytical Studies

HPLC has been used for measuring T and epi-T (17α-hydroxy-androst-4-en-3-one), in human urine (see also Chapter 9). For example, Amin et al. (1993) have reported the HPLC of anabolic steroids, including T, in urine with detection by sensitised terbium fluorescence. The authors showed that Forster energy transfer from (in this case) 4-en-3-oxosteroid donors to terbium ion acceptors can occur, provided that acceptor and donor are less than approximately 10 nm apart, this condition being fulfilled if both are sequestered in sodium dodecyl sulphate micelles. Optimum results were obtained using micellar chromatography when the mobile phase consisted of the detergent solution, with a C_{18} column and terbium nitrate solution being introduced into the eluent stream as it emerged from the column. Urine samples could be injected directly into the column, and detection limits down to 100 pg were achieved. This indicates another method where sensitivity of HPLC may be sufficient

In 1995, Navajas et al. described an HPLC method for T and epi-T that was simple, rapid, selective, sensitive and reproducible. Samples for analysis were prepared using a previous enzymatic hydrolysis of conjugates, followed by liquid–liquid extraction. HPLC was performed on a Hypersil BDS-C-18 column, with gradient elution and UV absorbance (240 nm) detection. The signal: noise ratio was 6:1 with detection limits 20 ng/mL and 300 ng/mL for T and epi-T, respectively.

More recently, Gonzalo-Lumbreras et al. (2003) have developed and validated an HPLC method for the measurement of T and epi-T in the urine of healthy males. The steroids were extracted either by the liquid–liquid extraction (urine samples from a healthy male population) or by SPE (urine samples from nine healthy men), These extraction methods were found to be interchangeable in relation to the values found for T and epi-T (quantification limits for these steroids were 8.6 and 5.4 ng/mL

for liquid–liquid extraction, and 7.3 and 5.7 ng/mL for SPE, respectively). Separation and quantification were achieved by HPLC on a Hypersil C_{18} column with water/acetonitrile (57: 43, v/v) as mobile phase and UV light (245 nm) detection.

6.6.4 Use of HPLC in Direct Quantification of Intact C_{19} Steroid Conjugates

Clearly, it is preferable to be able to measure intact steroid conjugates without the necessity for prior hydrolysis, and a number of groups have published assays for glucuronides and sulphates of use in metabolic studies or assay of biological fluids.

6.6.4.1 Glucuronides

In 1994, Iwata et al. described a highly sensitive method for the glucuronides of androsterone (A) and aetiocholanolone (Ae). The glucuronic acid moiety was directly derivatised by conjugation with 6,7-dimethoxy-1-methyl-2(1–4-quinoxalinone-3-propionylcarboxylic acid hydrazide. The resulting fluorescent derivatives were then separated on a C_{18} column using methanol/acetonitrile/0.5% methylamine (1:1:2, by vol.), and detection was achieved spectrofluorimetrically at 445 nm with excitation at 367 nm. This method of detection (130–480 fmol/5 μL urine) is clearly far more sensitive than the UV method described above.

Narayanan et al. (2000) have utilised HPLC as a tool in determining the kinetics of UDP-glucuronosyl transferases (UGTs) in rat liver and intestine. As the authors point out, earlier methods for glucuronides necessitated their hydrolysis before colorimetric analysis could be performed. Furthermore, these methods were unable to distinguish isomeric glucuronides or those of multiple receptor site substrates; hence the importance of this assay for intact glucuronides. The three ‘classical’ substrates chosen included T glucuronide (representing UGT isoform 2B1); all were estimated in incubation media containing microsomes of rat liver or intestine, the analysis time for T glucuronide being 12 min. The limit of detection was 0.125 nM, the isoform 2B1 only being associated with rat liver.

6.6.4.2 Sulphates

7-Oxo-DHEA is related metabolically to DHEA but is more effective than the latter steroid in various ways (Marwah et al., 1999) that include greater effects on induction of thermogenic enzymes, increased production of interleukin 1 by isolated T cells and increased enhancement of memory in old rats. 7-Oxo-DHEA is known not to be metabolised to androgens and oestrogens (unlike DHEA itself), but it was isolated from human urine several decades ago and is found in venous plasma, partly free and partly sulpho-conjugated. It appears that its biochemical origin has yet to be

elucidated. Marwah et al. (1999) have therefore developed and validated an assay for 7-oxo-DHEAS in human plasma. After addition of 17β-hydroxy-3β-methoxyandrost-5-en-7-one as internal standard, steroids were extracted by SPE with C_{18} cartridges. HPLC was performed at 40°C on a Zorbax analytical C_{18} column (protected with an appropriate guard column). Gradient elution was used with the mobile-phase composition: acetonitrile-methanol-5 mM-tetrabutyl ammonium dihydrogen phosphate (TBADHP) (30:10:60, by vol.) at time 0. The proportion of acetonitrile was gradually increased, while that of TBADHP was decreased to 75:10:15 (by vol) at 17 min and then returning to the initial composition by 20 min. With appropriate modifications, the authors were able to assay human plasma samples for 7-oxo-DHEA and 7-oxo-DHEA-3β-acetate, the latter being of use since the acetyl group acts to protect against oxidation of the 3β-OH group during synthesis; the acetyl group is readily hydrolysed *in vivo* when the compound is administered to human subjects. The detection limit for the method using UV light (360 nm) was 3 ng/mL; the standard curve was linear over the range 10–1,000 ng/mL and no appreciable degradation was evident on storage at –20°C for several months.

Closely related to 7-oxo-DHEA are the 7-hydroxylated DHEA derivatives and Jellinck et al. (2005) have utilised TLC and HPLC in studies on the relationship in rodent brain cells of 7-hydroxylation of DHEA and aromatisation. Different neuronal cell lines of mouse and rat brain were compared with embryonic rat hippocampal cells in primary culture. Incubations were performed with ^{3}H-labelled 4-androstenedione (4-A) and DHEA. After incubation, extracted metabolites (oestrogens from 4-A and 7-hydroxylated derivatives from DHEA) were separated by TLC and then by HPLC for further purification and identification. It was found that all brain types produced oestrogens from 4-A (although to different extents). However, although most mouse hippocampal cells were able to 7-hydroxylate DHEA, mouse brain BV2 microglia were virtually unable to do this but, instead, converted DHEA via 4-A to T. The authors believe that these results highlight the pivotal role of 3β-hydroxysteroid dehydrogenase/4-en-3-oxosteroid isomerase in regulating the conversion of DHEA to 4-A and thence to oestrogens via T, and it is suggested that differences in the metabolism of DHEA by brain cells may account for differences in their immunomodulatory and neuroprotective functions. Further studies on DHEA metabolism, involving identification of metabolites by LC-GC-MS are described in Section 6.7.

For the measurement of intact steroid sulphates (such as DHEAS), a major problem is its co-elution with serum proteins from pre-columns. In a study of this problem, Tagawa et al. (1998) used a polymer-coated mixed-function column as the pre-column for ion-pair chromatography with tetra-*n*-butylammonium (TBA) ion. The investigators used the 3-sulphates of oestriol, DHEA and pregnenolone in their experiments. Human serum (2.5 μL) was diluted with varying volumes of TBA ion (5–500 mM) included in the mobile phase for HPLC. It was shown that only in the presence of at least 100 mM TBA ions in the diluted serum were the steroid sulphates retained in the pre-column. It was therefore possible to prevent the co-elution of serum proteins and once these had been removed, column-switching HPLC was possible using a semi-microcolumn for analysis.

6.6.5 *High Temperature Liquid Chromatography*

The usefulness of HPLC and attempts to improve the resolution have been highlighted in previous sections and by Makin and Heftmann (1988) and Gower (1995). As Clark (2003) points out in her review, many attempts have been made to extend the use of HPLC by employing high temperatures for analysis and, as has been so advantageous in GC methodology, by using temperature programming. Now that the necessary programmable instrumentation and the required column stability at high temperatures are available, it is possible for analysts to obtain greatly improved resolution in shorter time spans than with ambient temperature HPLC (e.g. Djordjevic et al., 1999).

However, not only is temperature-programming (and hence heat-stable columns) necessary, so also is mobile-phase pre-heating, so that temperature mismatch between the latter phase and column stationary phase is avoided. With pre-heating, sharper peaks are obtained and the resolution is greatly improved (Clark, 2003). Figure 6.5

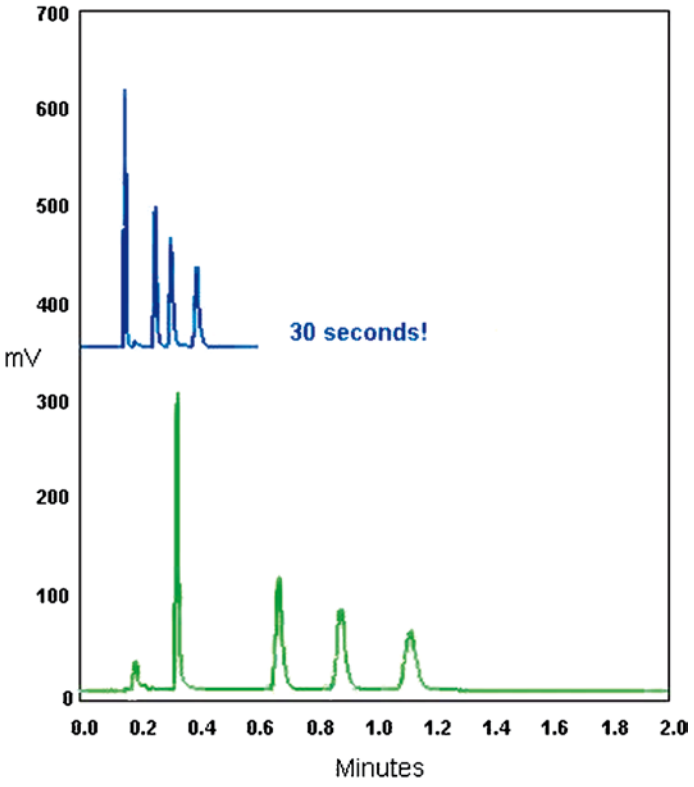

Fig. 6.5 Separation of steroids using water as a mobile phase. Column, Zirchrom PBD, 3µm, 100 × 4.6mm. Detection was with UV light 254 nm wavelength. For upper graph, flow rate was 6.0 ml/min; mobile phase water; temperature 200 degrees C. For lower graph, flow rate was 3.0 ml/min; mobile phase, acetonitrile; temperature 50 degrees C. Elution order was uracil, androstadienedione, androstenedione and epitestosterone. Reprinted from with permission of the author and publisher. (from Clarke, 2003, with permission of the author and The Biochemical Society, London)

illustrates the benefits in the separation of three C_{19} steroids, with uracil as internal standard. Further information concerning the instrumentation and benefits of HTPLC are discussed by Clark (2003).

6.6.6 *Separation of C_{19} Steroids Using Capillary Electrophoresis*

In their studies of T hydroxylation, Fernandez et al. (1996) have highlighted the need for suitable methods of separation of the numerous isomers likely to be formed. As these workers point out, reversed-phase HPLC methods were published in the 1980s but did not necessarily provide adequate resolution of all isomers (see also Section 6.6.2 and Fig. 6.6) Fernandez et al. (1996) therefore devised a capillary electrophoretic (CE) methodology. Initially, liver microsomes were incubated with T in a NADPH-generating system for 10 min and, after addition of internal standard, 11β-hydroxy T, liquid–liquid extraction of products was achieved with dichloromethane. It should be pointed out that previously Sonderfan et al. (1989) had demonstrated that, under the conditions used, neither 11α- nor 11β-hydroxy T were formed; hence the use of the latter steroid as internal standard. Unfortunately, due to constraints

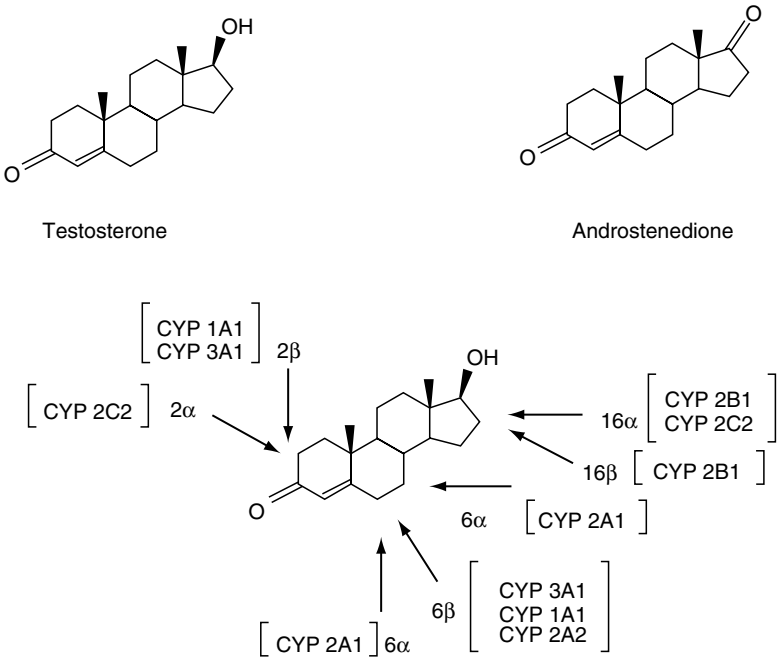

Fig. 6.6 Structures of testosterone, androstenedione and regioslectivity of testosterone oxidation by CYPs. Abbreviations: 6α, 6β, 7α, 16α, 16β, 11α, 11β, 2α, 2β, for the corresponding alcohols (from Fernandez et al., 1996, with permission of the authors and publishers, Elsevier Science)

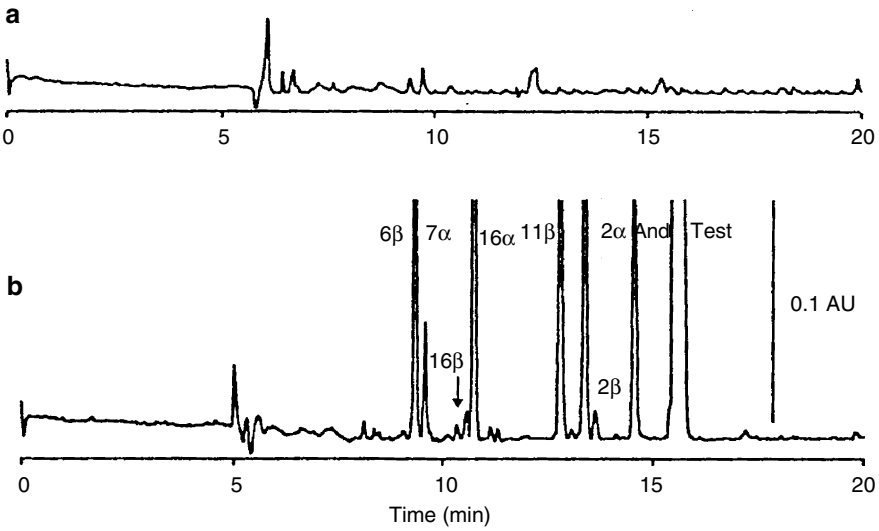

Fig. 6.7 Electropherograms of (**a**) control and PCM microsome mixture incubated without testosterone, (**b**) testosterone and its hydroxylated products catalysed by hepatic microsomes from control rats (electrophoresis conditions as in text) (from Fernandez et al., 1996, with permission of the authors and publishers, Elsevier Science)

on space, it is not possible here to describe the precise details of the conditions used for CE. Suffice it to say, however, that after painstaking modifications to the capillary utilised, the composition of the running buffer, etc., the optimum reproducible separation of T, 4-A and hydroxylated T derivatives was achieved (Fig. 6.7). The authors claimed that, at the time of writing, their method, once established and tested, was faster and simpler than previously published HPLC methods. A comprehensive review of the excellent resolving power of CE, working practices and the availability of equipment, has been published by Altria (2000).

6.7 Mass Spectrometry (MS), Gas Chromatography-Mass Spectrometry (GC-MS), Liquid Chromatography and Electrospray Ionisation (ESI)-MS-MS

6.7.1 Mass Spectrometry

MS, coupled with capillary GC or, more recently, with HPLC (LC-MS) is undoubtedly an extremely powerful technique for the quantification and characterisation of steroids (for earlier reviews, see Gower, 1995). Several types of MS have been developed and employed in steroid analysis during the past 20 years, such as metastable

peak monitoring, MS with selected ion monitoring (SIM), sometimes called mass fragmentography, and tandem MS (or MS-MS). The sensitivities that can be achieved by mass spectrometric methods are discussed in this volume. In this section, each method will be described briefly and illustrations presented which serve to highlight their use in the analytical studies of C_{19} steroids.

Honour (2003) has published an extremely useful review of many aspects of MS, including a survey of currently-available (2002) equipment (with commercial suppliers and costs), the various methods now in use (e.g. isotope-dilution-MS, tandem MS, etc.) and applications, in particular, to clinical chemistry, where through-puts of samples and rapidity of data handling are so important. Another extremely useful review is that by Griffiths et al. (2001) on electrospray and tandem MS in biochemistry.

6.7.2 *Derivatisation of C_{19} Steroids for Separation by Capillary GC*

It is probably true to say that most steroid researchers have used the *O*-methyl oximes (MO) for the derivatisation of oxo groups and the trimethylsilyl (TMS) ethers for hydroxyl groups. In the literature on the quantification of C_{19} steroids, it is still very common to find the combination of MO-TMS ethers for derivatisation (see, for example, Kwan et al., 1992; Table 6.3). The use of derivatives for androgens is twofold (i) to improve chromatographic characteristics by reducing adsorption, and thus loss or peak trailing, of steroids containing polar groups for example as a means of enhancing identification of structure than the underivatised steroids and (ii) the formation of enol-ethers, as in 4-en-3-oxo steroids, is prevented during the subsequent silylation procedure. For these reasons, it is usual to prepare the MOs of a mixture of steroids first and afterwards to prepare the TMS ethers. All steroidal oxo groups can be quantitatively converted to the MO derivatives, with the exception of the sterically hindered 11-oxo group as, for example, in 11-oxoandrosterone. Some hydroxyl groups are likewise difficult to convert to the TMS ether (for example, the 17α-hydroxyl in corticosteroids) and, to overcome the problem, it is usual to employ trimethylsilylimidazole as the silanising agent (see Gower, 1995 for further details).

Numerous other derivatives have been investigated, some being especially useful in that, in GC-MS, they yield a prominent $[M]^+$ or other characteristic ions of help in characterisation (Table 6.3). For example, considerably increased molecular weights are produced (483 in the case of the pentafluorobenzyl (PFB) oximes of T, with underivatised 17β-hydroxyl group). The preparation of the PFB oxime derivative from *O*-(pentafluorobenzyl) hydroxylamine is a simple reaction (Kwan et al., 1992). The value of this derivative for high-sensitivity analysis of molecules containing aldehyde and ketone functions was first recognised by Koshy et al. (1975) and by Nambara et al. (1975). The use of electron-capturing groups, including the pentafluorobenzyl moiety in negativeion chemical ionisation (NI CI)MS (Section 6.7.5.2) has been reported. De Boer et al. (1995) have published a detailed study of the MS characteristics of the PFB oxime of 19-nor T.

Table 6.3 Some characteristic ions useful in the quantification of C_{19} steroids and derivatives by GC-MS (SIM). Data compiled from Kwan et al., 1992 (from Gower, 1995, with permission of the publishers)

Steroid	Derivative	Ions useful for monitoring (*m/z*)
Testosterone	None	288(81)$[M]^+$; 246(50)
	3-oxo-17β-TMS ether	360(100)$[M]^+$; 270(62)$[M–90]^+$
	3-MO-17β-TMS ether	389(100)$[M]^+$; 358(20)$[M–31]^+$
	3-PFBO-17β-TMS ether	358(58)$[M–197]^+$; 268(55) $[M–197 – 90]^+$
Epitestosterone	None	288(90)$[M]^+$; 228(61)
	3-oxo-17α-TMS ether	360(100)$[M]^+$; 270(75)$[M–90]^+$
	3-MO-17α-TMS ether	389(100)$[M]^+$; 358(24)$[M–31]^+$
	3-PFBO-17α-TMS ether	356(26)$[M–197]^+$; 268(49) $[M–197 – 90]^+$
5α-DHT	None	290(92)$[M]^+$; 231(100)
	3-oxo-17β-TMS ether	347(80)$[M–15]^+$; 272(86)$[M–90]^+$
	3-MO-17β-TMS ether	391(84)$[M]^+$; 360(27)$[M–31]^+$
	3-PFBO-17β-TMS ether	360(10)$[M–197]^+$; 270(49) $[M–197–90]^+$
5β-DHT	3-MO-17β-TMS ether	360(100)$[M–31]^+$; 270(55) $[M–31–90]^+$
4-Androstenedione	None	286(100)$[M]^+$; 244(80)
	bisMO	344(100)$[M]^+$; 313(64)$[M–31]^+$
DHA	3β-TMS ether-17-MO	358(10)$[M–31]^+$; 268(30) $[M – 31 – 90]^+$
5β-Androstanedione	None	288(100)$[M]^+$; 244(38)
	bisMO	346(40)$[M]^+$; 315(100)$[M – 31]^+$
5α-Androstane-3α,17α-diol	bisTMS ether	436$[M]^+$; 346$[M – 90]^+$
5β-Androstane-3α,17α-diol	None	274(89)$[M – 15]^+$; 256(55)
	bisTMS ether	346(88)$[M – 90]^+$; 256(100) $[M – 90 – 90]^+$
5α-Androstane-3α,17β-diol	bisTMS ether	256(25)$[M – 90]^+$; 241(59) $[M – 90 – 90 – 15]^+$
5α-Androstane-3β, 17β-diol	bisTMS ether	346(41)$[M – 90]^+$; 241(36) $[M – 90 – 90 – 15]^+$
5-Androstene-3β,17α-diol	bisTMS ether	254(41)$[M – 90]^+$; 239(45) $[M – 90 – 15]^+$
5-Androstene-3β,17β-diol	bisTMS ether	344(27)$[M – 90]^+$; 254(26) $[M – 90 – 90]^+$
Androsterone	17-MO-3α-TMS ether	360(58)$[M – 31]^+$; 270(100) $[M – 31 – 90]^+$
Aetiocholanolone	17-MO-3α-TMS ether	360(44)$[M – 31]^+$; 270(100) $[M – 31 – 90]^+$
Epiaetiocholanolone	17-MO-3β-TMS ether	360(93)$[M – 31]^+$; 270(100) $[M – 31 – 90]^+$

For hydroxyl group derivatisation, an alternative to TMS ethers are the *tert*-butyldimethylsilyl (TBDMS) ethers. It is claimed that the latter provide 'cleaner' GC traces than TMS ethers, unless the silanising reagents for these are extensively purified before use. As noted below, the TBDMS ethers yield a pronounced $[M-57]^+$ as a result of facile loss of the *tert*-butyl group. Higashi and Shimada (2003) have reviewed the derivatisation methods available for neutral steroids to enhance their detection characteristics in liquid chromatography LC-MS (see Section 6.7.7). For the same technique, Mitamura and Shimada (2001) have summarised the derivatives available to the analysis of neurosteroids. In particular, the authors describe the derivatisation of pregnenolone-3-sulphate with 4-(*N*,*N*-dimethylaminosulphonyl)-7–2,1,3-benzoxadiazole. This gives good sensitivity during analysis by LC-ESI-MS Section 6.7.8.

Higashi and his group have described two derivatives that are effective in increasing the detectability in ESI-MS-MS. One of these (Higashi et al., 2005a), the permanently charged reagent 2-hydrazino-1-methyl pyridine (HMP), has been found useful in the LC-MS-MS analysis of androgens such as T and 5α-DHT in prostatic tissue (10 mg biopsy samples). These were initially extracted with methanol/water (3:7, v/v), then purified with an Oasis HLB cartridge and derivatised (HMP) before LC-MS-MS. The method was capable of measuring T and 5α-DHT with detection limits of 1 ng/g tissue from patients with BPH or prostatic cancer (see also Section 6.7.7).

The other derivative of particular use in NI-CI-MS for neurosteroid analysis (Higashi et al., 2005b) is 2-nitro-4-fluoromethylphenyl hydrazine (Higashi et al., 2005). The advantages of this derivative are described in detail in Section 6.7.5.2.

6.7.3 Use of Internal Standards in Quantification of C_{19} Steroids

In any quantitative method, especially one in which several purification steps are involved, each one resulting in analytical losses, internal standards are an absolute necessity. In order to quantify such losses, it is usual in many laboratories to add a suitable internal standard, preferably in the same biological form (e.g. glucuronide or sulphate) to the biological matrix prior to its extraction, whether by liquid or solid phase. Addition of high specific radioactivty steroids can be used as internal standard, although non-radioactive stable isotope-labelled compounds are preferable (see Section 6.7.4). If capillary GC is used for steroid separation before MS, then further standards must be added to allow for losses in the derivatisation process and the purification of derivatives, conveniently achieved, for example, using Lipidex chromatography (Shackleton and Honour, 1976). Following the recommendations of Shackleton and Whitney (1980), 5α-androstane-3α,17α-diol, a steroid of similar polarity to T and its metabolites, is added, together with stigmasterol for monitoring TMS ether formation and cholesteryl *n*-butanoate to monitor efficiency of sample injection to the GC columns. In our hands, overall recoveries averaged around 70–75% for the entire analytical procedure (liquid extraction, purification of Sephadex LH-20 columns, derivatisation, derivative purification and capillary GC).

In studies where GC with SIM was utilised to quantify androgens in human semen, we have found that 19-nor T (Kwan et al., 1992) satisfied the requirements for an

internal standard, namely: (a) it did not occur endogenously in this particular matrix, (b) its GC retention time (RT) was similar to, but not identical with, those of the analytes of interest (T and 5α-DHT in this application), and (c) whether derivatised or not, it showed a molecular ion which occurred either in appreciable abundance or as the base peak. It is obvious that an internal standard that is not resolved must have a different mass, whereas one which is resolved may have the same mass. For quantification by MS, calibration lines of the analytes of interest are required; this procedure has been described in detail earlier (Gower, 1995). The authentic steroid should be as pure as possible and should be included in increasing quantities over a limited range, together with a fixed quantity of the appropriate internal standard. However, the internal standard must be allowed to equilibrate with the biological matrix after addition. The time required for this is extremely difficult to determine but, commonly, 30–60 min at 37°C or overnight at 4°C are the conditions used. The accuracy of such assays is crucially dependent upon the purity of the analyte standards used. The purity of the internal standard is not so vital but may affect the precision of the assay.

6.7.4 Stable-isotope MS

During the past two or more decades, there has been an enormous expansion in the use of steroids labelled with stable isotopes such as ^{2}H, ^{13}C and ^{18}O. This expansion can be explained for least two reasons: (i) the necessity for an alternative to radioisotopic methods, especially in humans, when it may not only be unsafe but also unethical to administer ^{3}H- or ^{14}C-labelled steroids and (ii) the increasing availability of stable isotope-labelled steroids (whether commercially or synthesised by laboratory methods; e.g. Dehennin et al., 1980; Wudy, 1990). Early uses of stable-isotope MS have been reviewed by Gower (1995).

In biosynthetic studies, whether *in vivo* or *in vitro*, there is an obvious necessity to distinguish between endogenous steroids and those that are substrate-derived. Stable isotope-labelled steroids are ideal for this purpose because of the characteristic doubling of ions or mass shifts of, e.g. $[M + 2]^{+}$ produced in GC-MS analysis are consistent with the incorporation of one atom of ^{2}H into the metabolic product molecule. However, this is only true provided that the labels are in metabolically inert positions. Numerous studies have been published utilising ^{2}H-labelled steroid substrates, which have been synthesised 'in-house' largely by modifications of the methods devised by Dehennin et al. (1980) and Wudy (1990).

In our ongoing studies of equine steroid biochemistry (Houghton et al., 1990), [16,16-$^{2}H_{2}$]-labelled DHEA, T and 5α-androstane-3β,17β-diol have been synthesised by acid-catalysed isotope-exchange methods. The deuterated DHEA was perfused into a testicular artery of a pony stallion and shown to be metabolised into $^{2}H_{2}$-labelled T and 4-A, and their 19-hydroxy derivatives, as well as isomeric 5-androstenediols. Equine testicular minces *in vitro* were incubated with $^{2}H_{2}$-labelled and radio-labelled DHEA and 5-androstenediol. The metabolites (whose identity was confirmed by stable-isotope GC-MS) proved the inter-conversion of the two substrates and the

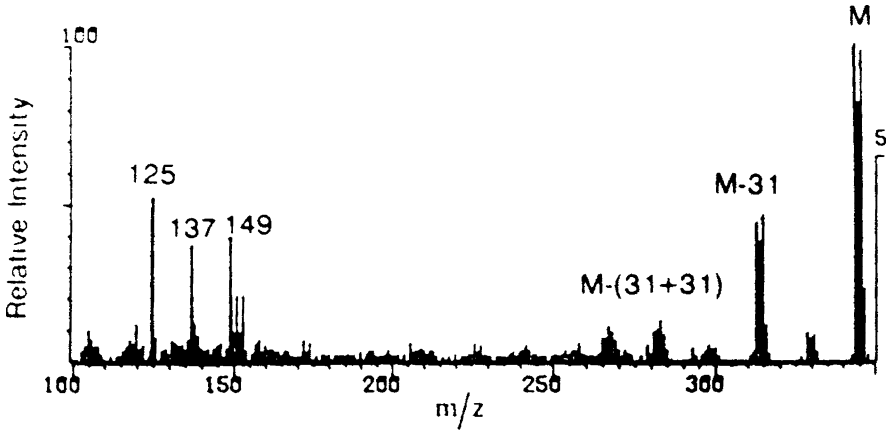

Fig. 6.8 The electron impact MS of the MO-TMS derivatives of 4-A and [16,16-$^2H_2$]4-A, isolated from perfused stallion testicular tissue (from Houghton et al., 1990, with permission of the publishers, Elsevier Science)

formation of T and 4-A. Figure 6.8 shows the characteristic doubling of ions in the spectrum of deuterated 4-A derived from [$^2H_2$]DHEA in the testicular perfusion study. It should be noted that deuterium-labelled internal standards are usually prepared by acid-catalysed deuterium exchange and thus care must be taken during the analytical process to avoid acid conditions which may lead to loss of deuterium.

6.7.5 Combined GC-MS-Selected Ion Monitoring

This technique has been used successfully for many years and is still being used extensively. It is necessary first to determine the way in which the steroid or steroid derivative (if subjected to GC first) fragments in the mass spectrometer. This information gives an overall view of the complete mass spectrum of the authentic compound or compound of biological origin (provided that sufficient is available). Out of this spectrum it is common practice to monitor for one prominent ion, but preferably for two or three appropriate ions of reasonable intensity. Numerous authors have published data on the principal mass spectral characteristic ions for C_{19} steroids and some of their derivatives; this applies particularly to the methyl oxime (MO)-trimethylsilyl (TMS) ethers. The ions that we have found useful in our own on-going studies of androgen analysis and biosynthesis are shown in Table 6.3 (Kwan et al., 1992). When both MO-TMS or PFB-oxime-TBDMS, for example, are present, as in the case of a C_{19} steroid with both oxo and hydroxyl groups derivatised, then $[M - 90 - 31]^+$ and $[M - 57 - 181]^+$, respectively, are commonly formed. The following examples, some of which include stable isotope-labelled internal standards, should serve to illustrate the principles outlined above, and the use of GC-MS-SIM in characterising the structures of several C_{19} steroids obtained from biological sources; many other examples are summarised in Table 6.4, including methodological details.

Table 6.4 Selected GC-MS methodologies, since 1992, for quantification of C_{19} steroids

Application	C_{19} steroids quantified	GC-MS method used; some extraction, purification and derivatisation methods	Additional methods used for comparison of results	References
Neurosteroidogenesis in isolated rat retinas	DHEA; DHEAS	SIM	None	Guarneri et al. (1994)
Plasma (normal children, various age groups)	T	Isotope dilution	IAs	Wudy et al. (1995)
7α-hydroxylation of DHA by *Fusarium moniliforme* microsomes *in vitro*	7α-hydroxy DHEA; 7β-hydroxy DHEA (minor product)	SIM	None	Cotillon et al. (1997)
Serum levels of 3β, 5-ene steroids of children (early neonatal period)	DHEA, DHEAS	SIM	None	Tagawa et al. (1997)
Urine steroid levels of pregnant women with extremely low serum estriol (ELSE); steroid sulphatase deficiency	DHEA, 16α-hydroxy-DHEA, 5-androstene-3β, 17β-diol, andro, aetio	SIM	None	Glass et al. (1998)
Urinary steroid levels in women with pre-menopausal breast disease (benign and malignant)	11-oxy- and 11-deoxy 17-oxosteroids	SIM	None	Lee et al. (1999)
Steroid profiling in amniotic fluid at mid-pregnancy	T, 4-A, 5α-DHT, DHA, androstanediol	Isotope dilution	None	Wudy et al. (1999)
T production rates in Cushing's syndrome; [$^2H_2$] T infused	T	Isotope-dilution	None	Vierhapper et al. (2000)
Blood, urinary T, steroid levels in the male blind mole rat	T, 4-A, 5α-DHT	SIM	RIA	Gottreich et al. (2000)
Solutions of authentic optimum procedure for steroids	T, T acetate	Membrane inlet (MI) MS	None	Lauritsen et al. (2000)
Serum steroid levels in patients with hyper- and hypothyroidism	DHEA, DHEAS	SIM (for DHA produced by DHEAS hydrolysis)	EIA (for DHA)	Tagawa et al. (2000)

Intracellular distribution of pregenelone metabolites during incubation of whole rat testes	T, DHEA, 5α-DHT, androstanediol, 5-androstenediol	SIM	None	Pertiwi et al. (2002)
Metabolism of [$^2H_3$]T in equine placental and allantoic tissues *in vitro*	19-hydroxy T	SIM (deuterium labelling)	None	Foster et al. (2002)
Urinary adrostanes in seven species of male great apes	T	Nanospray MS	RIA (for T) using 2 T antibodies	Hagey and Czekala (2003)
Serum steroids and immune system during and after normal pregnancy	Serum DHEA, DHEAS	ELISA (DHEA); GC-MS (DHEAS)	Cytokines (ELISA) measured after blood stimulated by phorbol derivatives	Tagawa et al. (2004)
Metabolic clearance and production rate in normal men; influence of ethnicity and age	Serum T	LC-MS-MS (with isotope dilution using d_3T)	RIA for d_3T plus d_0T	Wang et al. (2004a)
Newborn screening for CAH; second-tier steroid profiling	Blood-spot 4-A,17 -OH prog, cortisol	LC-MS-MS with deuterium-labelled-17 -OH prog as IS	None	Lacey et al. (2004)
Implications of 7-dehydrocholesterol-7-reductase deficiency (Smith-Lemli-Opitz syndrome (SLOS)) re neurosteroid production	Urinary DHAS, pregnenolone S and pregnanolones in post-pubertal females	GC-MS-SIM	None	Marcos et al. (2004)
Changes in rat brain and serum C_{19} steroids by immobilisation, stress and ethanol	T (rat brain and serum), 5α–A-3α,17β-diol (brain)	LC-MS; derivatisation: T, 2-hydrazino-1-Me-pyridine; 3α 17β-diol, p-nitrobenyl chloride. Derivatisation increases sensitivity 70 and 400 times, respectively	None	Higashi et al. (2006)

(continued)

Table 6.4 (continued)

Application	C_{19} steroids quantified	GC-MS method used; some extraction, purification and derivatisation methods	Additional methods used for comparison of results	References
Steroid measurement in newborns and children for diagnosis of CAH	17α-OH prog, T, 4-A	On-line extraction (cartridge); HPLC; MS-MS	IAs generally over-estimated, $r = 0.695$, $n = 97$. Recoveries (mean ± SD) were for 17α-OH prog. T and 4-A, 100(5)%, 102(2)%, 92(4)%. Lowest limit of detection 0.03–0.06 μg/L. Advantages: elimination of interferences; high throughput; short chromatography time	Rauh et al. (2006)
Measurement of low T values in sera from women and children	T	LC-MS-MS; serum (1 mL), deproteinised with acetonitrile containing $[^2H_3]$T. liquid–liquid extraction with CH_2Cl_2	LC-MS-MS (commercial kit); correlation 0.998 over concentration range 0.21–16.7 nM	Vicente et al. (2006)
Measurement of C_{19} steroids in human plasma	T	$[^2H_3]$ T (I.S) was added to plasma samples and SPE performed (C_{18} Sep Pak cartridge). Free steroids were eluted with ethyl acetate; sulphates were retained on cartridge, for further measurement. GC-MS SIM was performed after derivatisation (TBDMS ethers). The prominent ion monitored was at *m/z* 345 $[M-57]^+$ (see Table 6.3); IS gave *m/z* 349 for quantification	Recoveries for $T > 95\%$, lower limit of detection, 20 pg. Injection-column, or 0.69 mM/L, based on signal/noise ratio of 3:1	Masse and Wright (1996)

Analysis of human hair	T	Hair samples were washed with acetone, then ethanol, prior to being pulverised. The resulting powder was subjected to alkaline digestion, then extracted (liquid–liquid) after addition of $[^2H_3]$T (IS). Clean-up was by LH-20 column chromatography. After derivatisation (heptafluorobutyrates), GC-MS (SIM) was performed, monitoring at *m/z* 680 and 683 for T and IS, respectively	Quantities of T in male hair samples were higher ($p = 0.05$) than for female hair	Scherer et al. (1998)
Characterisation of CYP 3 4-A (6β-hydroxylase) by incubation of T with liver microsomes	T, 6β-OH-T	GC-MS (SIM) was used for separation and quantification (HP-SMS fused silica capillary column). RTs for T, methyl T and 6β–OH- T were 12.7, 12.8 and 13.4, respectively; ions used were *m/z* 288, 302 and 304, respectively		Testino et al. (1999)
Neurosteroids in rat brain (10 mg quantities)	DHEA (S); Preg (s)	After SPE extraction of brain tissues, free steroids and sulphates were separated. The latter were solvolysed. GC-MS SIM was performed after derivatisation (pentafluorobutyrates)	Values obtained were, for preg S and Preg, 8.26 and 4.17 ng/g, respectively and for DHEAS and DHEA, 2.47 and 0.45 ng/g, respectively	Liere et al. (2000)

(continued)

Table 6.4 (continued)

Application	C_{19} steroids quantified	GC-MS method used; some extraction, purification and derivatisation methods	Additional methods used for comparison of results	References
Neurosteroids in rat brains. Two groups of rats were used: I, normal controls, II acutely stressed previously. Grey matter and olfactory bulbs were excised and homogenised	Preg, DHEA	After addition of [$^2H_3$]T, homogenates were extracted with diethyl ether liquid–liquid). Initial purification was achieved by chromatography on micro columns of silsca gel. Oximes were prepared dimethyl isopropylsilyl imidazole. Quantification/characterisation were by GC-MS-MS		Shimada and Yago (2000)
Steroids in human testicular fluid	T, 5α-DHT, 5α-A-3α-17, β-diol, Oe-17β	Testicular fluid (20 μL portions) were extracted (diethyl ether). Purification was by HPLC (acetonitrite in H_2O, containing 1% formic acid, 70:30, v/v). Iso-cratic flow, with column effluents being monitored by MS-MS	Mean T concentrations by LC-MS-MS were 572 ± 102 ng/mL, similar to RIA results	Zhao et al. (2004)
Simultaneous estimation of steroids in rat brain and serum	T, DHT	LC-ESI-MS-MS	Extraction with MeOH–acetic acid; purification by SPE and derivatised with a permanently charged 2-hydrazino-1-methylpyridine. I. S. was 19,19–2H3-T. Assay CVs were <10%; recovery 98–103%	Higashi et al. (2008)

Estimation of androgen glucuronides and sulphates in human urine	T, epi T, A, epi A and DHEA sulphates-glucuronides	LC-ion trap MS-MS	Separation of glucuronides and sulphates with weak anion exchanger. SPE extraction	Strahm et al. (2008)
Monitoring urinary androgens in human hypogonadism	T, DHT in serum	UPLC-MS-MS	liquid–liquid extraction with MTBE. IS's were, respectively, 2H3-T and 2H3DHT for T and DHT. Derivatisation was achieved with POCA and final sample clean-up was performed with SPE. Validation over the ranges 0.2–40(T) and 0.01–2(DHT) ng/mL showed the method to be precise and accurate. Samples (300) could be analysed in 1 day	Licea-Perez et al. (2008)
Overexpression of refractory 5-steroid reductase, (5RD5AZ, type 3) in refractory prostate cancer (HPPC)	Conversion of T to DHT catalysed by 5-steroid reductase	LC-MS-MS	The findings suggest the activity of th novel type of SRD5A3 in HRDC cells. It indicates that this enzymatic activity could be a promising target for PC therapy	Uemura et al. (2008)

In some cases where methodological details are not given in the text (Sections 6.7.5–6.8), they appear in this table.
Andro, androsterone; aetio, aetiocholanolone; prog, progesterone; 17-OH-, 17-hydroxy-; IA, immunoassay, IS, internal standard; 5α–A-diol; 5α-androstanediol; MeOH, methanol; UPLC, ultraperformance liquid chromatography; MTBE, tertiarybutyl ether; POCA, 2,3-pyridinedicarboxylic anhydride.

6.7.5.1 Use of GC-MS-SIM

Introduction: Significance of Neuroactive Steroids and Their Quantification

It is now more than a quarter of a century since the significance of the neuroactive steroids (NASs) such as DHEA and pregnenolone and their sulphates began to be understood. Since then, a plethora of communications have been published that have thrown light on the biosynthesis, metabolism and the many important effects of NASs in the brain and peripheral nervous system (PNS). In Chapter 7 of this volume (Section 7.11.1.4), a detailed account of progesterone and its reduced metabolites and their psychopharmacological effects is given. In the present chapter, DHEA(S) are of particular relevance as they are C_{19} steroids.

The beneficial effects of, for example, DHEA are now thought to occur through neuronal excitability resulting in effects on the gamma-aminobutyric acid, Type A (GABA(A)) receptor, as well as on other neurotransmitters such as *N*-methyl diaspartate (NMDA) activities (e.g. Mo et al., 2004; Strous, 2005). Such effects can result in mood improvements, especially in middle-aged and elderly individuals (Strous, 2005). DHEA administration is also especially beneficial in schizophrenics, experiencing moderate to severe symptoms, or those maintained on an anti-psychiatric drug regime.

The beneficial effects of DHEA(S) have been proven (Silver et al., 2005) to be linked with cognitive functions in schizophrenic individuals. Significant improvements were shown in the executive function and memory, both for words and faces. Further interesting results (Doron et al., 2006) have shown that DHEA can act as a potential antidepressant in humans as well as in animal studies. In particular, DHEA administration can alleviate the distress induced during cocaine withdrawal and also reduce the incidence of relapses.

With this brief overview of the beneficial effects of the C_{19} NASs, DHEA and its sulphate, it is clear that accurate quantitative methods are of great importance. The biochemical importance of purification and HPLC quantification of NASs have been discussed in Section 6.6.2; the use of various types of mass spectrometry are discussed here and in later sections.

Specific ion monitoring (SIM) has been used in biochemical studies, not only to characterise metabolites formed after incubation of various precursors with tissue enzymes, but also in the quantification of such metabolites. For example, Kwan et al. (1997) characterised T and 5α-DHT formed during incubation of human testis preparations with pregnenolone. It was also possible to quantify these steroids by comparison of areas under the peaks obtained on GC. The metabolism of T by axillary coryneform bacterial species has also been studied using GC-SIM. In these studies, the PFB oxime-TBDMS ether derivatives were subjected to capillary GC-MS and the ions monitored were T, *m/z* 540 ($[M - 57]^+$); 5α(β)-DHT, 542 ($[M - 57]^+$); 4-A, 481 ($[M]^+$); and 5α(β)-androstanedione, 483 ($[M]^+$) (Mallet et al., 1991). Typical SIM traces for 4-A and 5α(β)-androstanedione showed good resolution of *syn*- and *anti*- isomers.

The use of GC-MS-SIM, with deuterium-labelled substrates, featured in the early investigations of Akwa et al. (1992) in this field of NAS biochemistry. Since then, Baulieu and Robel (1998) have published a review of this field of brain biochemistry. Rose et al. (1997) have also utilised GC-MS-SIM to identify and quantify 7-hydroxy derivatives as products of the incubation of DHEA and of pregnenolone. These are now considered to be important neurosteroids that influence, for example, cognition and behaviour. A review of the use of MS in quantifying neurosterols, such as cholesterol, and neurosteroids, such as DHEA and pregnenolone, has been published by Wang et al. (2007). Another review (Tsutsui et al., 2006) summarises the biosynthesis of neurosteroids and their sulphates in the brains of avian species, especifically the quail which, according to the authors is an excellent animal model. It appears that all the cytochrome P-450s necessary for cholesterol side-chain cleavage, 17α-hydroxylation, etc are present so that pregnenolone, DHEA, T and 5α-DHT can be produced. Furthermore, 7α-hydroxylase is present, so that the 7α-hydroxylase derivatives of DHEA and pregnenolone can be formed.

The cytochrome P-450, CYP 7b, involved in DHEA 7-hydroxylation is known to be strongly expressed in rat and mouse brain and is a cytochrome P-450 that is most similar to the steroidogenic P-450s found in other tissues. In the studies of Rose et al. (1997), mouse CYP 7b c-DNA was introduced into a vaccinia virus vector, and extracts of cells infected with this recombinant were incubated in the presence of NADPH with DHEA, pregnenolone or other C_{19} and C_{18} steroids. As far as C_{19} steroids were concerned, the expressed enzyme was most active with DHEA as substrate, activity with 5α-androstane-3β,17β-diol and T being less and negligible, respectively. As well as DHEA, pregnenolone served as a good substrate for the expressed enzyme, although it was less active against 25-hydroxycholesterol and 17β-oestradiol, and only minimally active against progesterone and corticosterone. After initial TLC, the DHEA metabolite showed identical retention time and mass fragments by GC-MS-SIM as 7α-hydroxy DHEA. Confirmation of the stereochemistry of the product was achieved (a) by TLC and (b) by incubating CYP 7b extracts with [7α-^{3}H] pregnenolone in the presence of NADPH, when large quantities of tritium were released into the medium. The authors concluded that CYP 7b (a 7α-hydroxylase) is important in cerebral tissues in the biosynthesis of the neurosteroids, 7α-hydroxy DHEA and 7α-hydroxypregnenolone (see also Section 6.3). Yau et al. (2006) have shown the importance of the latter steroid sulphate, but not pregnenolone itself, in improving spatial memory retention in cognitively impared old rats.

GC-MS with SIM has been used effectively for the quantification of the neurosteroids described above. For example, Liere et al. (2000) quantified several steroids from 10 mg quantities of rat brain (Table 6.4). Detection limits for pregnenolone, DHEA and their sulphate esters were 1 pg, and in a pilot study the authors have shown selectivity, extremely high sensitivity (femtomoles for quantification), linearity of calibration lines and good accuracy for both the free and sulphated NASs studied. Further work by the same group (Liere et al., 2004) has drawn attention to apparent discrepancies in analytical results for pregnenolone S in rodent brain tissue. If procedures for steroid sulphates (e.g. pregnenolone S and

DHEAS) included a hydrolysis (de-conjugation) step, the results indicated that substantial amounts of the conjugates were still present in the cerebral tissues. However, if steroid sulphates were analysed without prior hydrolysis, little or none was recorded. These results agree entirely with those obtained using an analytical protocol in which a cleavage/acylation derivatisation procedure was used after initial SPE of rodent brains. Liere et al. (2004) believe that the discrepancies between protocols with/without solvolysis could well be due to contamination with the lipoidal derivatives of pregnenolone and DHEA (L-Preg and L-DHEA). These are distinct from fatty acid esters but are more likely to be sulpho-lipids or steroid peroxides. Given the abundance in rodent brains of these novel lipoidal derivatives, it is clearly important to explore their function.

In many cases when SIM is used, studies of a particular precursor → product reaction can result in the characterisation of a specific enzyme. A case in point here, with regard to GC-MS-SIM (see Table 6.4) monitoring, is the characterisation of CYP 3A4 (6β-hydroxylase) metabolic activity in human and canine liver microsomes (Testino et al., 1999); the importance of hydroxylation of T has been referred to in Section 6.3. At the time of writing, it is clear that CYP 3A4 is being subjected to in-depth studies with respect to, for example, mechanisms of catalytic reaction, substrate binding (up to 3 mol of T being accepted) and the fact that the spin state changes from low to high as binding of T increases (Denisov et al., 2006, 2007). The heterotropic binding of xenobiotics (and also of T) has been explored by Lampe and Atkins (2006), using a time-resolves fluorescent methodology.

As noted above, CYP 3A4 catalyses the conversion oh T to the inactive 6β-hydroxy derivative. CYP 3A5 also has this capacity and its importance in the prevention of prostatic cancer has been studied by Moilanen et al. (2007). Since the prostate growth and development are T-dependent, the metabolising activity of the cyt P-450, CYP 3A5 is highly significant. This enzyme has been found both in luminal and basal epithelial cells of human prostate. Androgen response element was found in CYP 3A5 proximal promoter to which androgen receptor could bind. Androgen induction was abolished by the mutation of the response element. The authors suggest that CYP 3A5 is part of a regulatory feed-back loop that controls the exposure of prostate cells to T.

Song et al. (2003) have utilised GC-MS-SIM with deuterium-labelled internal standard to determine the hydroxylation of the 3-oxo-4-ene steroids, T, 4-A and progesterone, catalysed by recombinant human CYP1B1. In the preparation for hydroxylation incubations, human CYP1B1 and cytochrome P-450 reductase were co-expressed in *E. coli*. After reconstitution of the bacterial extracts, containing active CYP1B1 and cyt P-450 reductase, hydroxylation reactions for T, 4-A and progesterone were initiated by the addition of a NADPH-regenerating system. Termination of reactions and liquid–liquid extraction were performed with dichloromethane, and formation of TMS ethers of the reaction products was achieved with *N*,*O*-bis (TMS) trifluoroacetamide, containing 10% trimethylchorosilane. It was found that reaction conditions of 56°C for 10 min minimised the silylation of the 3-oxo groups of the steroid substrates; silylation occurred with increased time and temperature. Figure 6.9 clearly illustrates this point. Capillary GC-MS

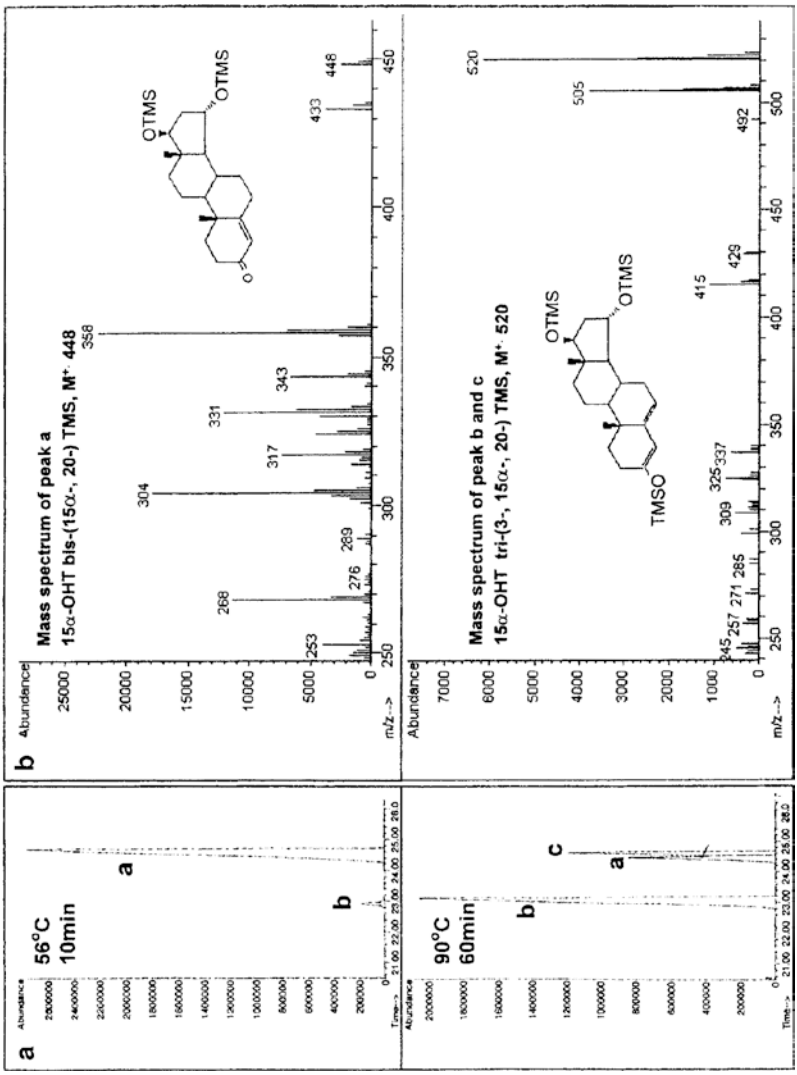

Fig. 6.9 Total ion chromatograms (TIC) and mass spectra of TMS derivatives of 15α-hydroxytestosterone (OHT). (**a**) TIC of TMS derivatives of 15α-OHT with different incubation conditions of 56°C for 10 min and 90°C for 60 min. Peak a is bis- (15α-, 20-) TMS derivative and peaks b and c are tri- (3-, 15α-, 20-) TMS derivatives. Peaks b and c are considered to be 3,5-dienol and 2,4-dienol isomers, respectively. (**b**) Mass spectra of bis- (peak a) and tris- (peaks b and c) TMS derivatives of 15α-OHT (from Song et al., 2003, with permission of authors and publishers, Elsevier Science)

Table 6.5 Retention times and characteristic mass fragments of TMS derivatives of 4-en-3-oxo C_{19} steroids and their hydroxylated metabolites

Compounds	Retention time (min)	Characteristic mass fragments (*m/z*)
Testosterone	20.43	129, 360, 73, 270, 345
2α-OHT	24.84	433, 73
2β-OHT	23.38	433, 73, 332
6β-OHT	21.85	73, 129, 392, 433, 448
7α-OHT	21.69	73, 358, 317, 433
15α-OHT	24.28	73, 217, 358, 191, 304
16α-OHT	25.98	73, 147, 191, 358, 448, 343
16β-OHT	24.28	73, 147, 191, 358, 448, 343
Progesterone	24.04	124, 272, 314, 229
d_9-progesterone	23.86	129, 279, 323, 233
6β-OHP	25.27	346, 387, 73, 402, 331
16α-OHP	27.19	73, 157, 312, 269, 297, 387, 402
21-OHP	29.48	299, 73, 271, 253, 387
Androstenedione	19.52	286, 124, 244
d_7-androstenedione	19.39	293, 129, 249
6β-OHA	20.63	359, 318, 73, 374
16α-OHA	24.07	303, 116, 73, 359

Underlined fragments were used for quantification.
OHA, hydroxyandrostenedione; OHP, hydroxyprogesterone; OHT, hydroxytestosterone (repoduced from Song et al., 2003, with permission of authors and publishers, Elsevier Science, B.V.)

was performed in the full-scan mode (*m/z* range 50–550) for the identification of hydroxylated steroids, with quantification of substrate steroids and products in the SIM mode. Table 6.5 summarises the GC retention times and mass fragments of T, 4-A and progesterone, and their hydroxy derivatives. Calibration lines for assays were linear over the range 0.125–5 µm except for 16α-hydroxy 4-A and 21-hydroxyprogesterone, which deviated from linearity at lower concentrations. As a routine assay, most compounds showed satisfactory within-day and between-day precision (CVs of <5% and <10%, respectively).

The analysis of human plasma for C_{19} steroids, in particular T, has always been of great importance in many clinical investigations, as emphasised earlier in this chapter Masse and Wright (1996). GC-MS with deuterium-labelled T as internal standard (isotope-dilution or ID-MS) has been shown to provide a rapid and convenient assay for plasma T. Masse and Wright (1996) have presented definitive methods for such assays and have discussed optimum extraction procedures, derivatisation, overall recovery precision, etc; the proposed method gave excellent accuracy and reproducibility (Table 6.4).

Another analytical paper utilising GC-MS-SIM is that by Choi et al. (2001), who quantified pregnenolone and T in clippings of finger and toe nails taken for the indication of androgenic status. The authors pointed out the merits of using nail clippings as a useful non-invasive technique, the ease with which they could be obtained and

the possible application of the technique in detecting steroid abuse in athletes. Nail clippings were washed initially three times with methanol to remove possible contamination from steroids in sweat and sebum. After NaOH digestion and liquid–liquid extraction with n-pentane, [$^2H_3$]T was added as internal standard. Derivatisation was achieved as mixed pentafluorodimethyl-trimethylsilyl ethers (flophenesyl-TMS ethers). This derivative has the advantage that it forms intense $[M]^+$ ions with minimal background noise, so that no further purification of the organic extract is required. In GC-MS-SIM, the ions selected for quantification of T, [$^2H_3$] T and pregnenolone were, respectively, *m/z* 584, 587 and 597, with GC retention times of 13.12, 13.08 and 14.91 min, respectively. The mean recoveries for T and pregnenolone were 89.8% and 86.7%, respectively, with limits of detection 0.1 pg/g and 0.2 pg/g, respectively (when 100 mg quantities of nail clippings were used for analysis). Good overall precision and accuracy, together with linearity of calibration lines, were obtained.

Closely related to the measurement of C_{19} steroids in human nails is the work of Wheeler et al. (1998) and of Scherer et al. (1998). The former group used scalp hair samples taken from 22 men (aged 21–65 years), 19 women (aged 17–55 years) and 6 children (aged 5–10 years). Hair samples were initially washed with water, dried and cut into short lengths. After treatment with diethyl ether for 15 sec., the samples were digested with hot 1 M NaOH solution. Following this procedure, liquid–liquid extraction was performed with diethyl ether. Extracts were dried down and reconstituted in BSA in phosphate buffered saline (pH 7.2). Purification was achieved with HPLC (Radial Medial Compression C_{18} cartridge), using acetonitrile-water (50:50, v/v) as eluting solvent. This system gave excellent separations of T, 4-A and 5α-DHT from each other and also from the internal standard 11β-hydroxy-4-A. The dried-down eluates, containing T, were reconstituted as above ready for RIA (method based on Wheeler and Luther (1983) and for GC-MS for validation, using [16,16,17]-$^2H_3$T as internal standard and TMS ethers for derivatives). Recoveries for the method (digestion, extraction and reconstitution) were 95.9 ± 2.9%, and for the entire protocol including HPLC, prior to RIA were 72.3 ± 2.7%. Hair T concentrations in men (12.9–77.7 pmol/g) were significantly ($p < 0.001$) higher than those in women (<0.9–10.8 pmol/g). There was no significant difference between T concentrations in women compared with children. Good correlations were recorded for results obtained with HPLC/GC-MS and HPLC/RIA at lower concentrations (e.g. 42.69 and 41.1 pmol/g, respectively, but not at higher values (e.g. 840.0 and 1324.8 pmol/g, respectively). In a shorter protocol, Wheeler (2006) omitted the HPLC step, the hair extracts being directly subjected to RIA. However, if a specific steroid is to be identified, then the HPLC step is required.

The protocols used by Scherer et al. (1998) for measuring hair samples are given in detail in Table 6.4.The authors recorded a significant difference ($p = 0.05$) for T concentration in male and female hair samples, values for men being 2.5–4.2 ng/g and for women 1.0–3.4 ng/g. Clearly, such methods and data are relevant to the doping scene when detection of anabolic steroid misuse is of extreme importance (see Chapter 9).

Choi and Chung (1999) measured, not only T in human hair but also eight steroids simultaneously, all related to androgen biosynthesis. The steroids studied were

Table 6.6 GC-SIM-MS data for the eight steroids quantified in human hair

Steroid	RRT[a]	Selected ion (m/z)[b]	Detection limit/ng g^{-1}	Calibration range/ng g^{-1}
4-A	0.39	430, 415, 358	0.1	0.5–50
Progesterone	0.59	458, 443, 386	0.5	0.5–50
Andro	0.71	586, 571, 329	0.02	0.5–50
Aetio	0.88	586, 571, 329	0.02	0.5–50
DHEA	0.69	584, 569, 327	0.2	1.0–20
5α-DHT	0.96	586, 571, 329	0.1	0.1–10
T	1.00	584, 569, 512	0.2	1.0–20
Pregnenolone	1.14	612, 597, 522	0.2	0.5–50

Steroids were derivatised as flophenesly-TMS ethers.
[a]Retention time relative to that of d_3-testosterone (13.03min).
[b]Quantitative ions are underlined.
Andro, androsterone; aetio, aetiocholanolone.
From Choi and Chung, 1999, with permission.

pregnenolone, progesterone, 5α-DHT, DHEA, T, 4-A, androsterone and aetiocholanolone. The methods used included washing the hair samples initially with organic solvents, alkaline digestion and liquid–liquid extraction of steroids. Derivatives used were the flophenesyl-TMS ethers for sensitive measurement with GC-MS-SIM. Table 6.6 summarises the GC retention times, ions utilised for quantification and detection limits for the eight steroids, and Table 6.7 shows the quantities found in male and female hair. Androsterone, aetiocholanolone, 4-A and progesterone were not detected. Choi et al. (2001) used similar techniques for GC-MS in order to measure T and pregnenolone in nails.

6.7.5.2 GC-MS-SIM (Chemical Ionisation-Negative Ion, CI-NI)

Several groups have utilised pentafluoro derivatives of C_{19} steroids and have found that, in the NI mode, chemical ionisation yielded highly sensitive responses. In their paper on an assay for plasma T, Legrand et al. (1995) have discussed the possible derivatisation procedures available. In their hands, the pentafluoropropionic acids (PFPA) gave optimum results for both T and [$^2H_3$]T (the internal standard), both yielding a single peak in the total ion chromatogram (TIC). The di-PFPA derivative yielded ions at *m/z* 560 and 563, respectively, for T and [$^2H_3$]T. The relative abundance of *m/z* 560 in the mass spectrum was about 34%, thus providing good sensitivity, and its high mass reducing any interference from co-extracted substances. The assay of Legrand et al. (1998) involved initial SPE on Extrulut columns, with T being extracted from the column by diethyl ether. After PFPA derivatisation, GC-MS could be performed without any further purification – a particularly useful feature of the assay. The overall extraction efficiency was ~95%, with linearity of calibration lines being achieved over the

Table 6.7 Steroids in human hair (data from Choi and Chung, 1999, with permission of authors and publishers, The Royal Society of Chemistry)

	Male hair/ng g^{-1}			Female hair/ng g^{-1}		
Steroid	Median	Range	Mean ± *s*	Median	Range	Mean ± *s*
DHEA	5.19	2.67–5.84	4.57 ± 0.79	2.18	1.02–3.54	2.25 ± 0.59
5α-DHT	0.50	0.38–0.83	0.57 ± 0.11	0.21	0.12–0.30	0.21 ± 0.04
T	2.51	2.03–2.54	2.36 ± 0.13	1.41	0.88–1.66	1.32 ± 0.19
Pregnenolone	1.82	0.83–3.75	2.13 ± 0.70	17.09	8.45–38.60	21.38 ± 7.32

range 1.7–71.5 mmol/L. Other requirements, normally expected for an acceptable assay, were found to be satisfactory, such as detection limit 3.5 fM injected, and signal/noise ratio 7.4. Comparison of results with those found using a commercial RIA kit for T (Immunotech International, Marseilles, France) showed good correlation, but GC-MS values were significantly lower ($p < 0.05$) than those obtained by RIA (see Section 6.10.10) for further comparisons of GC-MS and immunoassay results.

In 1997, Gower and co-workers also used CI-NI MS in the quantification of C_{19} steroid metabolites formed by incubating Coryneform bacteria spp., which had previously been isolated from the axillae of human male volunteers, and which had been proven as being involved in the formation of human under-arm odour (UAO) (Rennie et al., 1990). Bacterial isolates were incubated under appropriate conditions with (separately) androsterone sulphate (AS) and DHEAS as substrates. Steroid metabolites were obtained by liquid–liquid extraction and purified by HPLC. In pilot experiments, metabolites (derivatised as PFB oximes) were analysed by GC-MS monitoring in the EI mode but latterly, electron capture MS was employed for monitoring the ion $[M - HF]^-$ (i.e. $[M - 20]^-$) with essentially the same results but with greatly increased sensitivity. Table 6.8 summarises the *m/z* values monitored, with the corresponding GC retention times. In these studies, the *syn-* and *anti-* forms of the PFB oxime derivatives were well separated.

With the sensitivity of the method, it was possible to prove that both AS and DHEAS yielded T (5 and 60 pg, respectively) and 5α-DHT (a trace and 10 pg, respectively). In addition, both sulphates were converted into the free steroids, androsterone and DHEA, consistent with active bacterial sulphatase and other enzymes involved in the formation of T and 5α-DHT. In conjunction with these estimations, the two sulphates were shown to form various 16-androstene steroids, several of which are odorous and undoubtedly contribute to male underarm odour of bacterial origin.

Alomary et al. (2001) have reviewed the usefulness of EC-CI-MS in the quantification of neurosteroids and their sulphates. Higashi et al. (2005a, Section 6.7.2) have also noted that EC-MS is an extremely useful tool in measuring brain neurosteroid levels, when linked with LC (atmospheric pressure). The neurosteroids were

Table 6.8 Selected ion monitoring of the pentafluorobenzyl oximes (PFBO) of C_{19} ketonic steroids

Steroid	*m/z*	GC retention time, min (mean ± SD)
4-Androstenedione	656.21	15.83 ± 0.02(8); 16.27 ± 0.02(8)[a]
5α-DHT	579.30	13.48 ± 0.01(7); 13.80 ± 0.02(7)[a]
Androsterone	579.30	12.14 ± 0.03(8)
Testosterone	577.30	13.77 ± 0.01(6); 14.10 ± 0.02(6)[a]
DHEA	577.30	13.21 ± 0.01(10)
5α-Androst-2-en-17-one	447.22	8.47 ± 0.03(9)
5α-Androst-16-en-3-one	447.22	8.20 ± 0.03(10)
Androsta-4,16-dien-3-one	445.22	8.55 ± 0.03(10); 8.83 ± 0.04(10)[a]
5α-Androstan-3-one[b]	449.22	8.62 ± 0.02(10); 8.90 ± 0.02(10)[a]

The PFBO derivatives were analysed at $[M - 20]^-$ using negative ion chemical ionisation mass spectrometry (from Gower et al., 1997, with permission of the publishers, Pergamon).
[a] *syn*- and *anti*-forms.
[b] Internal standard.

derivatised with a highly electron affinitive reagent, 2-nitro-4-trifluoromethyl-phenyl hydrazine (NFP) to convert them to the corresponding hydrazones. A gain of 20-fold in sensitivity was thus achieved relative to measurements made for steroids by positive atmospheric pressure (CI-MS).

6.7.6 Tandem MS or MS-MS

This technique represents another of the advances made in the overall methodology of MS during past years. It has provided the analyst with an extremely powerful technique in that traces of a compound of interest in a complex mixture can be quantified. MS-MS has proved to be especially useful in the measurement of trace amounts of a drug or its metabolite(s) in a complex biological matrix (Houghton et al., 1991). The method is especially powerful as an analytical tool when linked with liquid and electrospray chromatography (see below). A common configuration is a triple-quadrupole instrument. Briefly, the principles of the method are that in the 'parent-ion mode', the analyte of interest is scanned over a required mass range (quadrupole Q1; Fig. 6.10) and the range of ions is subjected to collisionally activated dissociation in the CAD cell, Q2; a single ion can then be monitored by Q3. This technique is particularly useful since the output in the 'parent-ion mode' results in fragmentation of all the ions transmitted to the CAD cell, thus providing the common daughter ion that is transferred to quadrupole Q3. In this way, specific groups of drugs, or in the context of this chapter, of C_{19} steroids, which show a common fragment ion can be monitored. The alternative way of operating MS-MS is

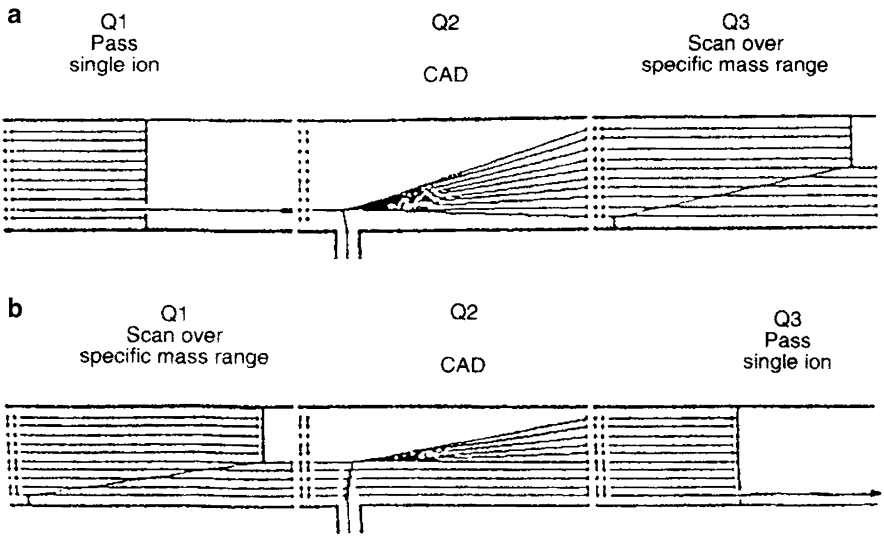

Fig. 6.10 Schematic diagram for production of (**a**) a daughter-ion and (**b**) a parent-ion spectrum using a triple-stage quadrupole MS; CAD, collisionally activated dissociation (from Houghton et al., 1991, with permission of the publisher The Royal Society of Chemistry, Cambridge, UK)

in the 'daughter-ion mode', which consists of the transmission of a single ion through the 'parent' quadrupole, Q1, and thence to the CAD cell, where fragmentation occurs. The fragment ions are then transferred to Q3 and scanned over a specific mass range to yield a 'daughter-ion' spectrum. The following examples and those in Table 6.4 illustrate the usefulness of MS-MS.

As noted above, neurosteroids are still of considerable interest to many groups of scientists, and an assay for pregnenolone and DHEA was published by Shimada and Yago (2000) using the grey matter and olfactory bulbs of rats (for details, see Table 6.4). Two groups of rats were used: Group I, normal and Group II, those that had been acutely stressed. It was found that pregnenolone in grey matter and olfactory bulbs increased three-fold after stress, whereas the DHEA content was not so clearly influenced.

6.7.7 Liquid Chromatography LC-MS-MS

Another exciting development that has been introduced into the MS field during the past 2 decades is that of combined HPLC and MS. Earlier references to the uses of this technique are given by Gower (1995) and more recent papers have been

reviewed by Shimada et al. (2001). In 2004, Zhao et al. used LC-MS-MS successfully in the quanification of T, 5α-DHT, 5α-androstane-3α,17β-diol and oestradiol -17β in human testicular fluid (see Table 6.4 for details). The mean intra-testicular T concentration for T, 572 ± 102 ng/mL, was similar to that obtained earlier by RIA (see Section 6.10.10). Raffaelli and Saba (2003) used one of the atmospheric pressure ionisation methods. By this means, LC-MS-MS could be applied to compounds that are not, or are only poorly amenable to thermospray. The uses of LC-ESI-MS-MS in quantification of intact steroid conjugates is discussed below.

LC-MS has also been utilised by Jellinck et al. (2006) for the identification of metabolites formed from DHEA in rodent cell lines. In the studies of the role of DHEA in neuroprotection, it was shown that 7α- and 7β-hydroxy DHEA were dominant metabolites of DHEA in all cell lines tested (see Section 6.6.2) with the notable exception of BV2 mouse microglia. These were unable to 7-hydroxylate DHEA but, instead, converted 4-androstenedione (4-A) to a major, previously unkown metabolite, that was characterised as the Δ^4 isomer of DHEA. In these studies, TLC and HPLC were utilised (Jellinck et al., 2005), followed by LC-MS, as well as with reverse isotope dilution analysis, involving co-crystallisation of the metabolic product with the authentic compound (Jellinck et al., 2006).

The interconversion of 7α-hydroxy and 7β-hydroxy DHEA catalysed by the human 11β-hydroxysteroid dehydrogenase has been studied by Muller et al. (2006). The interconversion takes place in liver, skin and brain, the enzyme involved being well-known for its catalytic activity in the interconversion of cortisone to cortisol (see Chapter 5). Further studies by Muller et al. (2006) showed that 7α- and 7β-hydroxy DHEA could each be converted into 7-oxo DHEA although with very different K_m values but at equivalent V_{max}. In contrast, the reduction of 7-oxo DHEA into a mixture of 7-hydroxy DHEA isomers catalysed by 11β-HSD1 exhibited the same K_m values although with significantly higher V_{max}. The authors suggest that in tissues where CYP7B1 is available to catalyse the formation of 7α-hydroxy DHEA, this may cause anti-glucocorticoid effects as it interferes with the 11β-HSD1-mediated cortisone reduction (see Chapter 5).

6.7.8 LC-Electrospray ionisation-MS-MS

In an extremely useful review, Griffiths et al. (2001) have highlighted the application of 'soft ionisation' methods (developed originally in the 1980s) to electrospray-MS-MS. The same group has (Liu et al., 2003a) reviewed this now well-established phenomenon of electrochemical reactions occurring within the capillary of the electrospray coupling. This group has proposed that on-column electrolysis of solvent can generate free-radicals that can subsequently initiate oxidation of unsaturated steroid sulphates, e.g. DHEAS, which possess a double bond at C-5. In these studies, HPLC capillary columns were tested for their efficiency in separating mixtures of the sulphates of pregenolone, 7-oxopregnenolone, DHEA and two isomeric pregnanolones. Figure 6.11a shows the procedure used for routine HPLC, where the mixture was injected into the pre-column

and, after HPLC, were automatically subjected to electrospray Ionisation (ESI) and then to MS-MS for identification. After detailed investigations, Liu et al. (2003a) showed that it was in the pre-column that free-radicals were generated electrochemically. Subsequently, these radicals caused oxidation of the unsaturated (not saturated) steroid sulphates so that $[M - H]^-$ ions expected from, e.g. DHEAS, in electrospray mass spectrometry were replaced by $[M - H]^-$ ions of the apparent oxidation products. Figure 6.11b depicts the set-up with the introduction of transfer tubing between unions 1 and 2 so that the formation of free radicals is prevented.

Griffiths et al. (2004) have studied the high-energy collision-induced dissociation (CID) of the Girard T (GT) and P (GP) hydrazone derivatives of neutral oxosteroids. Using the electrospray (ES) –MS-MS coupling mentioned above, it was found that the GT and GP hydrazones gave very intense M^+ ions, which fragmented within the ES interface and collision cell to yield characteristic fragment ions. High-energy CID spectra are obtained from the initial hydrazone derivatives that provide useful

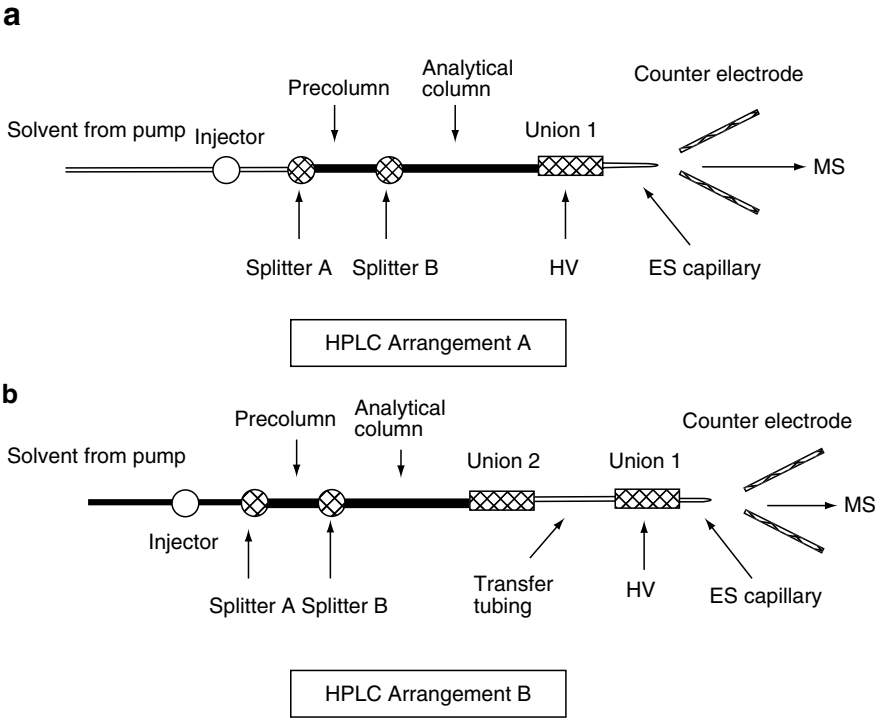

Fig. 6.11 Capillary column HPLC interfaced to ESMS. The HPLC pump, injector, splitters A and B, and unions 1 and 2 were made of stainless steel. The pre- and analytical columns consist of a fused-silica capillary packed with C18 particles. The ES capillary was made of a gold-coated, tapered, fused-silica capillary. High voltage was applied to union 1. In the absence of additional grounding, the pre- and analytical columns were at potentials above earth (from Liu et al., 2003a, with permission of authors and publisher, The American Chemical Society)

information concerning the structure of the originial oxosteroid. Clearly, the method could be of considerable use in biochemical applications. Recently, Josephs et al. (2004) have reported the creation and comparison of MS-MS spectral libraries using LC-MS-MS. Data-dependent scan-mode switching on both quadrupole ion trap and triple quadrupole mass spectrometers with ESI was utilised. The authors have systematically evaluated the effects on library search scores of changing the parameters for product CID on both instrumental set-ups. Using the information, a basis for determining a universal set of conditions can be built up for MS-MS libraries; 19 closely-related steroids were utilised in the study.

6.8 Quantification of Intact C_{19} Steroid Conjugates by MS Methods

6.8.1 Glucuronides

During the past few years, it has been possible to measure the concentration of intact C_{19} steroid glucuronides by, for example, GC-MS methods, several of which are summarised below or in Table 6.4. Such estimations of the glucuronides of 3α-androstanediol (3α-Adiol) and androsterone, as their serum concentrations, can now replace serum T measurements as markers for androgenic activity in women (Labrie et al., 2006). In these studies with 377 healthy post-menopausal women (aged 55–65 years) and 47 pre-menopausal women (aged 30–35 years), no correlation was found between serum T and the concentrations of 3α-Adiol G or of androsterone G, which account for the total pool of androgens. Further measurement of the pool based on measurements of the two C_{19} glucuronides cannot be replaced by the measurement of T or any other steroid, including DHEA(S). The authors suggest that measurement of the former glucuronides may result in identification of cases of true androgen deficiency. In these studies, serum T was quantified by GC-MS and the C_{19} glucuronides by LC-MS-MS.

The concentrations of a number of intact C_{19} steroid glucuronides in human urine have been measured by Choi et al. (2000) using a high temperature GC-MS with SIM. In all, nine glucuronides were quantified simultaneously, including androsterone, aetiocholanolone and their 11-oxo and 11β-hydroxy derivatives, 5α-DHT and T. Direct SPE of urine samples was achieved using Serolit PAD-1 resin, after which glucuronides were converted into the methyl ester-trimethylsilyl (TMS) ethers. GC was performed at temperature of 300–322°C (programmed) on a silico-steel-treated (cross-bonded dimethyl polysiloxane) stainless steel capillary column. Steroid glucuronides were monitored by GC-NI chemical ionisation (in scanning mode) at *m/z* 217, except for DHEA glucuronide (*m/z* 271). Figure 6.12 illustrates the excellent separation achieved over 15 min for all the nine glucuronides tested, with symmetrical peak shapes. Table 6.9 summarises the urinary concentrations of the glucuronides studied in five healthy male subjects. The same group (Choi et al., 2003)

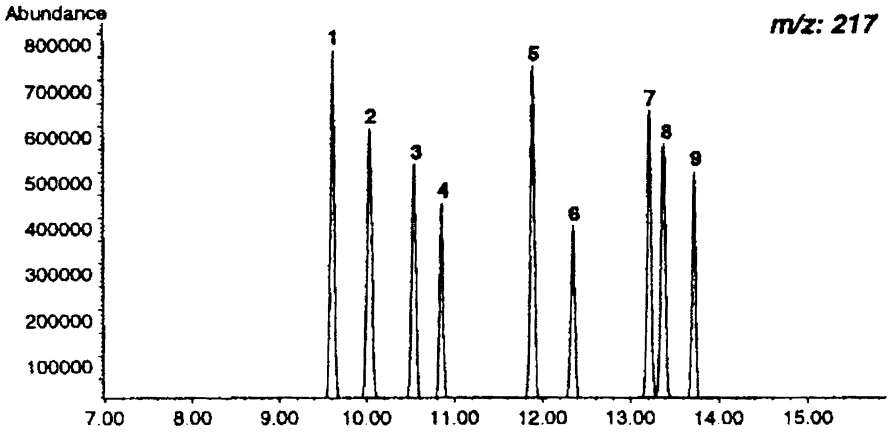

Fig. 6.12 Extracted ion chromatogram of a mixture of nine steroid glucuronides as their Me-TMS derivatives (ion at *m/z* 217) separated on MXT-1 (30 m × 0.28 mm ID and 0.25 μm film thickness) silico-steel-treated stainless-steel capillary column. Oven temperature was programmed from 300°C (4 min) to 322°C at 2°C/min. Peaks: 1 = androsterone-3-glucuronide; 2 = aetiocholanolone-3-glucuronide; 3 = 11-ketoandrosterone-3-glucuronide; 4 = 11-ketoaetiocholanolone-3-glucuronide; 5 = 11β-hydroxyandrosterone-3-glucuronide; 6 = 11β-hydroxy-aetiocholanolone-3-glucuronide; 7 = DHT-17-glucuronide; 8 = DHEA-3-glucuronide; 9 = testosterone-17-glucuronide (from Choi et al., 2000, with permission of the authors and publishers, Elsevier Science)

Table 6.9 Urinary concentrations of C_{19} steroid glucuronides of five normal male subjects

	Concentration (ng/mL)					
Glucuronide	A	B	C	D	E	Median
Andro	2,278.8	1,874.7	2,249.6	2,199.5	3,124.6	2,249.6
Aetio	1,854.3	1,569.2	2,301.4	1,892.8	2,855.2	1,892.8
11-Oxoandro	ND[a]	ND	ND	ND	ND	—
11-Oxoaetio	ND	ND	ND	ND	ND	—
11β-Hydroxyandro	823.7	943.6	1,208.4	1,064.1	987.1	987.1
11β-Hydroxyaetio	212.1	213.2	178.1	237.3	326.9	213.2
5α-DHT	27.1	24.4	15.1	21.3	31.7	24.4
DHEA	44.8	49.2	50.9	49.3	32.2	49.2
T	31.2	27.5	31.9	32.1	58.4	31.9

[a]ND, lower than limit of detection.
Andro, androsterone; aetio, aetiocholanolone. (from Choi et al., 2000, with permission of authors and the publishers.)

measured the T and 5α-DHT concentrations in urines from patients with BPH (see Table 6.4).

The use of serum glucuronides of androsterone and 5α-androstane-3α, 17β-diol as markers of androgen activity (instead of serum T) in women has been alluded to earlier (Section 6.3 and above). Quantification of the two glucuronides and serum T was achieved using LC-MS and GC-MS, respectively (Labrie et al., 2006).

The authors have emphasised the significance of their results in women with androgen deficiency in that rapid indication of the condition could help in designing the most appropriate therapy.

6.8.2 Sulphates

The extraction of C_{19} steroid conjugates has been discussed earlier (Section 6.4.3). Chattman et al. (1999) used the 'combinatorial approach' to extract both free steroid(s) and their conjugates using first, a non-polar solvent (for free steroids) followed by a polar solvent, e.g. chloroform-2-butanol (50:50, v/v) to extract conjugates. Both free steroid and sulphate extracts were subjected to preliminary ion scanning using a triple quadrupole mass spectrometer. Further, tandem MS afforded structural identification, and quantification of DHEA and DHEAS from urine and plasma was possible with a detection limit of 200 attoM/μL (approx. 80 fg/μL).

Zemaitis and Kroboth (1998) have published a simplified procedure for the measurement of serum DHEA and DHEAS simultaneously using GC-MS with selected ion monitoring (SRM-MS-MS or ion-trap MS). After addition of the internal standard, 3β-hydroxy-5-androsten-16-one methyl ester, serum samples were extracted by SPE using Oasis extraction tubes. Steroids were eluted with acetonitrile and, after evaporation, the sample was reconstituted in methanol, ready for analysis. Programmed capillary GC was performed to separate extracted steroids. The mass spectrometer used was a Finnigan ion-trap instrument, capable of performing selected reaction monitoring (SRM). As indicated above for GC-MS-MS (Section 6.7.6), it was possible to hold a parent ion of choice in the ion trap for further fragmentation via helium collision to yield a daughter spectrum, thus allowing high specificity for the chosen ion, even though present originally in a complex matrix.

The authors have emphasised several advantages of their assay, one being that it requires a single extraction and a single injection. The Oasis columns used in SPE were especially useful in that they offered the best balance between retention of the non-polar DHEA plus internal standard and the polar DHEAS. The second important advantage discussed was that it was unnecessary to derivatise DHEAS with perfluoroacylating reagents, a procedure which may result in sulphate displacement. Instead, DHEAS, when injected into the hot (270°C) injection port decomposed reproducibly with loss of the elements of sulphuric acid, yielding three isomers differing only in the location of the double bonds in rings A and B. The decomposition was shown to be reproducible within a given run and also between runs, providing that the injection port liner was changed between runs. The predominant peak (peak C in Fig. 6.13) and DHEA were eluted from the GC column before and after, respectively, the internal standard, so that the latter could be used in quantifying both steroids.

In the full-scan mode for DHEA, the parent ion was the base peak (*m/z* 288), while in the daughter spectrum, the base peak was at *m/z* 270, which was used for

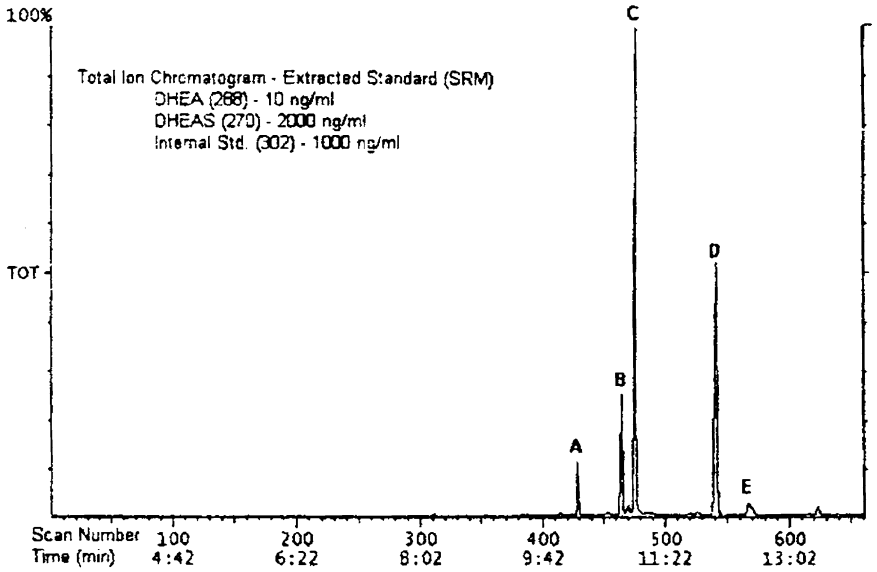

Fig. 6.13 Total ion chromatogram of an extracted serum standard containing dehydroepiandrosterone (DHEA), its sulphate (DHEAS) and internal standard, 3β-hydroxy-5-androsten-16-one. Peaks A, B, and C are derived from DHEAS, peak D is internal standard and E is DHEA (from Zemaitis and Kroboth, 1998, with permission of authors and the publisher, Elsevier Science)

quantification. For DHEAS, the predominant "desulphated" peak (isomer C) represents the result of loss of the sulphuric acid moiety at C-3 and double bond at C-5. In full-scan mode, all three isomers showed the same spectrum (as would be expected) with base peak at *m/z* 270; the daughter ion spectra were also similar, with base peak, *m/z* 199, subsequently used for quantification (Fig. 6.14). The daughter ion full-scan spectrum for the internal standard gave the base peak at *m/z* 270, which was used in calculating concentrations of DHEA and DHEAS from appropriate calibration curves.

Liu et al. (2003b) have quantified neurosteroids from rat brain using nano LC-ESI-MS-MS. These and conjugated steroids were obtained by solid-phase partition and cation- and anion-exchange chromatography. The intact sulphates of DHEA and of pregnenolone were not detected, 'contrary to expection' as the authors indicate in their paper. Earlier studies had shown the presence of these sulphates at concentrations above the limits of detection shown by Liu et al. (2003), i.e.0.3 ng/g wet brain. Cholesterol sulphate was detected in these studies at a level of 1.2 μg/g of wet brain. The neutral, unconjugated steroid fraction was derivatised with hydroxylamine hydrochloride to convert oxosteroids present into their oximes. Nano LC-ESI-MS-MS analysis showed the presence of pregnenolone, isomers of pregnanolone, progesterone, T and DHEA. Concentrations of these steroids were calculated by earlier addition in the analysis of [3,4] progesterone as internal standard and found to be in a range of 0.04–2 ng/g. The extreme importance of measuring

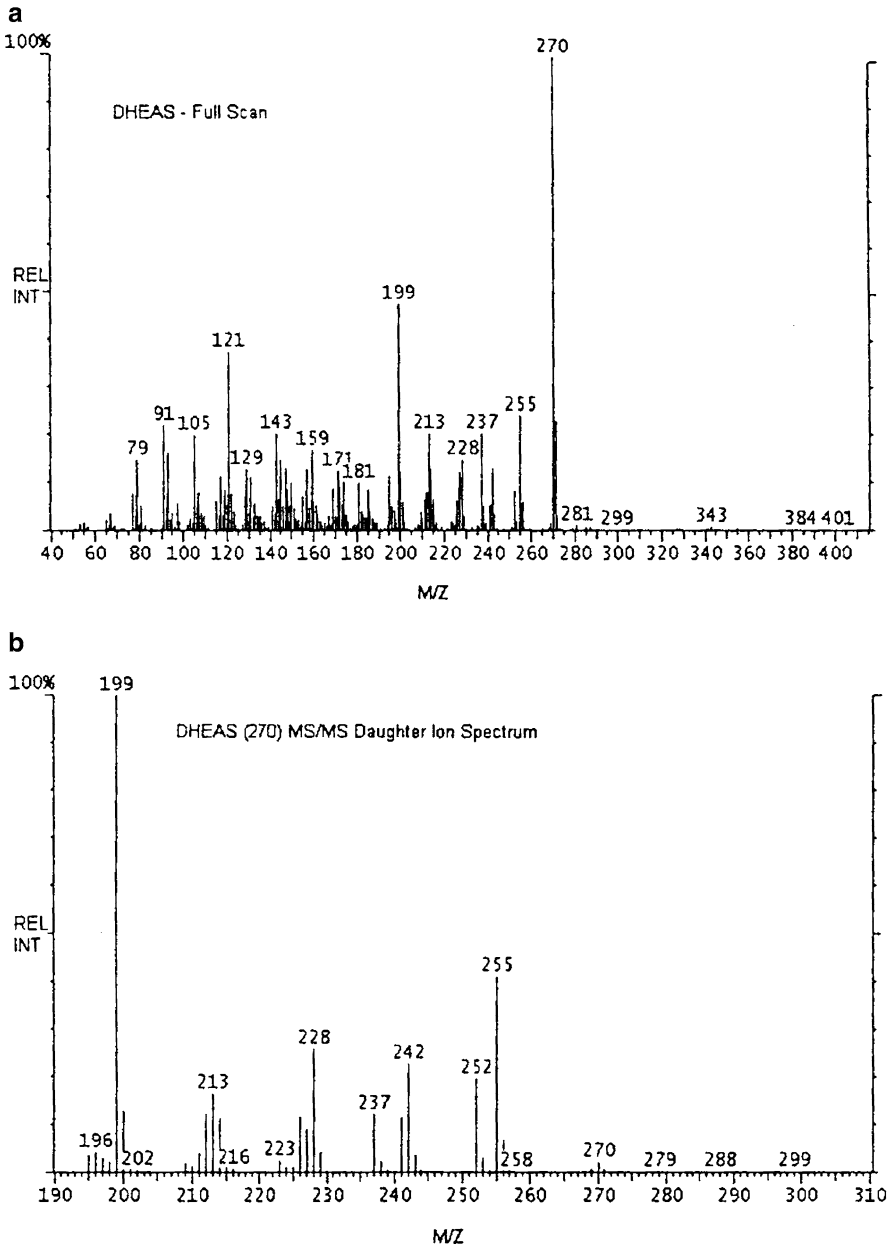

Fig. 6.14 Full-scan spectrum (**a**) and MS-MS daughter spectrum (**b**) of DHEAS (from Zemaitis and Kroboth, 1998, with permission of authors and the publisher, Elsevier Science)

accurately small quantities neurosteroids and their sulphates in the CNS has been highlighted by Gibbs et al. (2006).

A final example should serve to show the extreme usefulness of this technique. Mitamura et al. (2003) have measured 5-androstenediol-3-sulphate (5-AS) and DHEAS in human serum samples (100 μL). After the addition of deuterated 5-AS and DHEAS as internal standards, serum samples were de-proteinated (acetonitrite); the sulphates were obtained by SPE (Oasis HLB cartridge). After washing the conjugate fraction with hexane, LC-ESI-MS ion-trap in the negative-ion mode was carried out. The authors comment on the facts that no solvolysis was necessary nor any previous purification with HPLC. Concentrations of the sulphates in healthy men ($n = 14$) were 19.2–245.3 ng/mL for 5-AS and 0.175–5.16 ng/mL for DHEAS, respectively. For patients ($n = 19$) with prostate cancer, the corresponding values were 15.3–182.7 ng/mL (5-AS, not detectable in four samples) and 0.11–2.42 ng/mL, for DHEAS).

6.9 On-line Methodology for GC-MS Studies

During the past few years, the number of 'on-line' measurements of C_{19} steroids in analytical or biochemical applications has increased markedly. Choi et al. (2003) (Fig. 6.15) have highlighted the features of such methodologies, not least, the reduction in time-consuming pre-extraction/purification processes, such as the use of SPE, which can be performed automatically, the product then being valve-switched to chromatographic procedures and then to MS. One minute later, the valve was switched back to the loading position to equilibrate the SPE column for the next analysis. In their work, Choi et al. (2003) estimated the concentrations of T, 4-A and 5α-DHT in culture medium (human adrenocortical cancer cell line, H295) and also in serum. In brief, the sample solution was automatically loaded into the SPE column (a C4-alkyl diol silica-restricted-access) and then automatically valve-switched to the injection position so that reversed-phase (C_{18} column) gradient elution HPLC could begin. After 1 min, the valve was switched back to the loading position for re-equilibration of the SPE column prior to SPE of the next sample. Each eluted steroid was then transferred to an ESI interface, coupled to a triple-stage quadupole mass spectrometer (ESI-MS-MS). After optimisation tests, the $[M + H]^+$ ion was selected, and CAD gas flow and collision energy adjusted to give predominant product ion abundance. The usual parameters for validation of an assay, e.g. intra- and inter-day variations, were found to be satisfactory. The lowest points of calibration curves for standard solutions were 0.05 ng/mL for T and 4-A, and 1 ng/mL for 5α-DHT. A comparison of serum T levels was made with a RIA kit ('Coat-a-Count', for total T). It was found that, although the RIA method resulted in comparable lowest points on the calibration curves found for the ESI-MS-MS method, dynamic ranges were wider and the kit did not always distinguish between T and 5α-DHT (see Section 6.10.4 for further discussion of comparisons between IA and MS results). In the biosynthetic context, the importance of T hydroxylation derivatives has been emphasised several times in this chapter. Friedrich et al. (2003) have extensively reviewed the literature published on clinical, and other, aspects of these derivatives and methods available for assay. They have also described in detail the development and validation of a highly sensitive 'on-line' assay for 6β-hydroxy T (and other

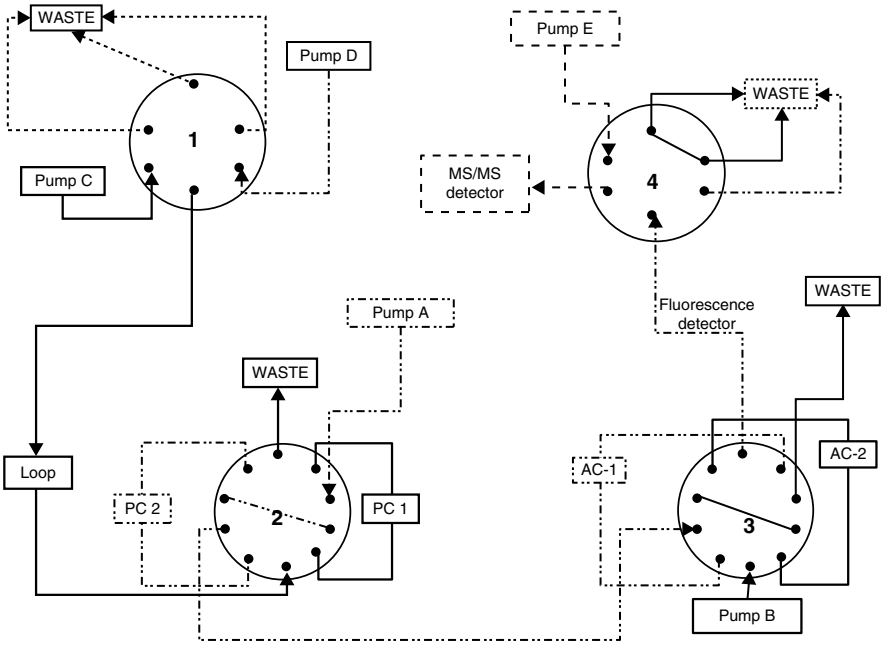

Fig. 6.15 Systematic diagrams for the two-valve column switching performed. The system consisted of the MF column (1), pre-column (2) and analytical column (3) for sample preparation. Shown are the flow paths during sample clean-up (**a**), concentration, (**b**), and separation and detection (**c**). Chromatographic conditions were as follows: solvent A, 10 mmol/L phosphate buffer (pH 5.4) in 100 mL/L acetonitrile; solvent B, 900 mL/L acetonitrile; flow rate, 500 µL/min; isocratic elution for 11 min, detection at 254 nm (**a** and **b**); flow rate, 100 µL/min; elution, linear gradient from 0% to 90% B over 11 min (**b**), UV, ultraviolet (from Choi et al., 2003, with permission of authors and publishers, Association of Clinical Chemistry, USA)

isomers) in human hepatocytes. Calibration curves were prepared, as well as 'simulated' standards with human hepatocytes in culture medium, containing T as substrate and clofibric acid as inducer of the required enzyme CYP3 4A. 'On-line' sample enrichment was achieved using a Bio Trap 500 MS™ extraction pre-column. Extraction was performed with triethyl ammonium formate buffer (pH 6.0) with back-flush of analyte (see Fig. 6.16) into the HPLC analytical C_{18} column for chromatographic separation using gradient elution with ternary mobile phase composed of methanol, tetrahydrofuran and water. Eluted steroids were initially monitored by their UV absorbance, but much greater sensitivity was achieved with MS-MS on an ion-trap mass spectrometer. This instrument was equipped with atmospheric pressure chemical ionisation source, operating in the SRM mode. The authors validated their method in the usual ways (inter- and intra-variation) and showed that the limit of quantitation was 20 ng/mL for an injection volume of 100 µL.

Highlighting the continued interest being shown in T hydroxylation as an example of extra-hepatic metabolism, Magnusson and Sandstrom (2004) have devised a highly sensitive method for separating and quantifying T metabolites in intestinal

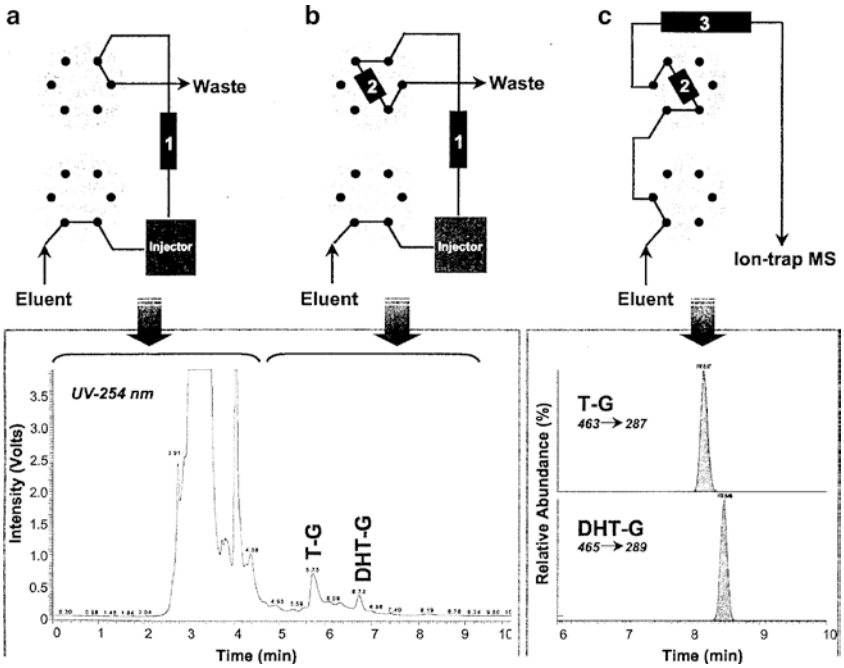

Fig. 6.16 Insert of column-switching scheme PC1/AC1-PC2/AC2 (from Friedrich et al., 2003, with permission original diagram published by Friedrich et al., 2002), with permission of authors and publisher, Elsevier Science)

wall preparations. LC/ESI/MS-MS was used, in conjunction with column switching, on-line, to clean-up and de-salt the samples. It was possible to separate and quantify 2α-, 2β-, 6α-, 6β-,7α-, 16α- and 16β-hydroxy derivatives of T and also of 4-A. The limits of quantification ranged from 0.3 to 3.33 nM for the various metabolites.

6.10 Immunoassays for C_{19} Steroids and Their Conjugates

6.10.1 Introduction

In the remaining sections of the present chapter, the various methods based on IAs will be described and discussed. Many of these, especially RIAs, are not new, having been used for several decades, and have been found particularly useful for routine and repetitive estimation of androgens in clinical laboratories. RIAs have also found use in many other applications, such as in analytical and biosynthetic studies *in vitro* or *in vivo*, when it is desired to estimate concentrations of various C_{19} steroids.

Numerous books and reviews have been published during the past decade (e.g. Price and Newman, 1997; Wild, 2005; Wheeler and Hutchinson, 2006), some covering fairly general aspects of IAs, and some more specific areas (mostly

reviews in this case), while others are distinctly biased towards practical aspects of the subject and, as such, constitute laboratory handbooks Gower (1995); Wheeler and Hutchinson (2006). Of particular interest are the discussions of trends towards the routine use of commercial kits, and in this context, the publication of The Immunoassay Kit Directory has been, and is still, invaluable to workers with IAs. In particular, the volume concerned with clinical chemistry (Wheeler, 1994) summarises the assay kits available for steroid hormones, the assay type (whether RIA, enzyme immunoassay (EIA), FIA (fluoroimmunoassay), etc.), with details of specificity, sensitivity, precision, cross-reaction, etc. Wheeler (2006) has also published a chapter concerned with estimation of T that provides detailed protocols to be followed in the laboratory context.

6.10.2 Synthesis of Potential Bridging Molecules

Marked developments have been made in the chemical syntheses of potential hapten conjugates for use in IAs for T and many other C_{19} steroids. The carboxymethyl oxime (CMO) of, for example, T, has been employed extensively, often linked with BSA (see also Section 6.8, where the use of the hemi-succinate (HS) derivative is also illustrated). Jana and Ali (1999a) have synthesised the geometrical isomers (3Z and 3E) of T-CMO and, after reverse phase HPLC, have explored their antibody binding characteristics. Using a standard EIA for T (see Section 6.10.7), the unfractionated mixture of oximes showed a cross-reactivity for T of 75%, whereas the purified 3Z- and 3E-isomers showed 124% and 26%, cross-reactivity, respectively. This was also increased for the histamine derivatives of the CMOs but the very marked difference for the isomers, noted above, was reduced. Suppression of the ionisation of the carboxyl group of the isomers by incubation of the antigen-antibody at pH 4.0 increased the cross-reactivity of the two oximes to 128% with respect to T. Clearly, the geometry and ionisation state of the CMO can exert a profound effect on their affinity for the antibody.

Fajkos et al. (1996) also experimented with the CMO derivative but used a five-step synthesis to yield (15E)-T-15- O-CMO and explored its properties as another potential conjugate for T in IAs. A novel conjugate for 7α-hydroxy DHEA in IAs was synthesised by Pouzar et al. (1998) in a complicated 11-step procedure. The compound produced was (19E)–3β, 7α-dihydroxy-17-oxo-androst-5-en-19-al-19-(O-CMO).

A more recent detailed study has been described by Basu et al. (2003) who used adipic acid dihydrazide as a linker between an amino group of the glycoenzyme horseradish peroxidase (HRP) and a carboxyl group of the steroid hemisuccinate (in this case, cortisol was used). The enzyme-conjugate so formed was successfully utilised in IAs. The authors emphasise the advantages of using hydrazide-containing reagents as they provide a built-in spacer that allows greater steric accessability. This point is borne out in practice by the greater sensitivity of the competitive enzyme-linked immunosorbent assay (ELISA)

described by Basu et al. (2003) and in chemiluminescent and fluorimetric IAs (Sections 6.10.8 and 6.10.9).

6.10.3 Sample Preparation (Extraction and Purification) in C_{19} Steroid IAs

6.10.3.1 Unconjugated Steroids

Prior to analysis of the required steroid (s) in a biological fluid, such as plasma, or serum or in tissue, it is often necessary to use liquid–liquid or solid-phase extraction (SPE) (see Section 6.4). This then provides a suitably 'cleaned-up' extract for IA analysis.

6.10.3.2 Conjugated Steroids

As Section 6.4.3 makes clear, the extraction and preparation of steroid conjugates for quantification requires a fairly complex and inevitably lengthy procedure involving hydrolysis, unless the intact conjugate can be measured. However, the use of affinity chromatography in the isolation from human sera of, for example, DHEA sulphate, a typical steroid conjugate, has been known for many years (Gower, 1995). The strategy for fractionating high-affinity antibodies by affinity chromatography has been reviewed by Giraudi and Baggiani (1996). This technique has been used effectively in IAs; for example, Luppa et al. (1996) have synthesised T-7α-(biotinyl-6-N-hexylamide) and have used this to obtain anti-T antibodies from a specific antiserum by affinity chromatography. The biotinylated T was attached to agarose-coupled streptavidin beads and a 20-fold increase in affinity of the purified antibody to the steroid, compared with the original antiserum. Several other biotinylated-T derivatives, linked via positions 3-, 7α-, 17α- and 19- have been synthesised by Hauptmann et al. (2000) and studied as potential immunochemical probes as ligands for biospecific interaction with monoclonal antibodies using, in these studies (Kaiser et al., 2000), microsensor devices, quartz crystal microbalance and surface plasmon resonance.

6.10.4 Direct (Non-extraction) Assays (Commercial Kits)

A very large number of kits now available for routine assessment of C_{19} steroids involve direct assays. The reasons for this are obvious when the often complex methodologies for extraction and purification of (especially) steroid conjugates are borne in mind. The time taken for such extraction inevitably reduces the through-put of samples for

analysis and eliminates the possibility of automated analysis. The volume edited by Wheeler (1994) summarises the variety of commercially available kits for quantification of numerous steroids, including the various C_{19} compounds with which the present chapter is concerned.

Many of the commercially available kits have been critically evaluated. It appears that one problem with potential effects on direct IAs is that of binding of steroid tracers by proteins in serum (Micallef et al., 1995). These authors found that ^{3}H-labelled T (as well as progesterone and oestradiol-17β) were all bound by proteins in every serum sample tested; ^{125}I-labelled analogues of these three steroids were affected likewise. Investigations showed that the addition of blocking agents caused substantially less binding of the ^{3}H-labelled steroids to serum proteins. The labelled analogues, however, were only slightly affected, and were still markedly bound. Hence, use of these in direct IAs of, for example, progesterone and oestradiol-17β produced anomalous results in some patient sera, compared with extraction RIAs. The blocking agents used in these studies were: for progesterone, danzanol; for oestradiol-17β, a mixture of danzanol and 5α-DHT in solution; and for T, a solution containing danzanol and oestradiol-17β.

A number of other evaluations have been published. In 1996, Wheeler et al. investigated the Ciba Corning ACS: 180 T assay. Comparisons of results were made with two other direct assay kits (DPC and Medgenix) as well as with an 'in-house' extraction assay and GC-MS targeted pools. The authors concluded that intra- and inter-assay imprecision for the various methods were <6% and <9%, respectively. Recoveries were quantitative but were reduced in the presence of increasing quantities of SHBG. No other interference was noted except that lipid caused an increase in results. With regard to other methods, the Ciba Corning ACS:180 was positively biased compared with the DPC and Medgenix assays and negatively biased compared with the 'in-house' extraction. RIA/GC-MS results showed that the ACS 180 method overestimated recovery by around 13%. However, this assay was fully automated and was especially useful in routine estimation of serum T as the first result was available in only 15 min.

Taieb et al. (2003) have also been concerned with investigating the performance of commercially available kits, in their ability to measure accurately T concentrations in human serum samples. This large survey involved testing 10 IAs and using 116 subjects (comprising men, women and children). Results obtained from all IAs (eight non-isotopic, two isotopic) were compared with values obtained by isotope dilution (ID- GC-MS; Section 6.7.4). Without going too deeply into the mass of results published, two important points emerge clearly: (1) in samples from women, seven out of ten IAs over-estimated T values by as much as 46%, compared with results using ID GC-MS, over the range 0.6–7.2 nmol/L; three IAs gave positive means of >2 nmol/L (range − 0.7–3.3 nmol/L); (2) in men, IA kits generally underestimated T values by some 12%, relative to the GC-MS method and, over the range 8.2–58.0 nmol/L, three of the assays gave mean differences of >4 nmol/L (range − 4.8–2.6 nmol/L). In this large multi-laboratory collaboration, the authors concluded

that none of the assay kits was sufficiently reliable for measuring low T values (0.17 nmol/L) in women or very low values (<0.17 nmol/L) in children.

Another extensive and in-depth survey has been carried out by Lamph et al. (2003). Eight commercial kits for T assay were evaluated; all were available and being used routinely in laboratories at that time. Evaluation protocols were as follows: method bias, imprecision, parallelism and recoveries. The products assessed were Abbot ARCHITECT™, Bayer ADVIA Centaur™, Beckman Coulter Access™, DPC Coat-A-Count™, DPC Immulite 2000™, Perkin Elmer Auto DELFIA™, Roche Elecsys™ and Tosoh AIA™. Some methods were calibrated against the accepted reference method ID-GC-MS (see Section 6.7.4) but gave very different results. All eight methods showed bias, either positive or negative. The total imprecision CVs were all found to be outside the manufacturers' claims. As the authors comment, this could be partly due to the fact that the kits were tested to the limit at low T levels, but could also reflect the overall poor performance. The problem found for low T levels is again consistent with the experiences of other laboratories. Commenting on the results obtained and the poor reliability of the commercial assay kits for T measurements in women, Herold and Fitzgerald (2003) question the use of assays that give results that do not agree well with corresponding concentrations determined by the ID GC-MS methodology.

In attempts to explain the discrepancies in T measurements with commercial kits (especially at low levels), Warner et al. (2006) have shown that addition of DHEAS interferes markedly with the Abbot Architect kit for serum T. Serum samples (81) that had been taken from women and assayed for DHEAS content, as well as T (both direct and extracted) were examined retrospectively. This analysis showed a relationship between interference and DHEAS ($r = 0.78$, $P < 0.0001$). Further studies to investigate possible cross-reactivity involved spiking with DHEAS, the free T diluent and serum obtained from one woman. Linear relationships were obtained between DHEAS added and the measured T values. Quantitatively, addition of every 1 µmol/L of DHEAS resulted in only 0.12 nmol/L of measured T.

Wang et al. (2004b) compared serum T values in 60 eugonadal and 62 hypogonadal men. Measurements were made in each case with four fully-automated commercially- available IA kits and two manual IA methods, one a commercial kit and the other, a research laboratory assay. All results were compared with those obtained using liquid chromatography-tandem mass spectrometry (LC-MS-MS). The authors concluded that, for samples containing T in the adult male range, 60% were within 20% of the LC-MS-MS values when measured by most automated and manual methods. There were, however, some biases found for different methods, some positively- and some negatively-biased. In contrast, for samples containing low (<3.47 nmol/L), over- and under-estimates were found with two methods providing results biased in both directions, compared with the LC-MS-MS values. The authors concluded that, given the imprecision, inaccuracy and biases, the IAs studied were incapable of providing sufficiently accurate results to distinguish between eugonadal and hypogonadal subjects. Further information about commercial kits is provided in Table 6.10.

Table 6.10 Selected references (since 1995) to immunoassays for C_{19} steroids and their conjugates

Application	C_{19} steroid(s) quantified	Type of assay	Separation/purification details	References
Less acidic forms of LH and steroid secretions in haemodialysis treatment	Serum T	FIA (and RIA)		Mitchell et al. (1994)
Adrenal C_{19} steroid levels in obese men	DHA, 4-A, T, 5-androstene-3β, 17β-diol	RIA	Extraction, chromatographic separation	Tchernof et al. (1995a)
Relationship of steroids to glucose tolerance and plasma insulin in men	DHA, 4-A, T, 5-androstene-3β, 17β-diol	RIA	Extraction, chromatographic separation	Tchernof et al. (1995b)
Behavioural effects of 3α, 17β-androstanediol administration in rats	Blood 3α,17β-androstanediol	RIA		Frye et al. (1996)
Relationship between blood/saliva hormones and body morphology	Serum and saliva T	RIA		Navarro et al. (1996)
Plasma and urinary steroids in an asynchronous fish	Plasma (male and female) and urine (males) T	RIA		Rocha and Reis-Henriques (1996)
Effects *in vitro* of gamma-hexachlorocyclohexane on steroid biosynthesis in goldfish	4-A, T, 11-oxo-T, A-trione	RIA	TLC separation after extraction	Kime and Singh (1996)
Relation between aggression in female barbary apes and faecal corticoids and 11-oxyaetio	Faecal 11-oxoaetio	EIA	HPLC used in separation of steroids in faecal extracts	Wallner et al. (1999)
Testicular mitosis, meiosis and apoptosis in mink	Serum T	EIA	SPE to obtain serum steroids	Blottner et al. (1999)
Seasonal and biological variations in blood steroids and hormones in healthy women	Free T, DHAS	EIA		Garde et al. (2000)

Seasonal variations in hypothalamus-pituitary-testicular axis in Japanese wood mouse	Plasma T	EIA	Kuwahara et al. (2000)
Thermacokinetic/dynamic modelling of T and LH supression by cetrorelix (LH-RH antagonist)	Plasma T	EIA	Pechstein et al. (2000)
Definition of serum steroid castrate levels	T	CIA (and RIA)	Oefelein et al. (2000)
Effects of quinal-phos in adult rats	Testicular morphology, T	CIA (and RIA)	Sarkar et al. (2000)
Pulmonary absorption of cetrorelix acetate in rats	Serum T	EIA	Lizio et al. (2001)
Effects of paint thinner exposure on hypothalamic catecholamines in male rats	Serum T	EIA	Yimaz et al. (2001)
Steroid hormones and lymphocytic corticoid receptors in Alzeimer's disease	Plasma DHAS	EIA	Armanini et al. (2003)
Seasonal timing of sperm production in deer	Serum T	EIA	Goeritz et al. (2003)
Faecal androgens in spotted hyenas	Faecal total androgen metabolites	EIA	Dioniak et al. (2004)
EEG spectra and steroid correlations in pre-pubertal children	Salivary T	CIA (and RIA)	Poblano et al. (2003)
Effects of photoperiod in rat testes	Serum T; testicular morphology	CIA	Kus et al. (2003)
Oral single-dose T triglyceride and T undecanoate in rabbits; pharmacokinetics	Serum T	FIA	Amory et al. (2003)

(continued)

Table 6.10 (continued)

Application	C_{19} steroid(s) quantified	Type of assay	Separation/purification details	References
Hormones in benign prostatic hyperplasia tissue	DHT, T	RIA	Chromatography on Celite mini-columns after tissue extraction	Sciarra et al. (1995)
Steroids in 17 specific regions of human female brain	T	RIA	Celite column chromatography after tissue extraction	Bixo et al. (1995)
Effects of castration on early development of male porcine accessary sex glands	Plasma T, DHAS	RIA		Raeside et al. (1997a)
Plasma steroid levels in female pacu	Plasma T	RIA		Gazola et al. (1996)
C19 steroids in newborn foals, post-natal decline in secretion	Serum DHA and 7-dehydro DHA	RIA (antibody for DHA cross-reacted >150% with 7-dehydro DHA)		Raeside et al. (1997b)
Develomental patterns of serum androstane-3α17, β-diol (3-G)	Serum androstanediol-3G	RIA (specific polyclonal antiserum)		Rittner et al. (1997)
Intra-tumour steroids in elderly breast cancer patients	T, DHT (plasma and tumour)	RIA		Secreto et al. (1996)
Sexual dimorphism in inhibins in boys and girls	Serum T	RIA		Bergada et al. (1999)
Seasonal variations and maturity changes/serum gonadal and thyroid hormones in the common Dentex	Serum T and 11-oxo-T	RIA		Pavlidis et al. (2000)
Secretion of inhibin B by pre-pubertal testicular cells in culture	T concentration in culture samples	RIA		Berensztein et al. (2000)

Effects of growth hormone in young men, women and in women taking oral contraceptives	Serum T	RIA	Eden Egstrom et al. (2000)
Influence of castration and oestrogen replacement in rams	Serum T	RIA	Pinckard et al. (2000)
Steroid hormones and natural sex changes in the Blackeye goby	Serum T, 11-oxo T	RIA	Kroon and Liley (2000)
Effects of androgens and ageing on vasoactive intestinal polypeptide in rat corpus cavernosum	Serum T (total and free); DHT	RIA	Shen et al. (2000)
Sex differences in sex hormone levels and glucose tolerance status in older men and women	Serum T (total); bio-available T by modified ammonium sulphate method	RIA	Goodman-Gruen and Barrett Connor (2000)
Effects of age on DHAS, IGF-1 and IL-6 levels in women	Serum DHAS	RIA	Haden et al. (2000)
Positive influence of 5α-androstane-3β,17β-diol on GABA(A) receptors in menopausal women	5α-Androstane-3β, 17β-diol; DHAS	RIA	Barbaccia et al. (2000)
Sulphated steroids in frog brain (telencephalon and hypothalamus)	DHAS	RIA	Mensah-Nyagan et al. (2000)
DHAS concentrations and cognitive performance in older men	Serum DHAS	RIA	Moffat et al. (2000)
Plasma and faecal steroids in domestic ganders – seasonal relationships	Plasma T; faecal T metabolites	RIA (T); EIA (T metabolites – antibodies against 17β-OH-C19 steroids)	Hirschenhauser et al. (2000)

(continued)

Table 6.10 (continued)

Application	C_{19} steroid(s) quantified	Type of assay	Separation/purification details	References
Seasonality in hairy-nosed wombats and faecal androgens	Faecal T, DHT and 5α-androstane-3β, 17β-diol	RIA		Hamilton et al. (2000)
Nickel-binding amino acids and inhibition of Ni2+ on T production in mice	T concentration in mouse primary Leydig cell cultures	RIA		Forgacs et al. (2001)
Association of selenoprotein P with T production in cultured Leydig cells	T in testicular cultures	RIA		Nishimua et al. (2001)
Neonatal hypothyroidism and Leydig cell number and steroids in plasma and interstitial fluid	T, DHT in rat plasma and testicular interstitial fluid	RIA		Maran et al. (2001)
Lactate and T production in rat Leydig cells	Culture medium T	RIA	Rat Leydig cells purified by Percoll density gradient centrifugation after enzymic separation of Leydig cells	Lin et al. (2001)
Regulation of T release in hyperprolactinaemia by testicular interstitial macrophages	T released by testicular interstitial or Leydig cells	RIA		Huang et al. (2003)
Production *in vivo* and *in vitro* of 15α-OH-T by the sea lamprey	15α-OH-T	RIA	HPLC and co-elution of testicular product with 15α-OH-T proved by TLC	Bryan et al. (2003)
Inhibition of 3β-OH steroid dehydrogenase in hormone-dependent cancer	Serum and tumour tissue 4-A, DHA	RIA	Reversed-phase HPLC	Geldof et al. (1995)

Reproducibility with time of urinary steroids in post-menopausal women	Urinary T, 5α-androstane-3β, 17β-diol	RIA	Enzyme hydrolysis, SPE, HPLC	Rinaldi et al. (2003)
Effects of C_{19} steroids levels on male patients with dermatophytosis	T, 4-A, DHAS	EIA	None	Hashemi et al. (2004)
Measurement of free T in women (normal and with androgen deficiency); comparison of methods	Serum T	Equilibrium dialysis; total T, SHBG	Direct RIA	Miller et al. (2004)
The androgen axis in recurrent prostate cancer	Tissue T, 5 –DHT, DHA, 4-A	RIA	Androgen receptor expression using monoclonal antibodies; PSA estimation	Mohler et al. (2004)

Literature searches for the year 2000 found a very large number of papers devoted to RIA of C_{19} steroids. Owing to the constraints on space, only a small proportion could be included in this table.

ELISA, enzyme-linked immunoabsorbent assay; EIA, enzyme immunoassay; RIA, radioimmunoassay; CIA, chemiluminescent immunoassay; FIA, (time-resolved) fluoroimmunoassay; OH, hydroxy-; LH, luteinising hormone; LH-RH, LH-releasing hormone.

6.10.5 Methods for Measurement of the Various Forms of Testosterone Occurring in Plasma

This important topic has been the subject of active discussion for many years (Gower, 1995; Wheeler, 1995). T occurs in plasma in several forms: free T (FT), albumin-bound (non-specific), SHBG-bound (specific) and total T (TT). Which of these should be measured in order to obtain as much useful clinical information as possible, especially in the context of an indicator of androgenic status, is clearly of considerable importance. Twenty years ago (Gower 1995), it was suggested that the albumin-bound fraction might be available to target tissues, as well as FT, since the steroid rapidly dissociates from the protein. The 'free' plus albumin-bound T is therefore called the non-SHBG-bound T. The latter fraction has also been suggested as being able to play a role in T action at the cellular level (Gower 1995), not least because SHBG receptors occur on the surface of plasma membranes of androgen receptor tissue cells. It has been suggested that there might be intracellular communication between T and its receptors (Gower, 1995).

The methods available for measurement of the various forms of T have been summarised by Gower (1995), Wheeler (1995), Vermeulen et al. (1999) and Wheeler (2006). These are equilibrium dialysis (EQD), ultra-centrifugation and steady-state gel filtration. The ammonium sulphate technique for precipitation of SHBG-bound T has also been used by a number of research groups (see below).

The accuracy of EQD has been questioned on the basis that quite serious errors can be caused by using (i) dilutions of plasma compared with the undiluted samples and (ii) impure radiolabelled tracer T in the dialysis procedures. For these reasons, a modified method was proposed (a) using a validated calculation to allow for dilution effects, (b) controlling pH (Gower, 1995; Obminski, 1998), (c) maintaining a constant F T concentration for dilutions, (d) measuring the water gain by the dialysis bag, and (e) rigorously purifying the [^{3}H]-T used as tracer. It was clear that pH affected the value of FT measured, the effect being greater in plasma samples having high SHBG-bound T than in those with low SHBG-bound T, and that the effect was even greater in a human serum albumin solution. Essentially, identical T values were obtained for diluted and undiluted plasma samples that had been charcoal-stripped, providing that the points (a)-(c) above were adhered to. The modified EQD method was found to be convenient with only 0.5 mL of plasma needed for analysis. Further, as the system reached equilibrium in 3 h, it was not necessary to time it precisely, so long as a 4-h period was allowed.

EQD has been used by Obminski (1998) in a detailed study of the effects of temperature and pH on the measurement of FT in human sera (see above). Undiluted serum was dialysed against buffer in micro-chambers so that the effects of temperature (36–39°C) and pH (6.9–7.4) could be monitored. It was found that a 3° rise in temperature made only a small (about 7%) increase in the FT content but that, at pH 6.9, the increase over the same temperature rise in FT was approximately 35%. In summary, Obminski (1998) found that, at 39°C and pH 6.9, the FT was 1.5-fold higher than that found at standard conditions of temperature and pH (37°C and 7.4),

implying that free T concentrations may increase in conditions of hyperthermia and acidosis, even though the total T concentrations may remain constant.

During the past decade, EQD has continued to be of use for measurement of FT in plasma. For example, Torma et al. (1995) evaluated a three-step method involving EQD to separate bound and unbound T in plasma samples from pre-menstrual women. After EQD, the resulting dialysate was extracted and separated from cross-reacting steroids by column chromatography. Finally, T was estimated by a sensitive RIA, the detection limit of which was 2.7 pmol/L. For the entire protocol, the detection limit was 6 pmol/L and the mean free T in 27 regularly menstruating women was 10.4 ± 4.9 pmol/L (range <6–19 pmol/L). Intra-assay CVs were 20.6, 16.8 and 9.5% for free T concentrations of 12.0, 21.4 and 31.4 pmole/L, respectively, while inter-assay CVs were 26.3 and 14.8% for free T concentrations of 10.4 and 21.3 pmol/L, respectively.

Sinha-Hikim et al. (1998) have used EQD in measurement of serum total and free T in healthy cycling women and in women with HIV. Using a larger volume of serum than usual, and making some modifications to the assay, the authors increased the sensitivity of the total T assay to 8 pmol/L and for the FT assay to 2 pmol/L. Studies of TT and FT in the sera of 34 healthy women in the follicular and luteal phases showed no significant differences but both demonstrated a modest pre-ovulatory increase 3 days prior to the LH peak. In HIV-infected women ($n = 37$), however, both serum FT and TT levels were significantly lower than in the normal subjects. Levels were also significantly lower in the HIV-infected women who were menstruating normally.

Another ‘older’ method is the precipitation of the SHBG-bound T with ammonium sulphate solution (Gower 1995). After centrifugation, the supernatant was extracted with ether. The dried-down residue, dissolved in isooctane, was subjected to chromatography on columns of Celite, and the eluted T fraction was measured by RIA. By comparing analytical and a computer-based predicted method for measurement of T, Pearce et al. (1989) concluded that the ammonium sulphate method was the only one that measured accurately non-SHBG-bound T (or bio-available T) in serum. Examples of the use of the method are given in Table 6.10.

Wheeler (2005) has noted that both EQD and the ultra-centrifugation methods significantly distort the equilibrium between bound and FT, due to gradual stripping of hormone from the binding proteins, an effect that increases with time. However, the steady-state equilibrium technique is free from this since free testosterone within the matrices of the gel forms an equilibrium with the free testosterone passing down the column, EQD and ultracentrifugation usually require diluted serum that itself may affect the equilibrium between bound and free hormone: SSGF uses neat serum. Nanjee and Wheeler (1985) have used the steady-state method (carried out on Sephadex mini-columns) routinely in their clinical laboratory and found it quite satisfactory for the measurement of FT in sera, not least because the analysis time is only about 1 h. The use of commercial kits, such as that provided by Diagnostic Products Corporation (DPC) for FT and other C_{19} steroids, will be discussed in detail in Section 6.10.4 (see also Table 6.11). There appears to be considerable doubt as to whether the kit actually measures serum FT (Vermeulen et al., 1999; Rosner, 2001; Wheeler, 2005).

Table 6.11 Selected references to indicate the success with which direct methods (kits) can measure concentrations of C_{19} steroids; corrrelations between kits and with other assay methods

Application	Steroid(s) assayed	Commercial kit used	Assay method for comparison	Extraction/purification methodology	Correlations between methods	References
Steroid concentrations in babies <6 months old	Plasma T		RIA	Extraction; column chromatography	Up to 3 months post-partum kit results were 3.8-fold greater than those of RIA; differences decreased with age	Fuqua et al. (1995)
Direct IA for T –comparison with GC-MS	Serum total T	Ciba Corning Diagnostic ACS–180 T kit	NI-CI GC-MS; (PFBO/ silyl ether derivatives)	liquid–liquid extraction	Adult male values, $r = 0.98$; adult females, $r = 0.31$	Fitzgerald and Herold (1996)
Comparison of direct methods	Plasma T	$A_x$sym	Classical ACS-180; Electsys (both for T)	None	Adult males: $A_x$sym vs. ACS-180, $r = 0.96$; $A_x$sym vs. Electsys, $r = 0.935$. Adult females: only slightly worse correlations (reference ranges for plasma T: males, 1.0–30.2; females, 0.9–3.1 nmol/L)	Gonzalez-Sagrado et al. (2000)
Evaluation of CIA analyser	Plasma T	CIA (Abbott Architect 2000)	DPC Immulite; Chiron Diagnostics ACS-180; EQD and IDGC-MS	None	For plasma T (nmol/L) Abbott Architect, 0.38; DPC Immulite, 0.37; Chiron ACS-180, 2.0 EQD and IDGC-MS, both showed CV < 6% vs. Abbott Architect	Ognibene et al. (2000)
Limitations of kits for IA of T	Serum T	9 kits used	EIA, CIA, RIA	Extraction needed for good results		Stanczyk et al. (2003)
T values in early infancy	Serum T	Direct CIA (Bayer ADVIA Centaur)	RIA	Solvent extraction for RIA	Bayer ADVIA reliable for neonate T. Ranges: 10 days old: 2.5–11.1 (m) and 1.7–5.6 (f), nmol/L; 10–50 days old: 0.2–17.2 (m) and 0.1–1.5 (f), nmol/L	Tomlinson et al. (2004)

EIA, enzyme immunoassay; CIA, chemiluminescent immunoassay; NI-CI, negative-ion chemical ionisation; PFBO, penta-fluorobenzyl oxime; *r*, correlation coefficient; CV, coefficient of variance; EQD, equilibrium dialysis; IDGC-MS, isotope dilution gas chromatography-mass spectrometry.

In addition to these, several mathematical methods have been proposed (Wheeler, 1995). For example, the 'Androgen Index' is a ratio of the plasma T and SHBG concentrations which, when multiplied by 100, yields values similar to the free T concentrations. The clinical usefulness of the Index and that it is a good indicator of the biologically active T are issues that have been discussed by Wheeler (1995; see also references therein). Other more complicated mathematical formulae have been suggested including that of Wheeler (1995). Generally, TT is measured together with SHBG (which is known to be highly correlated with the percentage FT). As for throughput of samples in clinical laboratories, Wheeler (1995) points out that some of the mathematically-derived methods are more likely to be used than the indirect ones, e.g. EQD, because of the time required for estimation.

In a critical evaluation of the various methods available for measurement of FT in serum, Vermeulen et al. (1999) have drawn conclusions confirming those of Wheeler (1995) and others. For example, EQD and the ammonium sulphate precipitation method appear to represent (among other techniques), reliable indices of FT. The problem is that they are time-consuming (as above). Further, the FT value obtained by calculation from TT and SHBG (obtained by RIA) was shown by these workers to be a simple, rapid and reliable index of FT, except in pregnancy when the oestradiol-17β present in sera, occupies a substantial proportion of the SHBG-binding sites and results in values which are lower than the apparent FT obtained by EQD. However, the values of non-specifically bound T (calculated from the FT concentration) correlated highly significantly with (r = 0.997), and were almost identical to, the values of non-SHBG-T, obtained by ammonium sulphate precipitation. These data are entirely consistent with the clinical value of FT calculated from SHBG.

Miller and co-workers (2004) point out the importance of recognising androgen deficiency in women. Hence, there is a need for accurate and well-validated measurements for serum FT levels; 147 women were selected for the studies. Serum FT values, determined by a direct RIA, were compared with those obtained by calculation from the law of mass action (requiring values for total T and SHBG) and also with values obtained by EQD. Finally, the androgen index (100 × T/SHBG) was calculated as this is known to correlate with FT concentrations (as described above). Some of the results are as follows: first, FT (calculated from the law of mass action) correlated extremely well with the EQD results (r = 0.99) although, as might be anticipated, the agreement depended strongly on values obtained for total T and SHBG, required for this calculation. Secondly, in contrast the direct RIA for FT showed high systematic bias and random variability, also with less sensitivity than the EQD results; correlation between the latter and the RIA results was poor (r = 081). These results, among others, indicate that the preferred methods for measurement of FT in women with androgen deficiency are the use of mass action equations and EQD.

Ly and Handelsman (2005) have discussed the above methods in the context of requirements necessary to provide suitable methods for large-scale studies (4,000 consecutive blood samples). The concentrations of FT, TT and SHBG were measured in each blood sample; the authors then describe their own assumption-free empirical equations for FT measurement. After comparison of results, it was concluded that the

empirical equations were routinely more accurate than laboratory measurements and the earlier empirical equations (see above) for FT measurement.

In attempts to assess hyperandrogenasmia in women, Mueller et al. (2006) have explored the role of calculated FT and bio-available T (BT). In this study, endocrinological parameters such as total T (TT) and FT were measured by analogue ligand IA methods, as well as 5α-DHT, DHEAS and SHBG. Calculated values of FT, bio-available (BT) and the free androgen index (FAI; see above) in hirsute women and those with polycystic ovarian syndrome (PCOS) were compared with those in control individuals. No differences were observed when the measured parameters were compared, but the calculated values were significantly increased in the hirsutism and PCOS groups compared with the same parameters in the control subject group. Mueller et al. (2006) suggested that their 'calculated parameter' method may be useful in replacing time-consuming and complicated methods for T measurement.

6.10.6 Enzyme-Linked Immunosorbent Assays

During the past decade, numerous developments and refinements to the assay have been published, resulting in far greater sensitivity and improved validation, as well as reduction in endogenous interference and in non-specific binding. In the case of ELISAs for T, a major recurring problem has been that of cross-reaction with 5α-DHT in particular and, to a lesser extent, with 5β-DHT. Attempts to improve the specifity of ELISAs for T estimation have been published by Jana and Ali (1999b). In their work, polyclonal antibodies raised against T-3-(*O*-carboxymethyl) (CMO) oxime were resolved on a homologous T-Sepharose matrix, using a gradient system cosisting of propionate slution and 1 M and 2 M propionic acid. More recently, Shrivastav et al. (2003) have published details of a competitive ELISA for direct estimation of T in serum. In brief, T-3-(*O*- carboxymethyl) oxime-BSA (T-3-O-CMO-BSA) was the immunogen and T-3-(O-CMO)-adipic acid dihydrazide-horseradish peroxidase (T-O-CMO-ADH-HRP) was used as tracer. The wells of a standard microtiter plate were coated with the T-antibody and standard T solutions or serum samples were incubated, together with the enzyme-conjugate for 60 min at 37°C. Bound enzyme activity was estimated by adding tetramethyl benzidine/H_2O_2 as substrate, and colour production obtained as normal. The authors pointed out the usefulness of their assay because charcoal-stripped serum was spiked with non-cross reacting C_{18}, C_{19}, C_{21} or C_{27} steroids and used for preparing standards. This procedure resulted in SHBG and other steroid-binding globulins being blocked. The sensitivity of the assay was 0.015 ng/mL, coefficients of variation for intra- and inter-assay were 7.8–11.8% and 4.8–10.4%, respectively and correlation of T concentrations in 100 individuals, compared with those obtained by RIA, were excellent ($r = 0.98$).

In such ELISAs, colorimetry is normally performed using commercial spectrometers but it is worth noting that Shishkin et al. (2000) have designed a portable photocolorimeter that operates in both the reflection and transmission modes for two systems of T immunoenzyme assays: microplate ELISA and membrane dot-ELISA

(detection thresholds 0.1 and 0.6 ng/mL, respectively). Readings obtained using this portable photoclorimeter correlated extremely well ($r = 0.999$) with those obtained with a conventional instrument. Further, the relative coefficients of variation over the extinction range 0.03–1.00 were 3.4% to 0.7%. The authors claim that the simplicity and versatility of their instrument makes it appropriate for use in both the laboratory and in the field.

Lewis et al. (1996) have devised ELISAs for intact steroid conjugates, notably, the sulphates of DHEA, androsterone and epi-androsterone. In these studies, mice were immunised by injection of DHEA-7-*O*-carboxymethyl oxime (CMO)–BSA or DHEA-7-hemisuccinate (HS)–BSA conjugates. The monoclonal antibodies produced were characterised and selected for maximum DHEAS binding. The authors found that, of these hybridomas, four clones from DHEA-3HS-BSA-immunised mice had acceptable criteria for development of an ELISA for plasma DHEAS. However, one hybridoma supernatant from mice immunised against DHEA-7-O-CMO-BSA showed 36% cross-reactivity to the sulphates of androsterone and epi-androsterone, suggesting the feasibility of devising assays for these conjugates in plasma, providing allowance had been made for the DHEAS contribution.

For the assays themselves, standard 96-well microtiter plates were used, to the wells of which was adsorbed DHEA-3-HS-thyroglobulin conjugate. DHEAS in solution or diluted plasma samples compete with this immobilised conjugate for antibody binding sites. Antibody was determined with a mouse Ig-peroxidase with further washing, and colour production was quantified by measurement of absorbance at 492 nm wavelength, following the addition of *ortho*-phenylenediamine. Lewis and Elder (2000) have abbreviated this ELISA to a one-step method, compared to the earlier two-step protocol. The monoclonal antibody supernatant at a Dilution similar to that used earlier was combined with the anti-mouse Ig-peroxidase at a 1:500 dilution, 50 μL of which was added to each well of the microtiter plate. After a single incubation of 1 h at room temperature, followed by washing, substrate was added with subsequent measurement of colour production. The authors have demonstrated excellent correlations for calibration curves obtained for both the one- and two-step formats.

A new immunofiltration assay for serum T, similar in some respects to an ELISA, has been described by Zherdev et al. (2003). First, T molecules, in solution, compete with the T-peroxidase conjugate for interaction with T-antibodies that had been bound previously to the staphylococcal protein A-polymethacrylate polyanion conjugate. The second step involves filtration of the reaction mixture through a membrane charged with immobilised poly (*N*-ethyl-4-vinylpyridium) polycation. During the filtration, a rapid separation of the polyanion-containing complexes occurs as a result of high-affinity electrostatic interactions. After removal of unbound compounds, the immobilised peroxidase is detected using a suitable substrate that forms an insoluble coloured product. The authors have listed several important characteristics of their assay: (1) speed (20 min), the assay being four times faster to perform than the ELISA microplate enzyme protocol, using the same immuno-reagents; (2) sensitivity (0.1 ng/mL) and reproducibility; (3) exclusion of the influence of immunoglobulins on the assay results by previously incubating the

antibody and the polyanion-protein A conjugate; (4) use of the latter conjugate as a universal reagent so that the necessity to modify specific antibodies for each IA can be eliminated; and (5) versatility of the assay, as it can be used both in the laboratory situation and in the field. See Table 6.11 for brief details of IAs.

6.10.7 Enzyme Immunoassays

The continuing problem, alluded to earlier, of cross-reaction between T and 5α-DHT in IAs has been addressed by Srivastava and Grover (1997) using a typical T-coated tube enzyme immunoassay (EIA) format, a structurally modified enzyme label (6-dehydro-DHT-17-hemisuccinate (6-DT-HS)–penicillinase) was synthesised and shown to have much lower cross-reactivity (25%) towards 5α-DHT compared with T-HS-penicillinase (45%). In contrast, the antiserum generated against T-3-O-CMO-BSA conjugate showed high cross-reaction 44.8%) towards 5α-DHT when evaluated with an RIA. The authors suggest that structural modifications to ring B of the steroid nucleus (i.e. by using 6-dehydro-DHT), is a novel approach to minimise interference by 5α-DHT cross-reactivity in IAs for T.

Further, in the context of T estimations by EIA, it is worth noting that Tejada et al. (1998) have studied the interference of the anti-hormone RV486. This compound, known as nifepristone, 11β-(4-dimethyl aminophenyl)–17β-hydroxy-17α-(prop-1-ynyl) oestra-4, 9-dien-3-one, is currently used in clinical practice as a contraceptive agent. However, it may interfere with T (and oestradiol-17β) estimations when using some commercial kits.

Tejada et al. (1998) found that, although the cross-reactivity was low for T (0.3%) and oestradiol-17β (0.16%), doses higher than 5 mg/kg might produce false-positive results in plasma T estimations by EIA (especially in castrated male and female mice). Oestradiol-17β assays could also be affected; even 48 h after blood had been taken for analysis.

Kronvist et al. (1997) have developed and evaluated two types of flexible, sensitive and very rapid flow injection EIAs. The typical protocols for this type of assay are well known when the signal is generated from the antibody-bound hapten-enzyme conjugate, described above. In the new competitive injection technique, the non-bound fraction was passed through an affinity column and allowed to react with an enzyme substrate emerging from a channel in a post-column reaction system. The enzyme product in this particular situation was *p*-aminophenol or 4-methyl umbelliferol, which were detected amperometrically or fluorometrically.

Illera et al. (2003) have designed an amplified EIA for serum 4-A that can be used in the diagnosis of cryptorchidism in male horses. Diagnosis is clearly very important in this condition, especially as the animals have no visible or palpable testes. As the stallion testis produces both T (in large quantities) and oestrone sulphate (to a much lesser extent), both hormones have been measured in the plasma as possible predictors of cryptorchidism. However, earlier studies have shown the

possibility of false negatives (Illera et al., 2003 and references therein). The EIA for 4-A devised by these authors follows a standard protocol with polyclonal antiserum being raised in male rabbits against 4-A-3-O-CMO or 4-A-6-HS, being conjugated with HRP. Column chromatography on Sephadex G-25 with phosphate buffer as eluant was used to purify the conjugate. Reactions were carried out in a polystyrene 96-flat-bottomed well microtiter plate. Blanks and standards were prepared and treated in the usual way for comparison with unknowns. Final colour was achieved by the addition of 3,3′,5,5′-tetramethylbenzidine, and reading of absorbance with an automatic microplate reader. Mean values for 4-A in serum for cryptorchids and stallions were, respectively, 0.51 ± 0.04 (n = 60) and 10.52 ± 1.36 (n = 25). Intra- and inter-assay CVs were <8.2% and <9.3%, respectively. Overall recoveries were around 96% and the lowest detection limit was 1.54 pg/mL, consistent with a highly sensitive assay as required for the small quantities of 4-A produced in the horse testis. Comparison of results by a commercial RIA kit showed close agreement (n = 90; r = 0.95; p = <0.001).

6.10.8 Chemiluminescent Immunoassays

Since the late 1970s, when chemiluminescent immunoassays (CIAs) began to be published in the literature, considerable advances have been made in improving sensitivity and specificity. Of the many modifications that have been described, two may be mentioned here. For example, a variety of formats for solid-phase separation techniques have been utilised, including second antibody-coated microtitre plates or polystyrene balls or antibodies coupled to a polymer containing magnetic particles. Chemiluminescent labels have been synthesised with improved luminescent properties (Gower 1995). During the past decade or so, three classes of such compounds have been synthesised: (a) aminophthalhydrazides, (b) aminonaphthol-hydrazides, and (c) acridinium esters.

Compounds of group (ii) provide good light emission, detection limits being usually less than 10^{-16} M. They are, however, extremely reactive – a disadvantage since they readily react with air or O_2 at the surface of the reaction liquid. The acridinium esters (group iii) were originally used in CIAs as high specific activity labels for hormones such as thyrotropin and for antibody labelling (Gower, 1995). More recently, they have found use in steroid hormone, e.g. progesterone, quantification.

Of the three groups of luminescent compounds, the aminophthalhydrazides are the most suitable, and consequently, the most used in CIAs. One example, used in earlier work is luminol (5-amino-2,3-dihydrophthalazine-1,4-dione). This, in common with other members of group (i), can be oxidised by peroxides at alkaline pHs (greater than pH 8), when light is produced, which can be measured to provide sensitivities in the femtomolar range or even lower. One of the problems with at least some members of this group is that the luminogenic properties are very largely lost once the label is linked to the steroid to be measured. This is especially true of

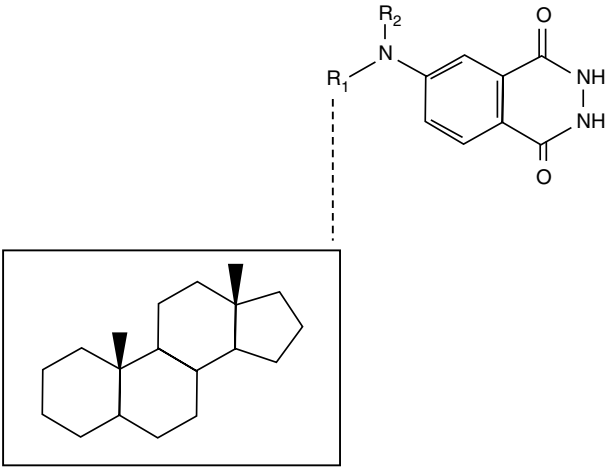

Fig. 6.17 Structures of isoluminol and isoluminol derivatives for the conjugation to steroid molecule to yield steroid chemiluminescent tracers. R_1, bridging arm; R_2, secondary arm. $R_1 = R_2 = H$, isoluminol; $R_1 = NH_2(CH_2)_2-$ $R_2 = CH_3CH_2-$, aminoethylethylisoluminol; $R_1 = NH_2(CH_2)_2-$, $R_2 = H$, aminoethylisoluminol; $R_1 = NH_2(CH_2)_4-$, $R_2 = CH_3-$, aminobutylmethylisoluminol; $R_1 = NH_2(CH_2)_4-$, $R_2 = H$, aminobutylisoluminol; $R_1 = NH_2(CH_2)_4-$, $R_2 = CH_3CH_2-$, aminobutylethylisoluminol; $R_1 = NH_2(CH_2)_6-$, $R_2 = CH_3CH_2-$, aminohexylethylisoluminol (from Pazzagli et al., 1983, with permission of authors and publisher Elsevier Science)

luminol, which loses more than 90% of its light production. It is more than 20 years since luminol was first used in a chemiluminescent EIA for DHEA and DHEA sulphate in human plasma. The enzyme label was peroxidase, which was linked to DHEA, separation of free and bound steroid-enzyme conjugate being by insolubilised antibody. This was prepared by coupling purified IgG of goat anti-rabbit IgG serum with Sepharose 4B or polystyrene tubes. The chemiluminescent reaction, using luminol and H_2O_2 as substrate, gave only 'faint chemiluminescence' according to the authors, but sensitivities of 2.5 pg/tube and 100 pg/tube for DHEA and its sulphate, respectively, were achieved. Good agreement was shown between these results and those by RIA.

Improved luminogenic properties have been recorded for isoluminol derivatives, which show adequate light production after being coupled to steroids. Detailed evaluations have been published for six isoluminol derivatives which can be conjugated to various steroids without the quantum yield of the chemiluminescent label being affected. This has been shown clearly to increase with increasing length of the second side chain (the 'bridging' arm to the steroid, Fig. 6.17; Gower, 1995).

The aminoethyl butyl (ABEI) and aminoethyl ethyl (AEEI) derivatives of isoluminol are considered as universal labelling compounds for steroids. The detection limit for ABEI (6-[N-(aminobutyl)-*N*-ethyl]amino-2,3-dihydrophthalazine-1,4-dione) is about 10^{-16} M when H_2O_2 and microperoxidase from horse heart are utilised for the oxidation procedure, and this has been used successfully in a solid-phase CIA for serum T. ABEI is also appropriate as a luminescent marker in a CIA for

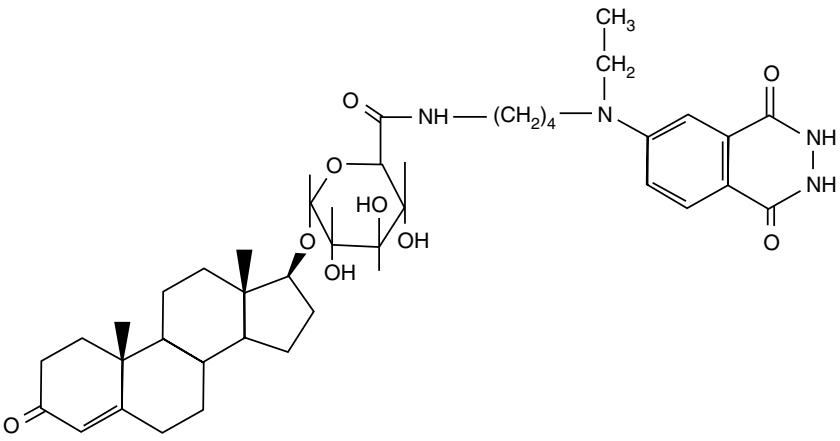

Fig. 6.18 Proposed structure for testosterone glucuronide aminobutylethyl-isoluminol conjugate for use in a CIA (from Vannuchi et al., 1983, with permission of authors and publisher, Elsevier Science)

urinary T glucuronide (Fig. 6.18; (Gower 1995). Standards and diluted urine samples, together with steroid-ABEI conjugate, were added to Lumacuvettes which had been coated with anti-testosterone glucuronide-IgG-BSA. The chemiluminescent reaction at alkaline pH in the presence of microperoxidase was initiated by the addition of H_2O_2, whereupon light emission was quantified in the luminometer. Correlation of results with an RIA method was excellent ($r = 0.95$), but there was cross-reaction with T itself (11.6%) and especially with 4-A (197.5%). However, as the authors point out, the mean 24-h excretion of free T (0.25 µg) and 4-A (2.9 µg) in adult female subjects is approximately 100-fold and 10-fold less, respectively, than that of the glucuronide (22 µg). The cross-reactions, therefore, were considered to pose little or no problem in clinical evaluations.

In a series of studies begun in the early 1980s (Gower 1995) and aimed at developing CIAs for steroid hormones, antibodies were covalently linked to polyacrylamide beads. The first antibodies were poly- or monoclonal and the second were goat anti-mouse IgG antibodies. This procedure eliminated problems encountered with regard to precision and quality control with the antibody-coated tube methods. A variety of steroids was used in these studies, including the 3-O-(CMO) derivative of T, conjugated to ABEI. In brief, diluted urines, plasma extracts and standards were added to Lumcuve HC polystyrene tubes and anti-T IgG-coupled beads plus T-3-O-(CMO)-ABEI chemiluminescent conjugate were added. After incubation and centrifugation, NaOH solution was added to the pellet and the light yield was determined in the standard oxidation system (microperoxidase and H_2O_2). The data compiled by Wheeler (1994) shows that some CIAs are available commercially (Table 6.11).

During the past decade, a number of techniques have been introduced and their use explored in, for example, the application of chemiluminescense in the detection

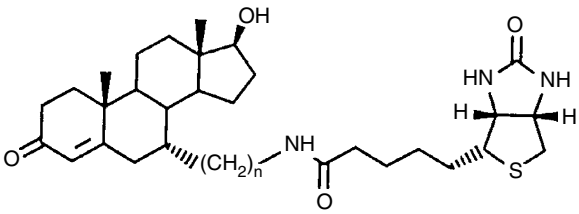

Fig. 6.19 Chemical structure of the 7α-Bio-T immunochemical tracers (from Luppa et al., 1997, with permission)

of analytes in the liquid phase and in the possible development of chemiluminescent sensors in IA (Garcia-Campano et al., 2000). One group (Luppa et al., 1997) has successfully synthesised ring core-biotinylated T tracers for use in a sensitive CIA for T in serum. The tracers were synthesised with bridges of varying lengths connecting the biotin moiety to the steroid (7α-C_4 -Bio-T), where n = 3, 6 or 11 (Fig. 6.19). This was then used, together with position-7-specific polyclonal anti-T antibody, to develop a novel labelled competitive IA. The authors found that bridges of length C_3 or C_6 were unsuitable for analogous IAs. It was also found that, if a second immobilised antibody and a streptovidin horseradish oxidase conjugate were used, a significant increase in chemiluminescent signal was generated. The working range of the assay was 0.2–20 nmol/L with limit of detection 0.125 nmol/L. Inter-assay variation was 13–16%, and good accuracy and recoveries (95%) were obtained using comparisons with GC-MS monitoring of reference samples. The authors concluded that, despite the imprecision of their assay needing improvement, the 7α-C_{11} -Bio-T tracer gave greater specificity because the length of connecting arm allows greater availability of the critical antigenic sites.

In 2003, an EIA for 19-nor T in bovine urine was published by Roda et al. The competitive assay involved the use of anti-19-nor-T polyclonal antibodies, raised in rabbits using the hemi-succinate of the steroid conjugated with ovalbumin. As a label, horseradish peroxidase (HRP) was also coupled to the 19-nor T. After immobilising antibodies on a 384-well black polystyrene microtiter plate, HRP-19-nor T was measured using an efficient chemiluminescent substrate. Only a 3-min incubation was necessary, after which emitted light was recorded using either a photo-multiplier tube-based microtiter plate reader or a sensitive back-illuminated cooled charge-coupled device (CCD) camera. The authors have validated their method with respect to accuracy (inter-assay CV < 10%), recovery (94–112%) and detection limit (0.3 ppb, i.e.1.1 × 10^{-9} mol/L. The authors have also emphasised some of the advantages of the assay. For example, the 384-well micro-tite plate reduces sample/reagent fivefold relative to conventional 96-well plates. Further, increased sensitivity (relative to colorimetry) was achieved as a result of enhanced detectability of the HRP-labelled tracer. Finally, the method allows greater through-put of samples compared with conventional colorimetric EIAs.

In the context of the CCD camera used by Roda et al. (2003), it is worth noting that Porakishvili et al. (2000) had previously designed a low-budget CCD camera

at approximately one-third the price of a conventional instrument. This group has emphasised that the sensitivity of CIAs can be 0–100 times greater than that obtained with ELISAs for chromogenic assays.

6.10.9 Time-Resolved Fluorimetric Immuno Assays

The underlying principles of this technique have been explained in an earlier chapter of this volume (Wheeler and Barnard see Chapter 4). In attempts to enhance the fluorescence of the sample, the properties of a variety of substances have been investigated and the most useful labels have been reviewed (Gower 1995). For fluorometric assays, organic labels such as coumarin derivatives and fluorescein have been used, although the latter, and many other fluorescent compounds, give high background signals. In time-resolved fluorimetry, the lanthanides (e.g. europium, samarium, terbium) and their chelates, have been used extensively. This group displays fluorescence with decay times of 50 μs. Since the europium chelates have very high specific activities, they are extremely efficient in providing fluorimetric immuno assays (FIAs) with sensitivities of 10^{-15}–10^{-16}mol/L. Examples of such assays have been published (see Gower, 1995 for references). The ions of other lanthanide have been used as labels, either separately or in conjunction with europium to provide double-label assays.

General procedures for time-resolved fluorimetric immuno assays (T-R FIAs) have been described by many researchers and several reviews have been published during the last decade (Gaillard et al., 1994; Dickson et al., 1995; Degan et al., 1999). The following descriptions of T-R FIAs for T and 11β-hydroxy-4-A should help to illustrate the practical issues. In a competitive double-antibody assay, highly specific monoclonal antibodies, raised against a conjugate, were used as the primary antibody. T was conjugated, via the 3-carboxymethyl oxime (CMO), to BSA, serving as immunogen. T in the standards and in plasma or other biological fluids, and labelled with the europium chelate, competes in binding to the primary antibody. The second antibody, anti-mouse IgG, which was coated on to the solid phase (usually a microtiter plate or strip) binds to the steroid-antibody complex so that the separation between the bound and the free antibody is achieved conveniently. After the immuno reaction had taken place, dissociation-enhanced-lanthanide fluoroimmuno-assay (DELFIA) enhancement solution was added to each well. In this way, the lanthanide is dissociated into solution and the enhanced fluorescence measured in the time-resolved fluorimeter.

As noted earlier, various derivatives are used in IA procedures to link the steroid to protein or to the fluorescent label, e.g. CMOs, hemisuccinates (HS), carboxyethyl ethers and thioethers. Attempts have been made to increase the length of spacer arms by introducing aliphatic reactive groups, e.g. alkyl oximes, into them; these studies have been described and evaluated (Gower 1995). The europium chelate-labelled alkyl oxime of T was synthesised and tested in the DELFIA procedure described above. Both the *O*-(4-aminobutyl)- and *O*-(6-aminohexyl)-oxime derivatives

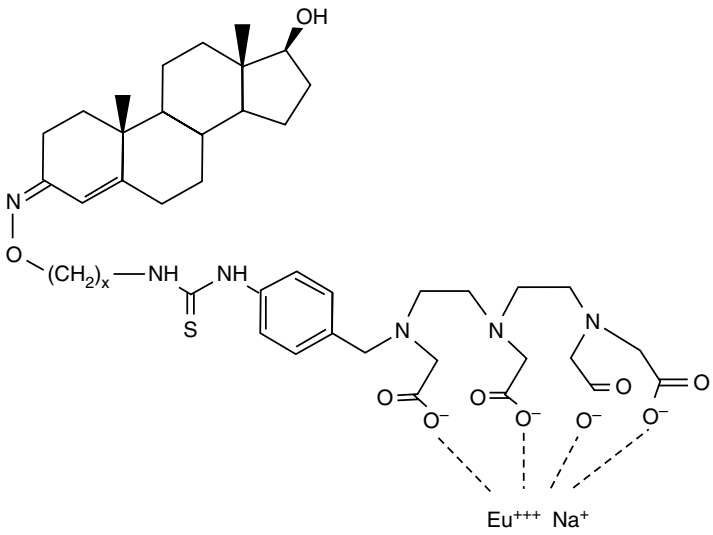

Fig. 6.20 Structure for europium and labelled testosterone alkyl oxime derivatives. x = 4, *O*-(4-aminobutyl)- and x = 6, *O*-(6-aminohexyl)-oximes (from Mikola et al., 1993, with permission of authors and publisher, Elsevier Science)

(ABO and AHO) (Fig. 6.20) as europium chelates were highly specifically bound by the primary antibody, signals being in the range 22–29 × 10^3 cps compared with the instrumental background and non-specific binding values of 300–900 cps. However, the 3-CMO derivative with its shorter spacer arm gave a less sensitive assay than its ABO and AHO counterparts. The major problem encountered was to obtain adequate purification of the 'tracer', i.e. steroid derivative-europium chelate. TLC and HPLC (reversed-phase C_{18} column) did not provide sufficient purity and it was only after chromatography on a Trisacryl GF05M column that the 'tracer' was pure enough to result in a successful and reproducible FIA. Several commercial FIA assay kits are available for steroid hormones (Wheeler, 1994; see also Section 6.10.4 and Table 6.11).

The T-R FIA published by Yamada et al. (1997) for estimation of T in rainbow trout largely follows the procedures noted above. T-3-CMO-BSA was immobilised by adsorption to microtiter plate wells. The competitive assay involved the use of two antibodies and was performed with T-3-CMO-BSA, T-extract for analysis, T antibodies and europium ion-labelled secondary antibodies. Measurements were made with a T-R fluorimeter, using the DELFIA system (see above). The authors showed that the assay range was 1.5 pg/mL to 25 ng/mL, with intra- and inter-assay CVs ranging between 6.2 and 6.38%, and 2.96 and 6.29%, respectively. The assay was found to be sensitive, and as efficient as RIAs.

The work of Tschop et al. (1998) focussed on devising a T-R FIA for T in saliva. In these studies, the rapid and sensitive saliva T assay was ideal for monitoring T replacement therapy in eight hypogonadal men (divided into

treatment groups). Groups I and II had received intramuscularly a single dose of T buciclate (200 mg and 600 mg for Groups I and II, respectively). Saliva and blood samples were taken at fairly frequent intervals for up to a week and then weekly for up to 3 months. Evaluation of the T-R FIA showed a detection limit of 16 pmol/L saliva, with good intra- and inter-coefficients of variation (8.9% with T concentrations 302 pmol/L and 8.7% at T concentrations 305 pmol/L, respectively). Two other important features of the assay were (a) consistent results being obtained compared with an established RIA and (b) only 180 μL saliva being required for extraction and duplicate determination. The authors found that the smaller (200 mg) dose of T buciclate did not result in increase to normal T levels either in saliva or serum, whereas the 600 mg dose caused T levels to increase rapidly to maximal levels that were maintained for up to 12 weeks post-injection. Maximal levels of T in salvia and serum were 303 pmol/L and 13.1 nmol/L, respectively, in study weeks 6 and 7.

Fiet et al. (2004) have developed a TR-FIA for serum T measurement, using a biotinylated T as tracer. The biotin molecule was separated from C-3 of the T molecule by a long tracer arm consisting of biotinylamino decane CMO. The assay was shown to be extremely sensitive (at least equivalent to 21 pg T/mL of serum), and specificity was assured by including a purification step of Celite chromatography on new mini-columns. CVs for the FIA were 2.17% and 5.07% for serum T levels in normal and T-treated hypogonadal men, respectively. Other parameters were found to be satisfactory, as anticipated for an acceptable IA. Comparison of results with GC-MS showed no significant differences ($r = 0.992$).

Ibrahim et al. (2003) have devised and validated a T-R FIA for 11β-hydroxy-4-A in plasma. Such an assay is necessary because plasma 11β-hydroxy 4-A levels are high in patients with 21-hydroxylase deficiency (i.e. Cushing's syndrome and in hyperandrogenism of adrenal origin). However, in cases where the plasma 4-A concentrations are raised, it is useful also to measure the 11β-hydroxy derivative levels to ascertain whether the origin of the hyperandrogenism is of the adrenals or ovaries.

The 11β-biotin conjugate of 11β-hydroxy-4-A was synthesised 'in-house' and T-R fluorescence measured after addition of Eu^{3+}-streptavidin to microtiter wells. Plasma samples were first extracted, after which a purification step was effected with Celite to ensure removal of other interfering steroids from the assay. A similar procedure was used for RIA of 11β-hydroxy-4-A employing a tritiated label. Although the correlation coefficient between the two assays was 0.965, the FIA technique was found to be more sensitive and to have a greater degree of precision.

6.10.10 Radioimmunoassays

Radio immunoassay (RIA) has become routine in steroid hormone estimations as judged by the enormous numbers of RIAs reported (Table 6.10). Inevitably, there have been changes and improvements in methodologies. Earlier changes, that have taken

place since the early RIAs appeared, have been reviewed, and comparisons between the various IA methodologies available. For example, the relative merits of RIAs for steroids using ^{125}I- or ^{3}H-ligands have been compared, and important topics such as design of antibody specificity, have been reviewed (see Gower, 1995 for references).

One of the requirements for RIA is the separation of bound and free steroids, and the numerous methods devised and evaluated for this purpose, e.g. dextran-coated charcoal, have been discussed earlier (Gower, 1995). In the RIA for T in human plasma (ether-extracted), a typical standard assay procedure was adopted, incorporating antiserum raised against T-O-CMO-BSA and [^{3}H]T of high specific radioactivity. This pseudohomogeneous RIA, relying on the new separation technique (called ligand differentiation immunoassay, LIDIA) was evaluated extensively with regard to sensitivity, accuracy, precision, etc. The 3-CMO derivative of T is now used extensively and references to it are included in the examples given below and in Table 6.10. In view of the potential biochemical and clinical importance of steroid conjugates, the availability of RIAs for these can be of great importance. Wheeler (1994) has listed 21 commercially-available kits for DHEA sulphate, of which the majority (18) are RIAs, with three EIAs but only one RIA for 5α-androstane-3α,17β-diol-17-glucuronide.

Table 6.10 summarises a selection of the very large number of RIAs that have been utilised in the past decade for estimation of C_{19} steroids and their conjugates (directly) in numerous fields of study, whether clinically related or biosynthetic and metabolic. For example, a differential extraction method for simultaneous direct RIA of T and 5α-DHT, and of 5α-androstane-3α,17β-diol glucuronide has been published by Brind et al. (1996). Using human serum samples, free steroids and conjugates were extracted with solid-phase gravity-flow extraction columns. Free T and 5α-DHT were eluted with diethyl ether, and the glucuronide with diethyl ether containing 2% acetic acid. The glucuronide fractions were washed with 1% aqueous acetic acid to remove cross-contaminating steroid sulphates. Standard RIA methodologies were employed to determine the two free steroids, T and 5α-DHT, and androstanediol glucuronide, but androsterone glucuronide was measured by RIA that involved the use of ^{3}H-androsterone tracer, together with an anti-serum raised against DHEAS that cross-reacted.

An example of the use of RIA in the clinical context is the blood-spot T assay developed by Walker et al. (1999). The assay involved the use of an antiserum, raised against T-O-CMO-BSA conjugate and with [^{125}I]-T-3-CMO-histamine as tracer. The blood-spot technique is particularly useful in infants and small children and when repeated estimations are required during monitoring of T levels, e.g. for children on replacement therapy for congenital adrenal hyperplasia. In this condition, adequate treatment is indicated in the presence of consistently low levels of plasma T being found in the presence of moderately elevated early morning 17α-hydroxyprogesterone. The authors carefully validated their method to show a limit of detection of 0.4 nmol/L for plasma T. Between-batch precision was <15% for values of 0.9–2.0 nmol/L and <10% for values >20 nmol/L. Overall recovery was 100.9% when 4 nmol/L T was spiked into blood samples from 16 women (unspiked T values 0.4–3.7 nmol/L).

Another example of the use of RIA in the clinical situation relates to the work of Monti et al. (1998b), in which the correlation between androgens and their receptor concentrations in benign prostatic hyperplasia (BPH) was confirmed. Prostatic samples were sectioned into periurethral, intermediate and sub-capsular zones, and

cytosolic and nuclear androgen receptors (AR_C and AR_N, respectively) were measured, and also concentrations of T, 5α-DHT and 5α-androstane-3α,17α-diol (androstanediol) by RIA, after tissue extraction and purification on Celite microcolumns. The authors found linear correlations between 5α-DHT and androstanediol, between T and 5α-DHT, and between androstanediol and AR_N concentrations. The periurethral regions contained the highest concentrations of the C_{19} steroids and also of AR_N. Since this region of the prostate is the site of the primitive BPH nodule, the authors suggest that the high concentration of androgens found there may be involved in growth promotion leading to urinary flow obstruction.

The biochemistry and significance of the two 7-hydroxy DHEA isomers have been discussed in earlier sections and a review is available (Hampl et al., 2003). Lapcik et al. (1998, 1999) have described and validated RIAs for 7α- and 7β-hydroxy DHEA. Polyclonal rabbit antisera were raised against the appropriate 19-O-CMO-BSA conjugate and bridge and homologous [^{125}I]–iodo-tyrosine methyl ester used as tracer. Alternative methodology was also used involving tritium-labelled tracer but the sensitivity of the assay in this case was lower. The identity of the immuno-reactive material was confirmed using HPLC to separate the 7α- and 7β-isomers. As these are known to occur in plasma not only as free (unconjugated) steroids but also as fatty acid-esterified and sulphate-conjugated forms, these had to be fractionated before RIA could be performed. For this reason, plasma samples were first extracted with a mixture of iso-octane and ethyl acetate followed by partition of the extract between iso-octane–methanol–water mixture and, finally, the ester and sulphate fractions were hydrolysed and solvolysed, respectively (Corpechot et al., 1983). The usual parameters expected for an acceptable assay were found to be satisfactory. Mean values for 7α- and 7β-hydroxy DHEA and DHEAS in healthy subjects were 2.33, 2.26 and 7.69 nM. The same group (Bicikova et al., 2004) has shown the value of plasma 7α–hydroxy DHEA and certain amino thiols as being discriminatory tools in Altzeimer's disease and vascular dementia. Plasma levels of pregnenolone S, 17α–pregnenolone and DHEAS were independent of the diagnosis (see also Table 6.10).

An RIA for intact 3α-androstanediol glucuronide (3α-Adiol G) has been developed by Rittner et al. (1997). As this steroid conjugate is a marker of androgen metabolism in peripheral tissues (see Sections 6.3 and 6.8.1), such an assay is clinically useful, especially as it can be performed rapidly on the intact glucuronide without prior hydrolysis. Serum samples studied were from 146 boys and 137 girls (aged from 1 month to 20 years), as well as from 28 adults. A non-extraction, solid-phase RIA was set up, using a polyclonal antiserum specific for 3α-Adiol G. Intra- and inter- assay CVs were 5.1–10.1% and 2.7–9.0% respectively. There was a marked sex- and age-dependence, with serum levels increasing throughout childhood and adolescence, and with males having higher values than females. There was a significant correlation with the pubertal stage and in children with congenital adrenal hyperlasia both in clinical and metabolic states (i.e. with 17α-hydroxyprogesterone serum levels). The authors suggest that, given their reference values obtained from a large number of children and adolescents, a single serum 3α-Adiol G measurement could well be helpful in high-lighting disorders in androgen status and in disorders in puberty or sexual development.

6.11 Summary and Perspectives for Future Studies

In writing this, the second edition of a chapter on estimation of C_{19} steroids, it is clear that, during the past decade, so much new material has to be included. Many of the aspirations expressed then for the advances in methodologies etc. have been realised. In general, sensitivities, CVs and reproducibilities of assays have improved, while estimations of intact steroid conjugates, some of which now proven to be clinical 'markers', are available. A number of papers have been devoted to the development of 'on-line' estimations, which include extraction, purification and estimation of various C_{19} steroids from biological fluids.With the very real prospect now of such 'on-line' methodologies being available for intact conjugates, coupled with greatly improved data handling systems, inevitably means greater through-put of results in clinical laboratories. A final thought is concerned with the way in which research into the presence biosynthesis and possible modes of action of C_{19} steroids ('neurosteroids') in brain and spinal cord has developed over the last 2 decades. For example, DHEA and pregnenolone and their sulphates are present in brain tissue of rodents, but only in small quantities relative to the corresponding lipoidal derivatives of these steroids. The exact nature of these compounds is still not clear, at the time of writing but their significance and biochemical actions should be of considerable importance.

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Chapter 7
Analysis of Progestagens

P.J. Wood and D.B. Gower

7.1 Introduction

This chapter covers the analysis of steroids with progesterone-like activity, classified as "progestagens". Steroids in this group include the naturally occurring C_{21} steroids, progesterone (4-pregnene-3,20-dione) and its metabolites, together with synthetic steroids, such as norgestrel norethisterone (NE), and medroxyprogesterone acetate which also have progestational activity.

The structures of some synthetic progestagens are shown in Fig. 7.1.

7.2 Progesterone

7.2.1 Discovery and Isolation

Progesterone was first discovered in the 1920s when Corner and Allen (1929) reported that an extract prepared from swine corpora lutea caused progestational changes in uterine endometrium of castrated rabbits. The fat-soluble hormone responsible was isolated in pure form from sow's ovaries by Allen and Wintersteiner (1934), and the structure of this new hormone "progesterone" was described in the same year (Butenandt and Schmidt, 1934 ; Fernholz, 1934).

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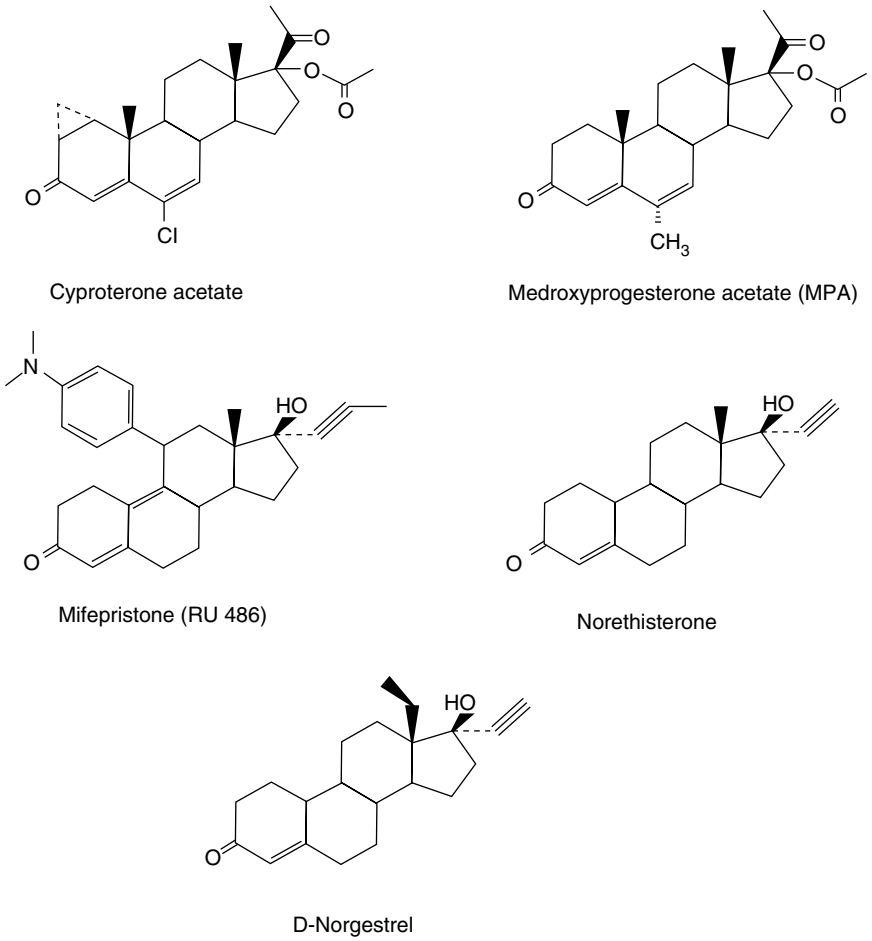

Fig. 7.1 Structures of some synthetic progestagens

7.2.2 Biosynthesis

Progesterone is accepted as being formed in the cellular endoplasmic reticulum from its immediate precursor, pregnenolone by the actions of 5-ene-3β-hydroxysteroid dehydrogenase (HSD or oxido-reductase)/4,5-isomerase. Pregnenolone itself, is known to be produced from cholesterol in the mitochondria by side-chain cleavage of the 20,22-dihydroxy derivative, catalysed by a cytochrome P-450 – now known as CYP 11A1. Further details of the various cytochromes P-450 involved in steroid hormone biosynthesis and mechanism of action are provided in Chapters 5 and 6.

7.2.3 Secretion

Progesterone is secreted by ovarian granulosa cells prior to ovulation under the influence of luteinising hormone released from the anterior pituitary. Secretion of progesterone into the circulation may also be facilitated by the theca interna cells, which lie in close proximity to the granulosa cells in the follicle (Ryan and Smith, 1965). Follicular fluid progesterone concentrations are raised just prior to ovulation, and production increases further with the formation of the corpus luteum. Progesterone production by the corpus luteum reaches a maximum of up to 30 mg/day 7–8 days after follicular rupture, at a time when functional activity of the corpus luteum is at a maximum (Zander et al., 1958).

The corpus luteum plays an essential role in providing progesterone to maintain early pregnancy (Csapo et al., 1973) and continues to produce progesterone throughout pregnancy (Lemaire et al., 1971). Studies show that the foetus cannot metabolise pregnenolone to progesterone and that the feto/placental unit relies on production of the steroid by placental mitochondria. Placental synthesis of progesterone increases in early pregnancy so that in the second and third trimester the placenta is the main source (Lebech, 1971), producing 200–500 mg/day.

The main site of action of progesterone is the uterine endometrium, where, during the luteal phase, it increases vascularity, glandular development and secretion, and reduces myometrial activity. These influences prepare the uterus for implantation of the fertilised ovum and provide support for the developing foetus.

Progesterone measurements are used routinely to check that ovulation has occurred, and that the corpus luteum is functioning normally.

A rise in plasma progesterone to over 30 nmol/L for a mid-luteal sample (day 21 for a regular 28 day cycle) indicates that ovulation has occurred. If it is uncertain which day of the cycle a sample has been taken, then a progesterone concentration above 16 nmol/L is consistent with ovulation.

Strictly speaking, the progesterone rise confirms the formation of the corpus luteum rather than ovulation. Since it is possible for the corpus luteum to form without the release of the ovum (the "unruptured luteinised follicle syndrome;" Marik and Hulka, 1978) plasma progesterone may also be elevated in this situation, although levels achieved are usually lower than those for healthy ovulation.

7.2.4 Measurements for the Timing of Ovulation

Measurements of progesterone are also used during *in-vitro* fertilisation therapy, where rising levels indicate that ovulation has occurred or is about to occur.

In addition to the ovary and corpus luteum, the adrenal also secretes progesterone in relatively small amounts (Short, 1960). However, adrenal production of progesterone can be much higher in patients with congenital adrenal hyperplasia (Janne et al., 1975).

7.2.5 *Metabolism*

As a result of many decades of experimentation with human and animal tissues, the pathways of progesterone metabolism have been well-established. These are similar to those occurring for C_{21} corticosteroids (Chapter 5) and also, but to a more limited extent, for C_{19} steroids such as 4-androstenedione (Chapter 6). The sequence for progesterone, illustrated in Fig. 7.2, is as follows: (i) reduction in Ring A, catalysed by 4-ene-5α- and 5β-reductases, the products being 5α- and 5β-pregnane-3,20-diones (the 5β-isomer predominating); these are also known as 5α- and 5β-dihydroprogesterones; (ii) reduction at C-3, catalysed by the 3α- and 3β-hydroxysteroid dehydrogenases (HSD) (also referred to as oxido-reductases). The resulting products are

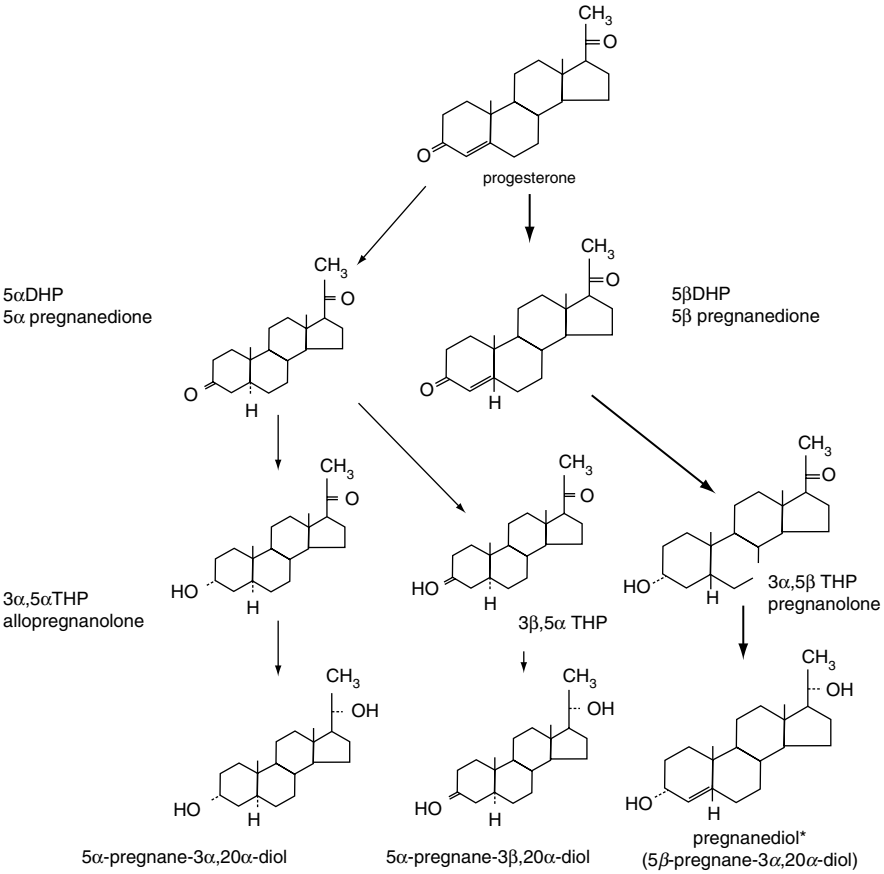

Fig. 7.2 Metabolism of progesterone. Heavy lines indicate major pathways; □ indicates major urinary metabolite; DHP = dihydroprogesterone; THP = tetrahydroprogesterone (adapted from Gower, 1984, with permission of the publishers Blackwell, Oxford, London, Edinburgh, Boston, Palo Alto, Melboune)

isomeric pregnanolones, also known as tetrahydroprogesterones. The 3α,5α-isomer is currently often referred to as allopregnanolone.

This isomer and 3α-hydroxy-5β-isomer are known to have extremely important effects in the brain and peripheral nervous system (PNS) (see Section 7.11). (iii) reduction at C-20, catalysed by 20α- and 20β-HSD (or oxido-reductases) to form isomeric pregnanediols, of which the 3α, 5β, 20α-isomer predominates. It is generally referred to as pregnanediol as it is the major urinary metabolite of progesterone. Similar reactions (i–iii) occur for 17α-hydroxyprogesterone, resulting finally in the formation of isomeric pregnanetriols, of which the most important quantitatively is 5β-pregnane-3α, 17α,20α-triol (commonly referred to as pregnanetriol), the major urinary metabolite of 17α-hydroxyprogesterone.

Both pregnanediol and pregnanetriol are excreted in the urine as glucuronide conjugates. So also are the two corresponding principal pregnanolones, described above, and the corresponding metabolite of 17α-hydroxyprogesterone, 3α, and 17α-dihydroxy-5β-pregnan-20-one.

7.3 Early Methods for Serum Progesterone Measurement

Before the 1960s no method had sufficient sensitivity to measure progesterone in serum, and endogenous progesterone production was assessed by measurement of its main metabolite, pregnanediol, in urine (see Section 7.10.3)

However, during the 1960s, methods for serum progesterone measurement based on bioassay, gas chromatography, and isotope dilution analysis were developed.

7.3.1 Bioassays

The physiological basis of progesterone bioassay relied on the effect of progesterone on the uterine endometrium or on the maintenance of pregnancy in laboratory animals.

Earlier bioassays for progesterone measurement have been reviewed by Miyake (1962). The methods were relatively sensitive, but suffered from lack of specificity and were influenced by varying concentrations of progesterone metabolites. These assays were not suitable for routine progesterone measurements although they still have value for the assessment of progestational effects of synthetic progestagens and antiprogestagens in the pharmaceutical industry.

7.3.2 Spectrophotometry

Early spectrophotometric methods utilised UV absorption of progesterone at 240 nM, associated with the Δ^4-3-oxo bond in the steroid nucleus. These methods,

reviewed by van der Molen (1963) were extremely lengthy, cumbersome, and insensitive.

Derivatisation of the Δ^4-3-oxo group to form dinitrophenyl hydrazones (Hilliard et al., 1961) thiosemicarbazones (Sommerville et al., 1963) or isonicotinic acid hydrazones (Sommerville and Deshpande, 1958) was used together with the correction procedure described by Allen (1950) to compensate for the interferences. However, these methods could not detect less than 0.1–0.5 μg of progesterone.

7.3.3 Fluorimetry

Attempts to measure progesterone using fluorimetric assays were hampered by interference and lack of specificity.

Short and Levett (1962) used potassium hydroxide/sulphuric acid-induced fluorescence to develop a method which could detect 50 ng progesterone, allowing analysis of a 20 mL blood sample. However, the method lacked specificity, particularly for follicular phase samples. Enzymatic conversion of progesterone to 20β hydroxy-pregn-4-ene-3one prior to induction of fluorescence was used to develop a method with a detection limit of 3–5 ng progesterone (Heap, 1964).

7.3.4 Double Isotope Derivatisation

This method for steroid measurement was developed during the 1960s, and involves the use of radioactive reagents which react with steroids. The labelled product could then be isolated and measured. To correct for losses, a known amount of [^{3}H]- or [^{14}C]-labelled steroid was added to the plasma sample. After extraction and purification, the labelled and unlabelled steroid samples were derivatised using the reagents which contained a different isotope. Following further purification, relative amounts of the two isotopes were then determined by liquid scintillation counting on a beta scintillation counter with a twin channel facility. The initial mass of progesterone could then be calculated.

Labelling reagents used include sodium [^{3}H] borohydride (Woolever and Goldfien, 1963), [^{35}S]-thiosemicarbazide (Riondel et al., 1965) and [^{3}H] acetic anhydride (Wiest et al., 1966).

In general, these methods required extensive purification procedures to ensure specificity, and were not suitable for routine serum progesterone analyses.

7.3.5 Competitive Protein Binding

The principle of competitive protein binding assays and radioimmunoassays (RIAs) was described by Yalow and Berson (1960) who examined insulin binding by

endogenous antibodies, and by Ekins (1960) who studied thyroxine binding to thyroxine binding globulin in serum. Ekins introduced the term 'saturation analysis' to describe these methods, which rely on competition between the labelled and unlabelled analyte for a limited amount of binding reagent. The binding reagent used was not sufficient to bind all the analyte, and was therefore 'saturated'. After separation of bound and unbound analyte, the percentage of label contained in the bound fraction was inversely proportional to the concentration of analyte in the standard or sample.

Competitive protein binding (CPB) assays use a specific binding reagent such as a plasma protein, whereas RIAs use an antibody raised against the analyte as a specific binding reagent.

CPB determination of progesterone in blood has been described by several investigators (Murphy, 1967; Neill et al., 1967; Yoshimi and Lipsett, 1968; Hagerman and Williams, 1969; Johansson, 1969a, b). All these methods relied upon competition between progesterone extracted from serum and radiolabelled steroid (progesterone or corticosteroid) to corticosteroid binding globulin (CBG) derived from human or dog plasma. Early assays used [^{14}C]-labelled steroids and tedious dialysis or gel filtration to separate bound and free steroid fractions, but later the use of [^{3}H] tracers and fluorisil or charcoal (Murphy 1967) to absorb free steroid made methods a more practical proposition. Precise methods with detection limits of 0.5 ng were achieved with a tritium tracer, but specificity depended on separation of progesterone from other steroids by a chromatographic step prior to CPB assay. Methods were still being refined in the 1970s; for example the use of pregnant guinea pig serum as a binding reagent enabled Pichon and Milgrom (1973) to develop a CPB assay which did not require the preliminary chromatographic separation of progesterone.

Since the binding proteins were present at relatively low titre, it was necessary to prepare new batches frequently and this required re-optimisation of the assay each time.

7.4 Radioimmunoassay

7.4.1 Early RIAs

The first RIAs for progesterone were published in the early 1970s, but these assays were non-specific and still relied on chromatographic separation of progesterone from other steroids. For example, Abraham et al. (1971) used celite column chromatography followed by RIA using [1,2-^{3}H]-17-hydroxy progesterone label and an antiserum raised against 11-deoxycortisol-21-hemisuccinate.

Steroids are not large enough to elicit an antibody response on their own, and need to be conjugated to larger, more immunogenic carrier proteins such as bovine serum albumin or keyhole limpet haemocyanin to be able to produce an antibody response in the host animal. The small molecule to be linked to protein is termed the 'hapten' and the process is known as 'haptenisation'.

Early RIAs for progesterone used haptens linked to carrier proteins through their functional groups: this produced low-specificity antisera since the functional determinants were 'masked'. Antisera raised to conjugates linked to carrier protein through the D ring do not recognise changes in functional groups on this ring. For example, antisera to a progesterone-20-carboxymethyloxime (CMO) conjugate showed extensive cross reactivity with C21-steroids such as deoxycorticosterone and also to C_{19} steroids such as testosterone.

The introduction of reactive groups into progesterone at sites remote from functional groups yielded antisera which showed much lower cross-reactivity with related steroids. Lindner et al. (1970) and Niswender and Midgely (1970) obtained useful antisera by linking progesterone to carrier protein through positions C6 or C11 on the B ring.

Antiserum specificity is greatest when 11-conjugates are used for immunisation since these antigens leave both the 17 side-chain and A/B ring junction free for antibody recognition

Most high-specificity antisera used in progesterone immunoassays are generated with progesterone-11-linked immunogens.

By 1973 the components of the "classical" radioimmunoassay for progesterone with the required sensitivity, precision, specificity and accuracy had been identified. A typical assay system would use solvent extraction, Tritium-labelled progesterone, an antiserum raised against an 11-linked progesterone immunogen and separation of bound from free tracer with dextran-coated charcoal (Thorneycroft and Stone, 1972).

Further improvements in methodology focussed on optimisation of the solvent extraction and the use of [125-I]-labelled steroids to increase sample throughput and assay cost-effectiveness.

7.4.2 Solvent Extraction

Extraction of progesterone from serum using diethyl ether gave a recovery of approximately 90%, but the formation of peroxides in the solvent influenced the dose-response curve. The use of small bottles of HPLC grade diethyl ether provided the most practical solution to this problem and avoided the need for frequent redistillation. Light petroleum ether was also used, but this reagent has a variable mixture of components with a variable extraction efficiency for progesterone and for other unwanted polar steroids which cross-reacted in the assay.

Correction of individual results for extraction efficiency was rarely used as it was impractical for routine methods.

7.4.3 Direct (Non-Extraction) Immunoassays

The need for solvent extraction placed limitations on sample throughput and the establishment of automated assay methods. However the introduction of non-extraction

methods caused additional problems which had to be addressed in order to establish valid assays.

Solvent extraction of progesterone with a non-polar solvent has advantages of firstly, removing other more polar hydroxylated steroids and steroid conjugates, giving the method greater specificity. Secondly, the process of extraction dissociates progesterone from the binding with CBG and therefore removes the potential interference of binding proteins in the immunoassay. Thirdly, the influence of other non-specific interference in the plasma sample (termed "matrix effects") is also reduced.

Haynes et al. (1980) established a direct assay for plasma progesterone which utilised cortisol to displace progesterone from binding to CBG. Charcoal-stripped serum was used as a source of progesterone free matrix to make up assay standards. Although this method showed good agreement with an extraction method, not all direct assays gave such good agreement when compared with extraction assay results. Positive bias due to interference from more polar steroids and metabolites can be a problem, and sometimes calibrator values were adjusted to compensate for this effect.

The recovery shown by samples spiked with a known amount of progesterone is always a good test of assay validity.

7.4.4 [^{125}I] Radioligands

The preparation of [^{125}I] labelled steroid radioligands results in tracers with higher specific activity compared with [^{3}H]-radioligands. However, this increase in specific activity does not always produce assays with greater sensitivity. The main advantage in using [^{125}I] radioligands is the convenience and speed of gamma counting, which can be performed on multi-head gamma counters. By comparison, tritium counting required lengthy counting times in a beta scintillation counter (typically overnight to count an assay) and involved the use and disposal of large volumes of scintillation fluid.

When a radiolabelled steroid cannot be purchased it is possible to prepare iodinated tracers in-house, but this requires skilled staff and special facilities.

There are two widely-used methods for the production of [^{125}I] labelled steroid tracers. The first method involves linking a steroid derivative such as a carboxymethyl oxime to tyrosine methyl ester. The resulting conjugate can then be iodinated in microgram amounts and the product is purified. This approach was first described by Oliver et al. (1968) for a digoxin assay, and was adapted to steroid iodination by Warren and Fotherby (1975). The method does have drawbacks, including instability of the tracer and the formation of di-iodo derivatives with reduced immunoreactivity.

The most convenient and widely used steroid iodination method is the two-step synthesis first described by Hunter et al. (1975). Firstly, histamine is iodinated and then the radiolabelled iodohistamine is linked to the steroid oxime or hemisuccinate derivative. The steroid tracer is purified by thin layer chromatography on silica gel plates.

Although the use of iodinated steroid tracers was a great advance in assay technology, they did not always perform as expected in immunoassay systems. A key factor in the development of an assay is the choice of antiserum in terms of the site of derivatisation of the steroid used to produce the hapten for immunisation, and the steroid derivative used to produce the tracer. In some assay systems, higher affinity of the antiserum for a tracer linked through the same position as that used for antiserum production (termed a "homologous" assay system) leads to a loss of sensitivity (the "bridge effect"). This increased affinity for the tracer makes it more difficult for the non-derivatised antigen to compete for binding. Cameron et al. (1975) reported a bridge effect for a progesterone assay which used an antiserum raised to a progesterone-11α-hemisuccinate-BSA conjugate and a progesterone-11α-hemisuccinyl-[^{125}I]-histamine tracer.

Nevertheless, there are no hard and fast rules for antiserum/tracer combinations to produce sensitive steroid assays. Some steroid antisera give acceptable assays using homologous systems – for example, some antisera raised against steroid-3-conjugates and using 3-linked iodohistamine tracers do not show bridge effect problems. This type of assay format has been successful for cortisol, testosterone and norgestrel. For progesterone however, the 3/3 system resulted in extensive cross-reactivity with pregnenolone and 5α- and 5β-reduced metabolites and was not acceptable in terms of assay specificity.

A more recent study by Karir et al. (2006) examined the influence of structural modifications in the [^{125}I] tracer on progesterone assays. Assays using a heterologous bridge radioligand (progesterone 11α-hemiphthalate-[^{125}I]-tyrosine methyl ester with an antiserum against progesterone-11α-hemisuccinate – BSA), or a heterologous site radioligand (progesterone-3-(*o*-carboxymethyl) oxime-[^{125}I]-histamine with an antiserum against progesterone-11α-hemisuccinate-BSA) gave the most sensitive displacement curves compared with homologous 11α-hemisuccinate or 3-CMO tracer and antibody pairings.

The method described by Corrie et al. (1981) represented a high point for a routine progesterone radioimmunoassay. Use of [^{125}I]-labelled progesterone 11α-glucuronide-tyramine conjugate gave steep and sensitive dose response curves with several progesterone-11α-hemisuccinate antisera, with specificities very similar to those obtained with a tritium tracer. This method, which employed hexane extraction, was modified by Ratcliffe et al. (1982) to a direct assay system with improved precision. Standards were prepared in pooled male serum, and either danazol at pH 7.4, or 8-anilino-1-napthalene sulphonic acid (ANS) at pH 4.0 was used to displace progesterone from binding proteins.

7.5 Non-Radioactive Labels for Progesterone Immunoassay

7.5.1 Enzyme Immunoassay

Enzymes have potential advantages as labels in immunoassays since they avoid the hazard of radioactivity and produce a coloured endpoint which can be measured on

comparatively simple and widely available equipment. Their use in manual assays has been slow to be taken up by routine laboratories however. Extra steps required for signal generation compared to [^{125}I] labels, a limited assay working range because of maximum OD readings of 2.0 on most plate readers or spectrophotometers, and the use of carcinogenic substrates (for example, 0-phenylenediamine with horseradish peroxidise) have all contributed to the lack of widespread adoption of enzyme labels.

Early serum progesterone enzyme immunoassays (EIAs) were less sensitive than corresponding RIAs (Dray et al., 1975). This was due in part to the use of steroid-enzyme conjugates with high average molar incorporation ratios (MIRs) of up to 20, which produced less sensitive displacement curves. Theoretically, the use of steroid-enzyme labels with an MIR of 1 should provide the most sensitive EIA, although in practice, average MIRs a little higher than this are used.

Methods for the preparation of hapten-enzyme conjugates have been reviewed by Gosling (1997) and by Dent (2001).

Steroid-enzyme conjugates can be prepared using the mixed anhydride reaction of Erlanger et al. (1957), as modified by Dawson et al. (1978) with the use of dimethyformamide as co-solvent and *N*-methyl morpholine as the acid acceptor. The product is dialysed and then chromatographed on Sephadex G-25 to remove excess steroid. Alternatively, steroid-enzyme conjugates can be synthesised by the "active ester" procedure. This uses carbodiimide to link the steroid oxime or hemisuccinate to *N*-hydroxysuccinimide, and the *N*-hydroxysuccinimide ester is then reacted with the enzyme (Hosada et al., 1985).

For tube-based EIA's the antiserum can be coated directly onto polystyrene tubes, or it can be incorporated into a solid phase such as cellulose, Sepharose or polystyrene beads. The great majority of commercial EIA kits for serum progesterone now use microtitre plates with the antiserum immobilised onto the microtitre wells.

Optimisation of an EIA involves using antibody concentrations which are varied around the concentration used in the corresponding RIA, and performing a series of "hill-climbing" studies to determine the optimum enzyme label concentration, progesterone standard range and incubation time.

Enhanced assay sensitivity and speed for EIAs can be achieved by the use of enzyme amplification techniques to enhance the signal generated by an alkaline phosphatase enzyme label (Self, 1985). This principle has been used to develop an assay for progesterone in milk (Stanley et al., 1986).

A further advance in EIA technology was demonstrated with the development of the "OVEIA" test by Boots-Celltech, Ltd. (Baker et al., 1984). This method was a dual-analyte colorimetric EIA which measured the oestradiol-3-glucuronide/pregnanediol-3-glucuronide ratio in urine to determine the fertile period of the ovulatory cycle. Urine samples were incubated with oestrone-3-glucuronide antiserum and a mixed steroid antigen (oestrone-3-glucuronide chemically coupled to pregnanediol-3-glucuronide) in microtitre plate wells coated with anti-pregnanediol-3-glucuronide antiserum. Following a 1 h incubation, wells were washed and an enzyme-conjugated monoclonal antibody to oestrone-3-glucuronide was added. Wells were incubated for 20 min, washed, enzyme substrate added and absorbance at 620 nm was determined after a further 20 min. Peak ratios dropped sharply with

significant increases in plasma LH prior to ovulation, and results correlated with follicular phase plasma oestradiol and with ovarian follicular volume (Anthony et al., 1988).

EIA assay formats for progesterone continue to be updated and improved. One example of this is the progesterone method developed recently by Basu et al. (2006), who studied six different combinations for immunogens used to produce antibodies and enzyme conjugates used as labels. The optimum pairing was the enzyme conjugate 17α-hydroxyprogesterone-3CMO-alkaline phosphatase and an antiserum raised against the immunogen progesterone-3CMO-BSA. This direct assay required no displacement reagent and would detect 0.11 ng/mL (0.35 nmol/L) progesterone. Intra- and inter-assay precisions ranged from 5.1% to 9.6%, and the method showed close correlation with RIA ($r = 0.97$).

The same research group went on to use two different enzymes, alkaline phosphatase (AP) and horse radish peroxidise (HRP) in labels for progesterone and human chorionic gonadotropin (HCG) to develop a dual EIA for these analytes (Basu et al., 2007). Microtitre plate wells were coated with anti-progesterone and anti-HCG antiserum and 750 μL of serum or combined standard were added together with a combined conjugate reagent consisting of 17α-hydroxyprogesterone – AP and HCG-biotin. After incubation and washing, ALP substrate and HRP-streptavidin were added. After 30 min the absorbance at 405 nm was measured to provide progesterone results. Following a further wash, wells were incubated with HRP substrate and H_2O_2 and then the reaction was stopped with H_2SO_4 and the absorbance at 460 nm measured to give the HCG result. Results for this dual assay showed good correlation with the corresponding single analyte assays.

7.5.2 Time-Resolved Fluorescence

The value of conventional fluorescence labels is severely limited by problems of light scattering, background fluorescence and quenching, which can reduce potential sensitivity by a factor of 1,000 (Soini and Hemmila, 1979).

Time-resolved fluorescence using a lanthanide ion such as Europium^{3+} greatly improves the sensitivity of fluorescence measurements, and has been used to develop the DELFIA (dissociation-enhanced lanthanide fluorescence immunoassay) system. The principle of DELFIA methods has been reviewed by Wood and Barnard (1997).

Of all the non-radioactive label immunoassay systems available, DELFIA methods offer the most convenient alternatives to [^{125}I] for the development of in-house steroid assays. In the author's experience, reliable steroid assays can be developed by preparation of a steroid-biotin conjugate and the use of anti-steroid antibody coated onto microtitre plate strip wells. Biotin-steroid conjugates can be synthesised from the steroid oxime or hemisuccinate derivative using *N*-hydroxysuccinimide in the activated ester procedure to link to biotinimidocaproyl hydrazide (Dressendorfer et al., 1992). Conjugates can be purified by thin layer chromatography.

Streptavidin can be labelled in-house using the DELFIA labelling reagent N^1-(*p*-isothiocyanato-benzyl)-diethylene triamine tetra acetic acid-Eu^{3+} which is supplied by Perkin Elmer, Ltd. This provides a "universal" detection system that can be used with all biotin-steroid labels.

DELFIA kits for serum progesterone measurement are available for use in manual or "Auto-DELFIA" assays (Section 7.6).

The Brahms "Kryptor" serum progesterone assay uses time-resolved amplified cryptate emission (TRACE) technology to provide an automated "homogeneous" immunoassay which does not require separation of bound and free label. The anti-progesterone antibody is linked to a europium cryptate complex, and progesterone is labelled with "XL665", an allophycocyanine. When progesterone label is bound to the antibody, energy transfer occurs between the europium cryptate and XL665, which then emits light at a longer wavelength (665 nm) than that of the cryptate emission (620 nm). The ratio of the fluorescence at the two wavelengths is measured by time-resolved fluorescence and provides an index of bound progesterone label which is inversely proportional to the sample progesterone concentration.

7.5.3 Chemiluminescence

Chemiluminescence has been utilised to enhance the detection of a steroid-enzyme label in an EIA, or chemiluminescent steroid conjugates can be used to develop immuno chemiluminescence assays (CIAs). The application of chemiluminescence techniques to immunoassays has been reviewed by Weeks (1997).

An example of the application of chemiluminescence to provide a sensitive detection system for EIA is the use of adamantyl-1,2-dioxetane arylphosphate (AMPPD) which gives a long-lasting chemiluminescence when dephosphorylated by alkaline phosphatase labels. This reaction forms the basis of the "Immulite" analyser systems (DPC, Ltd.; Section 7.6).

Chemiluminescent steroid conjugates have been utilised to develop CIAs which have proved to be viable alternatives to enzyme/colorimetric or time-resolved fluorescence assays. Acridinium ester labels have been used extensively in manual and automated immunoassay systems. Addition of hydrogen peroxide generates an unstable dioxetanone intermediate which dissociates to form the activated 10-methyl acridone. This molecule is free from the steroid residue and protein binding, and so quenching is avoided when light emission occurs. Early CIAs for progesterone were described by Richardson et al. (1985) and also by Miller et al. (1988), who used an assay design incorporating bridge heterology. Acridinium ester chemiluminescence technology forms the basis for the Bayer "Centaur" immunoassay analysers (Section 7.6).

Another variant on the use of chemiluminescence in immunoassay is the development of electrochemiluminescence assays or ECLIAs. This principle is the basis of the Roche "Elecsys" analysers (Section 7.6). Progesterone in the serum sample is incubated with a biotinylated monoclonal anti-progesterone antibody, and ruthenium

complex-labelled progesterone. Streptavidin-coated paramagnetic microparticles are then added to immobilise the antibody. The micoparticles are captured onto an electrode, washed, and then a voltage is applied to induce chemiluminescence. The electrochemically active substances involved are the ruthenium label and tripropylamine (TPA). When the electrical potential is applied the ruthenium label is oxidised and, simultaneously, the TPA is oxidised to a radical cation which reacts with oxidised ruthenium to form an excited state and this then decays with the emission of light.

7.5.4 Surface Plasmon Resonance

Microchip technology has an enormous potential for immunoassays of the future. The principle of surface plasmon resonance (SPR) is based on the internal reflection of monochromatic light by a high refractive index, transparent medium, the outer surface of which is coated with a thin layer (approximately 50 nm) of a suitable material such as gold or silver. When the angle of incidence of the light beam is altered and reflected light is recorded, there is a sudden disappearance of internally reflected light, after which it reappears. This absorption of light energy is due to coupling with electrons in the metal film which sets up resonant oscillations in the "plasmon" or cloud of electrons at the metal surface – hence the term surface plasmon resonance. If other parameters are constant, a change in refractive index at the surface of the metal ions causes a change in the angle of incident light at which SPR occurs. Protein molecules bound at the metal surface perturb the SPR, and small molecules such as steroids can be quantified using displacement techniques with anti-steroid antisera and immobilised steroid-protein conjugates.

The "Biocor"™ biosensor (Pharmacia, Ltd.) has provided a convenient system for developing SPR techniques for progesterone measurement. Using this instrument, Gillis et al. (2002) developed an inhibition immunoassay for milk progesterone in which progesterone was immobilised by covalent linking to the carboxymethyl dextran matrix of the sensor chip. A fixed amount of monoclonal anti-progesterone antibody was then mixed with the sample and injected over the progesterone sensor surface. The SPR signal was dependent on the amount of antibody binding to the progesterone surface, which was inversely proportional to the progesterone concentration in the test sample. The assay had a working range of 0.5–50 ng/mL (1.6–160 nmol/L) with a detection limit of 3.6 ng/mL (11.4 nmol/L) for milk samples. Further studies involving a reduction in the concentration of anti-progesterone antibody improved the assay detection limit to 0.4–0.6 ng/mL (1.3–1.9 nmol/L; Gillis et al., 2006).

Changes in the length of the link between progesterone and ovalbumin in progesterone-ovalbumin conjugates used in an SPR assay were studied by Wu et al. (2002). Increasing the number of carbon atoms in the link from 4 to 11 or 18 resulted in a large increase in antibody binding capacity and assay response for the SPR method. The SPR assay performed equally well when compared to a conventional EIA method, but had advantages of speed and simplicity.

The potential sensitivity of these systems has been demonstrated by Yuan et al. (2007), who have developed a progesterone assay with a detection limit of 4.9 pg/

mL (15.6 pmol/L). This inhibition immunoassay uses SPR together with a progesterone-oligoethylene glycol-ovalbumin conjugate immobilised onto a mixed self-assembly monolayer with 10 nmol/L nanogold particles.

7.6 Automated Immunoassays for Progesterone

Automated progesterone immunoassays are available on several clinical chemistry systems. Examples of the labels and assay principles used are listed in Table 7.1.

Four manufacturers of automated serum progesterone assays employ EIA with fluorescence or chemiluminescence measurements with the remaining four manufacturers divided between chemiluminescence and time resolved fluorescence systems. Detection limits quoted range from 0.2 nmol/L (Kryptor) to 0.8 nmol/L (DELFIA), although often the precision at low progesterone concentrations leaves room for improvement.

7.7 Chromatography/Mass Spectrometry for Progesterone Measurement

7.7.1 Gas–Liquid Chromatography/Mass Spectrometry

Immunoassays for progesterone offer convenient and rapid measurements, but results obtained often show bias and lack of specificity compared with

Table 7.1 Automated immunoassays for serum progesterone

Manufacturer/system	Type	Label system	Detection limit ng/ml (nmol/L)
Abbott "Axsym"	EIA/fluorescence	ALP/4-methyl umbiliferyl phosphate	0.2 (0.6)
Bayer Advia "Centaur"	CIA	Acridinium ester	0.12 (0.4)
Beckman "Access/ DXI"	EIA/ chemiluminescence	ALP/dioxetane phosphate	0.08 (0.25)
Brahms "Kryptor"	TRFIA	Eu^{3+}cryptate/ allophycocyanin	0.06 (0.2)
DPC "Immulite" systems	EIA/ chemiluminescence	ALP/AMPPD	0.1 (0.3)
Perkin-Elmer "Auto-DELFIA"	TRFIA	Eu^{3+}-progesterone	0.25 (0.8)
Roche "Elecsys" systems	ECLIA	Ru^{2+}-progesterone/ tripropylamine	0.15 (0.5)
Tosoh "AIA" systems	EIA/fluorescence	ALP/4-methyl umbiliferyl phosphate	0.1 (0.3)

chromatographic-MS methods. This problem was highlighted by Boudou et al. (2001) who studied 12 different non-isotopic immunoassays for serum progesterone and compared results on 99 human serum samples with those obtained by gas–liquid chromatography/mass spectrometry (GC-MS). In general, immunoassay results showed a wider distribution than GC-MS values, with evidence of calibration bias and lack of specificity. When the influence of antibodies, control and preparation of calibrators, blocking agents and choice of tracer were examined, none of these showed a significant effect on assay performance when studied seperately.

GC-MS is not used routinely to measure plasma progesterone, but GC-MS methods have found extensive application in the determination of progesterone and related steroids in a variety of sample matrices.

Heptafluorobutyryl derivatives were used by Casademont et al. (1996) for the GC-MS analysis of progesterone and 11 other anabolic steroids in calf urine. Urine steroids were extracted with octadecylsilica columns and the extracts purified in two steps using basic alumina and silica solid phase extraction (SPE) cartridges. The purified extracts were derivatised with heptafluorobutyric anhydride and analysed by GC-MS in single ion monitoring (SIM) mode using [3-^{2}H] testosterone as an internal standard.

A method for the analysis of human hair samples for eight steroids including progesterone was developed by Choi and Chung (1999). Following alkaline digestion and liquid–liquid extraction, steroids were converted to mixed pentafluorophenyldimethylsilyl-trimethysilyl (flophemesyl-TMS) derivatives prior to analysis by GC-MS in SIM mode. Detection limits for the eight steroids ranged from 0.02–0.5 ng/g.

An optimised trimethylsilyl derivatisation protocol was used by Song et al. (2003) to analyse 3-keto-4-ene steroids including testosterone, progesterone and androstenedione produced by recombinant human cytochrome P450 1B1. Formation of the TMS ester by the 3-keto group was minimised by performing the silylation reaction at 56°C for 10 min. Steroid-TMS derivatives were then analysed by GC-MS in SIM mode. Within-and between-day CVs were less than 5% and 10%, respectively.

7.7.2 Liquid Chromatography–Tandem Mass Spectrometry

Liquid chromatography–tandem mass spectrometry (LC-MS/MS) is not used widely for routine serum progesterone measurement at present, but such methods offer advantages for rapid and specific quantitation of serum progesterone concentrations without the need for sample derivatisation. The increasing use of LC-MS/MS in routine clinical chemistry laboratories will enable methods for progesterone and other steroids to be established.

An example of the use of this technique is provided by Wu et al. (2000) who have developed a convenient LC-MS/MS method for serum progesterone and norgestrel which used norethisterone as an internal standard. The method employed atmospheric pressure chemical ionisation and multiple reaction monitoring (MRM) mode for quantitation. A transition of *m*/*z* 315/97 was selected for progesterone, and the method could detect 0.2 ng/mL (0.6 nmol/L) progesterone with a linear range of 0.2–50 ng/mL (0.6–160 nmol/L).

Isotope dilution–tandem MS was utilised by Soldin et al. (2005) to measure progesterone and seven other steroids in plasma during pregnancy and 1 year post-partum. The method used deuterium-labelled internal standards and atmospheric pressure ionisation.

Analysis of animal muscle tissue for ten anabolic steroids including progesterone has been reported by Xu et al. (2006). Following enzymolysis of tissue samples, steroids were extracted with *t*-butyl methyl ether and purified by reverse solid phase extraction. Extracts were then measured by LC-MS/MS in MRM mode. Detection limits for the ten steroids ranged from 0.12–0.54 μg/kg.

A candidate reference method for the determination of progesterone in human serum by isotope dilution LC-MS/MS has been proposed by Tai et al. (2006). Internal standard ([2-^{13}C] progesterone) was added to the sample which was then purified using liquid–liquid extraction prior to reverse phase LC-MS/MS. The method was validated using a lyophilised human serum reference material for progesterone (Certified Reference Material (CRM) 347) with a certified value determined by a GC-MS reference method. Recovery of progesterone from female serum samples ranged from 100.1% to 100.9%, and between-run CV's of 0.3–0.5% were achieved. The detection limit, corresponding to a signal-to-noise ratio of 3, was 1.8 pg progesterone.

7.8 Plasma Free Progesterone

Approximately 2% of progesterone in plasma is unbound or "free", and this is in equilibrium with progesterone bound with high avidity to CBG, and with a proportion bound with low avidity to albumin.

It is widely accepted that it is the free steroid concentration in the circulation that is responsible for the biological activity of steroids. However, the role of protein-bound steroids remains controversial (Pardridge, 1981). In his review on the measurement of free hormones in blood, Ekins (1990) proposed that the steroid binding proteins have a role in determining the tissue-specific distribution of steroids. Tissues with extensive capillary beds such as liver and placenta may be able to extract more of the protein bound steroid fraction. In addition, the pattern of hormone transport within the body may be altered by changes in binding protein concentration which accompanies a specific physiological stimulus.

The earliest methods for the determination of free progesterone in plasma used the principle of equilibrium dialysis. An example is the method reported by Yannone et al. (1969). Plasma samples were diluted in saline, equilibrated with [^{3}H] progesterone and then dialysed against saline at 37°C. Aliquots of the fractions inside and outside the dialysis bag were then taken for liquid scintillation counting. The percentage bound was calculated by the formula:

$$\%\,\text{Steroid Bound} = 100[1-(\text{dpm outside/dpm inside} \times \text{volume inside/volume outside})]$$

These methods were inaccurate since no correction was made for the sample dilution, the possible effects of dialysis against a hypotonic solution, or the possibility of impurities in the [^{3}H] progesterone tracer.

Steady state gel filtration has been applied to the estimation of plasma free progesterone concentrations (Greenstein et al., 1977; Anderson et al., 1985). Plasma aliquots were equilibrated with radiolabelled progesterone and applied to a small Sephadex G-50 column maintained at 37°C in a water bath. The ratio of radioactivity in the first (protein bound) to the second (free progesterone) peaks was used to calculate the percentage of free progesterone in the sample. Optimisation of the column length was essential since it needed to be long enough to resolve the two fractions while minimising the risk of re-equilibration of the bound fraction which would increase with increasing column length.

The use of filtration through cellulose ester filters to absorb free progesterone has been described by Batra et al. (1976). Their results were high compared with those reported by other groups, and were probably compromised by re-equilibration of the bound fraction during the procedure.

Willcox et al. (1985) applied rate dialysis to determine free and protein-bound progesterone using two half cells separated by a dialysis membrane. Each side was filled with an aliquot of the sample, and radiolabelled progesterone was added to one side. Calculation of the free progesterone concentration assumed that transfer across the membrane was a first order rate process, although in practice calibration of the cells using solutions of known free steroid concentrations was necessary.

Centrifugal ultrafiltration-dialysis as described by Hammond et al. (1980) allows the estimation of free progesterone to be made under conditions that are more similar to those which exist *in-vivo*. The method used [^{3}H] progesterone and [^{14}C] glucose tracers which were equilibrated with the sample, and this was then centrifuged for 1 h at 37°C and 3,000 g in a small dialysis cell made from 10 mm glass tubing with a dialysis membrane stretched across one end. The results obtained by Hammond et al. are in good agreement with those obtained by steady state gel filtration (Greenstein et al., 1977; Anderson et al., 1985), rate dialysis (Willcox et al., 1985) and more rigorous equilibrium dialysis (Rosenthal et al., 1969) (Table 7.2) with a consensus value of approximately 2% for plasma free progesterone.

Methods using radiolabelled progesterone are susceptible to large errors caused by slight impurities in the tracer. If progesterone is 98% bound in plasma, a 1% impurity in the tracer that does not bind to CBG would cause a 50% error in the result.

Table 7.2 Plasma free progesterone estimates

Authors	Estimate (% free)	Method
Anderson et al. (1985)	2.32 ± 0.4	Steady state gel filtration
Greenstein et al. (1977)	1.99 ± 0.11	Steady state gel filtration
Hammond et al. (1980)	2.64 ± 0.11	Centrifugal ultrafiltration
Rosenthal et al. (1969)	1.8	Equilibrium dialysis
Yannone et al. (1989)	6.5–10	Equilibrium dialysis
Batra et al. (1976)	6–13	Filtration disc device
Willcox et al. (1985)	2–3	Rate dialysis

Methods of choice for plasma free progesterone would be the use of ultrafiltration or gel filtration techniques with direct measurement of free progesterone concentrations using a sensitive immunoassay. This would avoid the problems caused by minor impurities in the radiolabelled steroid.

It is clear that there are major difficulties in establishing valid methods for plasma free progesterone measurements, making them unsuitable for routine use. Where plasma free progesterone has been studied, there is no evidence that its measurement to assess ovulation offers any advantages over plasma total progesterone. Minassian and Wu (1993), for example, studied free and protein bound progesterone during normal and luteal phase defective cycles. Plasma free progesterone correlated with total progesterone in both the follicular and luteal phases, with no changes in percentage free progesterone throughout the cycle. Although both total and free progesterones were lower in luteal phase defective cycles the percentage free progesterone did not change, and the data did not support any association between luteal phase defective cycles and changes in free, biologically active progesterone.

In practice, the requirements for assay sensitivity and specificity for measurement of plasma free progesterone concentrations are the same as those needed for salivary progesterone assays. For this reason, recent studies have relied on salivary progesterone measurements as an indirect but more convenient assessment of plasma free progesterone.

7.9 Salivary Progesterone

Unconjugated steroids present in the free form in plasma diffuse freely through the acinar cells of the salivary glands, and so salivary concentrations are independent of salivary flow rate (Vining et al., 1983). This contrasts with the situation for conjugated steroids such as dehydroepiandrosterone sulphate (DHAS) which enters the saliva via ultrafiltration through the tight junctions between acinar cells, making their concentrations in saliva very dependent on salivary flow rate.

Salivary unconjugated steroid concentrations can provide a useful reflection of plasma free concentrations, although results in plasma and saliva may differ because of steroid metabolism by the salivary glands. In the case of progesterone,

the salivary glands, particularly the sub-mandibular glands, can metabolise progesterone to 20α-hydroxy-4-pregnen-3-one. However, the extent of this metabolism is minor and it is not considered to be an important source of error in salivary progesterone measurements (Blom et al., 1993).

Salivary progesterone concentrations in women are approximately 30–80 pg/mL (95–250 pmol/L) in the follicular phase and 130–450 pg/mL (400–1,400 pmol/L) in the luteal phase. Post-menopausal women have levels of <50 pg/mL (<160 pmol/L) and men have values of <60 pg/mL (<190 pmol/L). Therefore progesterone assays used for the analysis of saliva need to be 50–100 times more sensitive than plasma assays to measure low salivary concentrations reliably.

Early salivary progesterone assays used RIA with solvent extraction and a tritiated progesterone label (Walker et al., 1979). Since saliva has much lower levels of protein than plasma, with very low concentrations of specific binding proteins, direct, non-extraction RIAs have been employed successfully (Lenton et al., 1988; Vienravi et al., 1994; Lu et al., 1997). Several direct non-radioisotopic immunoassays for salivary progesterone have been reported, including EIAs (Tallon et al., 1984; O'Rorke et al., 1994) and CIA (De Boever et al., 1986). Salivary progesterone assay kits are now available commercially; these use microtitre plate technology with either EIA methods (American Laboratory Products Co, IDS, Ltd., Salimetrics, Inc.) or CIA reagents (IBL Hamburg).

Gas chromatography/mass spectrometry has been applied successfully to salivary progesterone measurements. For example, Leith et al. (1986) used selective ion monitoring of the 3-enol heptafluorobutyrate derivative with [7,7-^{2}H]-progesterone as the internal standard. The method would detect 10 pg/mL (32 pmol/L) progesterone, and results on samples from adolescent girls showed good correlation with RIA.

Salivary samples may be collected into microfuge tubes or "Salivettes" (Sarstedt, Ltd.). However, Salivettes with a cotton wad insert should not be used for salivary progesterone measurement since interference from plant sterols in the cotton causes spuriously high results (Shirtcliff et al., 2001). Salivary samples should not be collected within 30 min of eating or brushing the teeth to avoid possible increases due to micro-injury to the oral cavity and contamination of the sample with blood (Kivlighan et al., 2005). Progesterone in saliva is stable for 3–4 days at room temperature, for 1–2 weeks at 4°C, and indefinitely at −20°C. Repeated freezing and thawing for five cycles has been reported to reduce the progesterone concentration by only 5% (Groschl et al., 2001a). In the author's experience it is essential to freeze and thaw saliva samples and to centrifuge them prior to analysis to ensure valid recovery of salivary steroids.

Sequential sampling of salivary progesterone during the menstrual cycle has been used extensively in research studies as a non-invasive alternative to plasma measurements. This has been particularly useful for the study of girls during and after menarche, where there are major ethical objections to blood sampling (Read et al., 1984; Groschl et al., 2001b).

Despite the wealth of literature on sequential salivary progesterone measurements in the study of the menstrual cycle, they are not used in routine diagnostic laboratories

to check for ovulation or to assess luteal function. The main problem is that salivary progesterone results show much greater variation over a 24 h period than those shown by plasma concentrations. In a detailed study, Delfs et al. (1994) looked at 24 h profiles of salivary progesterone and concluded that a single plasma progesterone determination reflected 24 h progesterone secretion more accurately than repeated salivary samples measured in the same individual. Similarly, one major manufacturer of salivary progesterone kits comments in the literature on salivary progesterone that "single samples in most cases will give arbitrary results which are difficult to reproduce". They suggest taking five samples over a period of 2 h, and then analysing a pool made from the five samples, thus saving analysis costs. It is understandable therefore, faced with this rather cumbersome strategy, that routine diagnostic laboratories continue to measure plasma progesterone.

Another area where salivary progesterone measurements have provided interesting data is in the study of the effects of topically applied progesterone creams, which have been claimed to reverse post-menopausal osteoporosis (Lee, 1990). Although salivary progesterone concentrations showed a fivefold increase in response to the application of progesterone cream, plasma concentrations showed little or no increase (Cooper et al., 1998; Carey et al., 2000; O'Leary et al., 2000). The plasma concentrations of progesterone achieved would not be sufficient to prevent osteoporosis, and the clinical value of progesterone cream continues to be controversial.

7.10 Pregnenolone and Metabolites of Progesterone in Plasma and Urine

7.10.1 Pregnenolone

Measurement of plasma pregnenolone, the precursor of progesterone, has applications in the study of progestagen increases during pregnancy and in the investigation of adrenal enzyme deficiencies in congenital adrenal hyperplasia. Together with dehydroepiandrosterone and dehydroepiandrosterone sulphate, pregnenolone and pregnenolone sulphate were the first "neurosteroids" to be identified in rat brain (Corpechot et al., 1983) where they act as a counterbalance to anxiety-inducing steroids such as allopregnanolone (Section 7.11). Decreased plasma pregnenolone concentrations have been reported in patients with schizophrenia (Ritsner et al., 2007), and subjects with social phobia (Heydari and Melledo, 2002).

Methods used to assay plasma pregnenolone incude RIA, a combination of chromatography with RIA, and tandem mass spectrometry.

In their study of plasma pregnenolone and progesterone metabolite increases during pregnancy, Murphy and Allison (2000) utilised a pregnenolone RIA which did not cross-react with dihydro- and tetrahydro-progesterone metabolites. Plasma pregnenolone concentrations rose from mean basal values of 1.9 nmol/L (follicular phase) and 4.2 nmol/L (luteal phase) to 14.7 and 42.9 nmol/L in the second and third trimester of pregnancy (Murphy et al., 2001).

A combination of chromatography and RIA was used by Riepe et al. (2001), who extracted plasma with dichloromethane and separated pregnenolone from cross-reacting steroids using automated Sephadex LH-20 multi-column chromatography. Pregnenolone was then measured by RIA using an antiserum raised against pregnenolone-20-CMO-BSA, giving a detection limit of 0.15 nmol/L.

Solid phase extraction with derivatisation of extracts to form oximes was employed by Kushnir et al. (2006). Samples were then analysed by tandem mass spectrometry with electrospray ionisation in positive mode and multiple rate monitoring acquisition. The method showed within- and between-run imprecisions of <7.1% and <11% respectively.

7.10.2 5α and 5β Pregnanediones (5α and 5β Dihydroprogesterones) and Pregnanolones (Tetrahydroprogesterones)

This group of reduced progesterone metabolites has an important role in pregnancy and parturition, and also has functions as neurosteroids (Section 7.11).

Plasma concentrations of these steroids increase during pregnancy and fall following delivery, the largest rise being shown by 5α-pregnanedione (5α-dihydroprogesterone) which increases approximately 20-fold compared with normal luteal phase levels. Plasma 5β-pregnanedione (5β-dihydroprogesterone) and 3β,5β-, 3α,5α-, and 3β, 5α-pregnanolones (tetrahydroprogesterones) increase by 5- to 10-fold by 37 weeks gestation (Murphy et al., 2001). There was evidence that patients who developed depression during pregnancy had higher concentrations of progesterone metabolites, with significant increases in 5α-pregnanedione at 27 and 37 weeks gestation.

High pregnancy levels of 5α-pregnanedione cannot be accounted for totally by the metabolism of maternal plasma progesterone, and may be supplemented by placental conversion of 5α-pregnan-3α and -3β-ol-20 ones (5α, 3α- and 5α, 3β-tetrahydroprogesterones) produced by the foetus (Dombroski et al., 1997).

In addition to its roles in pregnancy and as a neurosteroid, 5α-pregnanedione also has been implicated in the promotion of breast neoplasia and metastasis (Weiler and Wiebe, 2000).

Studies of the *in-vitro* effect of progesterone and its reduced metabolites on myometrium in pregnancy have demonstrated that 5β-reduced metabolites in particular had a greater potency than progesterone in eliciting a relaxation effect (Perusquia and Jasso-Kamel, 2001). A fall in 5β-pregnanedione may play a role in the onset of spontaneous labour, since concentrations showed a twofold fall in association with labour, parallel to decreased expression of 5β-reductase mRNA (Sheehan et al., 2005).

Plasma allopregnanolone and pregnanolone concentrations increase in the luteal phase of the menstrual cycle as a result of production by the corpus luteum (Ottander et al., 2005), and increase during pregnancy (Murphy et al., 2001).

Changes in plasma levels of pregnanolones (tetrahydroprogesterones) have been described in patients with obesity (Predieri et al., 2007), anorexia and bulimia

nervosa (Monteleone et al., 2001), chronic fatigue syndrome (Murphy et al., 2004), depression (Hardoy et al., 2006), post-partum "blues" (Nappi et al., 2001), premature ovarian failure (Bernardi et al., 1998) and children with precocious pubarche (Iughetti et al., 2005).

Methods for the measurement of pregnanediones and pregnanolones include the use of specific RIA's, HPLC separation followed by RIA using broad cross-reactivity antisera, and GC-MS.

Zwirner et al. (1983) assayed 5α-pregnane dione in plasma using ether extraction and a simple column chromatographic separation followed by RIA with an antiserum showing only 1.8% cross-reactivity with 5β-pregnane dione.

Measurement of plasma reduced metabolites of progesterone by HPLC separation followed by RIA with a broad specificity antiserum has been described by Murphy and Allison (2000). Plasma (100 μL in late pregnancy) was spiked with [^{3}H] progesterone and extracted with toluene, the dried extract taken up in dichloromethane, and steroids separated by HPLC. The HPLC eluate was split, one third was taken for determination of recovery and two thirds were taken into 50 assay tubes for RIA using an anti-progesterone antiserum that cross-reacted with five dihydro- and tetrahydro-metabolites. CV's ranged from <11% for metabolite concentrations over 40 ng/mL to less than 20% for levels below 0.5 ng/mL. Detection limits ranged from 7 to 33 pg, with values of around 20 pg for most metabolites.

Gas chromatography with electron capture, negative chemical ionisation and mass spectrometry was employed by Gilbert Evans et al. (2005) to monitor pregnane diones and pregnanolones during pregnancy and the post-partum period.

A detailed study of the influence of gender, age and endocrine stimulation and suppression tests on circulating allopregnanolone levels was undertaken by Genazzani et al. (1998). Plasma samples were extracted with ether and the dried extracts dissolved in assay buffer and applied to a C18 "Sep-Pak" cartridge prior to elution with methanol. Dried extracts were then analysed by RIA with a detection limit of 15–20 pg/tube, and an inter-assay CV of 9.1%. Men showed an age-related decrease in plasma allopregnanolone, whereas levels in women were independent of age until the menopause, when concentrations fell. Dexamethasone suppression and GnRH and ACTH stimulation tests indicated that both the ovaries and adrenal were sources of allopregnanolone in pre-menopausal women.

7.10.3 Pregnanediol

Pregnanediol (5β-pregnane-3α,20α diol) is the major metabolic reduction product of progesterone. It is excreted as the glucuronide conjugate in relatively large amounts (10–40 mg/24 h) during pregnancy, and in smaller amounts in the luteal phase of the menstrual cycle. Lower but measurable urine pregnanediol glucuronide (PG) concentrations are found in men and in women in the follicular phase of the cycle or post-menopause.

Low concentrations of pregnanediol stereoisomers 5α-pregnane-3α,20α diol (allopregnanediol) and 5α-pregnane-3β,20α diol have been identified in pregnancy urine and ovarian vein plasma (Kalliala et al., 1970), but they are excreted in low amounts, and their significance is unclear.

Urinary PG measurements have been used extensively for the non-invasive assessment of the menstrual cycle luteal phase, and a wide variety of chromatographic and immunoassay methods have been developed.

Early RIAs were able to analyse PG directly in diluted urine, and relied on the use of tritiated label, liquid phase incubation and separation with dextran-coated charcoal (Collins et al., 1979). These assays were followed by EIAs using colorimetry (Mitsuma et al., 1989), or fluorimetric measurement (Shah et al., 1984), chemiluminescence immunoassays (Eshhar et al., 1981; Magini et al., 1989) and time-resolved fluorescence immunoassay (Kesner et al., 1994).

In general, PG results using these methods showed good correlation with serum progesterone levels. Results for early morning urine samples were found to correlate well with 24 h urine excretion (Samarajeewa et al.,1983), making a simple non-invasive assessment of corpus luteum function possible.

Novel simultaneous time-resolved fluorescence immunoassays for PG and oestrone glucuronide (EG) have been described by Barnard and Kohen (1998). The two assays are performed in the same microtitre well, and utilise two different immunoassay strategies. The EG method uses an anti-idiotype antibody as a surrogate antigen, and this is labelled with samarium. The PG method is a conventional competitive immunoassay using europium-labelled PG as the label. 10 μL of undiluted urine or mixed standard was added to the streptavidin-coated wells of a microtitre plate. 100 μL of assay buffer containing the two labels were added, followed by 100 μL of assay buffer containing the two biotinylated monoclonal anti-steroid glucuronide antibodies. After a 1 h incubation the plate was washed, enhancement solution added, and the europium and samarium labels were counted using a multiple-wavelength time-resolved fluorimeter (Perkin–Elmer "Victor" multi-label counter). CV's of 5–8% were achieved across the relevant working ranges for each analyte.

Early gas chromatographic methods for PG determination required a preliminary hydrolysis step followed by solvent extraction of the free pregnanediol, making them time-consuming and inconvenient. Shackleton et al. (1983) avoided this problem by the use of mass spectrometry with fast atom bombardment (FAB) and negative ion ionisation. PG is a highly polar molecule, and is not ionised by traditional techniques such as electron impact ionisation. The FAB ionisation technique in negative ion mode produces $[M–H]^+$ ions almost exclusively without co-production of fragment ions, giving a high detection sensitivity. Moneti et al. (1985), adapted this technique to provide a practical and reliable method for routine PG analysis, and demonstrated good agreement with results from a conventional GLC method and a chemiluminescence immunoassay.

7.11 Progesterone and Its Metabolites in the Brain and Peripheral Nervous System

7.11.1 Introduction

It is now over 25 years since researchers discovered that steroid hormones could be biosynthesised and subsequently metabolised in the brain and various nervous tissues. With the passage of time and a great deal of experimental data being obtained, neurosteroids (as they were called) have been further classified into two groups (Mellon (1994); Baulieu et al. (2001) for a reviews of much of the earlier work): (i) neuroactive and (ii) neuro inactive steroids. The former designation refers to steroids that are synthesised in the brain, where they are active, or in other nervous tissues (PNS). Alternatively, the steroids may be synthesised in 'classical' endocrine organs, then being imported to the brain where they can be active in numerous ways (see Section 7.11.3). A further possibility has been suggested in that cholesterol or cholesterol esters may be imported to the brain and then converted enzymatically to neurosteroids (see below). In contrast to Group (i) above, the neuro-inactive steroids may be formed in brain or PNS but have no biological or other actions there. However, it should be noted that Group (ii) 'Neuro inactive steroids' is not now mentioned as a separate group (Dubrovsky, 2005).

7.11.2 Biosynthesis of Progesterone and Other Neuroactive Steroids in Brain and PNS

Progesterone is now accepted as being formed in the brain from cholesterol and its immediate precursor, pregnenolone, by the enzyme-catalysed reactions described in Section 7.2.2 for progesterone biosynthesis in ovarian tissues. Reviews detailing properties of cytochrome P-450s in the brain are available (Warner and Gustafsson, 1995; Baulieu, 1998; Baulieu et al., 2001; Ebner et al., 2006.). The reductive processes for progesterone (described in Section 7.2.5) are also known to occur in the brain and PNS, 3α-hydroxy-5α-pregn-20-one (3α-pregnanolone or allopregnanolone) and the 3α, 5β-isomer, having especially important effects in the PNS (see below). In the past few years, several groups have studied in detail the progesterone-reducing enzymes, using tissue preparations of hypothalamus of male and female rats. Detailed studies of the enzymes involved and possible changes in activity with sexual activity have been published (Eechaute et al., 1999; Lephart et al., 2001; Gao et al., 2002).

7.11.3 Some Effects of Progesterone and Its Reduced Metabolites on Neurotransmitter Receptors in Brain and PNS

As their name implies, neuroactive steroids (NASs) have the ability to modify neural activities by binding to, and modulating allosterically, the various neural transmitter receptors. Among these are the gamma-aminobutyric acid, type A (GABA(A)), Sigma and *N*-methyl di-aspartate (NMDA) receptor complexes. In addition to these modulation effects, reduced progesterone metabolites passing into the neuron can be oxidised in Ring A, allowing binding to the intra-cellular progesterone receptor, so causing regulation of gene expression.

In this context, Ghoumari et al. (2003) have shown that progesterone markedly stimulates myelination in organotypic slice culture of 7-day-old rat and mouse cerebellum (myelination was evaluated by fluorescence analysis of myelin basic protein [MBP]). The increase in MBP involved the classical progesterone receptor. Significantly, progesterone was metabolised in culture slices to 5α-dihydroprogesterone and 5α-allopregnanolone, the latter metabolite being well-known as a GABA(A) activator. It was suggested by the authors that the stimulation of MBP expression and subsequent increase in cerebellum myelination were caused by the intra-cellular progesterone receptor plus the membrane GABA(A) receptors. For further description of the actions of neuro-transmitter receptors see the reviews by Baulieu (1998), Zinder and Dar (1999), Wang et al. (2000), Czlonkowska et al. (2001), Liu et al. (2002), Laufer et al. (2005), Lambert et al. (2003), Rupprecht (2003), Turkmen et al. (2004), Dubrovsky (2005), Biggio et al. (2006), Maguire and Mody (2007).

As far as NMDA is concerned, the effects on this selective receptor antagonist by 3α-hydroxy-5β-pregnanolone have been studied by Lapchack (2004). NMDA receptors have also been shown to have a role in the neuroactive steroid modulation of GABAergic neurotransmission in the central amygdala (Wang et al., 2007). The PNS is also involved with neuroactive steroids; progesterone is synthesised in Schwann cells and can promote the formation of myelin in the PNS.

7.11.3.1 Selected Examples of Psychopharmocological and Neuro-Psychiatric Effects

The range of effects by progesterone and its di- and tetrahydro-reduced metabolites is now extremely large and, for this reason, only a summary can be given here:

(i) Anti-convulsant effects in immature rats by allopregnanolone (Lonsdale et al., 2006; Mares et al., 2006); effects on seizure susceptibility – a useful potential in the treatment of neuropsychiatric disorders such as epilepsy (Beyenburg et al., 2001)

(ii) Reducing social isolation stresses in rats immediately after weaning (Serra et al., 2007); control of, and importance in, stress in humans (Strous et al., 2006); correlation between regulation of GABA(A) receptors and relevance to

ovarian cycle stress (Maguire and Mody, 2007); modulation of social phobia via involvement of GABA(A) receptors (Laufer et al., 2005)

(iii) Positively useful effects in cases of learning and memory difficulties (Vallee et al., 2001) and in Alzheimer's disease (Marx et al., 2006)

(iv) Inhibition by progesterone treatment of inflammatory agents accompanying traumatic brain damage (Pettrus et al., 2005; Meffre et al., 2007)

(v) Sedation and anaesthetic actions (van Broekhoven et al., 2007); correlations with aggressive behaviour (Pinna et al., 2005), and depression (van Broekhoven and Verkes, 2003)

(vi) Intra-hippocampal pregnanolone administration to rats reduces chronic alcohol consumption (Martin-Garcia et al., 2007)

(vii) Increased myelination in cerebellum and PNS by progesterone, 5α-dihydroprogesterone and 5α-allopregnanolone (Ghoumari et al., 2003)

7.11.4 Identification of Progesterone and Its Reduced Metabolites in Brain Tissues and the PNS

Because of the large number of important effects of this group of steroids, it is clearly necessary to devise suitably specific and sensitive quantitative methods for their estimation.

In Chapter 6, the measurement by HPLC of several steroids, such as DHEA and pregnenolone (and their sulphates) is described. In recent studies several groups have utilised liquid chromatography and electron spray techniques, coupled with tandem mass spectrometry (LC-ESI-MS-MS) for measurement of numerous steroids, including progesterone and isomeric pregnanolones in brain tissues and serum (e.g. Ebner et al., 2006; Higashi et al., 2007a, b). As a result of such measurements, it has been possible to build-up a picture (now realised as being very complex) of steroid biosynthesis and metabolism in rat brain.

7.11.4.1 Steroid Profiling in Brain and Serum

This technique published earlier (e.g. Shackleton et al., 1980) has the advantage that a large number of steroids can be separated from a suitable extract of tissue, blood, urine or saliva. Shackleton et al. (1980) used steroid profiling, (initially with GC and later coupled with MS) to profile steroids in urine samples from healthy individuals as well as from patients with endocrine abnormalities, such as congenital adrenal hyperplasia. Since these earlier days, steroid profiling has been used extensively and has recently been employed to 'scan' numerous neurosteroids in brain tissues and plasma samples.

In 2000, Liere et al. published a validation of what they considered to be an extremely sensitive method for quantifying both free and sulphate-conjugated neurosteroids in rat brain. After separation of free steroids and conjugates by solid

phase extraction of the appropriate tissue preparation, the sulphate fraction was solvolysed. Derivatisation was achieved by treatment with heptabutyric anhydride, separation and simultaneous quantification of a large number of steroids being performed by HPLC, coupled with GC-MS (selected ion monitoring, SIM). Detection limits were 1 pg for DHEA, pregnenolone and their sulphates and, in the context of this chapter, 2 pg for progesterone and 5 pg for 3α,5α-tetrahydroprogesterone. For progesterone, this represents an actual content of 1.95 (mean value) ng/g, 10 mg rat brain being used for analysis. Linearity and accuracy were excellent, with femtomole contents measurable in simultaneous quantification.

In 2006, Ebner et al. published an in-depth study of neurosteroids, their precursors and metabolites in male rat brain. Tissues were homogenised, and free steroids extracted followed by the conjugates (SPE, Oasis cartridge). This latter fraction was subsequently solvolysed. After derivatisation as methyl oximes-trimethyl silyl ethers (MO-TMS ethers) or heptafluorobutyrates (HFB), an initial scan of steroids present in the fractions was performed using GC-MS with SIM; 32 steroids were present in the 'free' fraction, 23 in the sulphate fraction. It was significant that, in the 'free' fraction, corticosterone, 3α,5α-tetrahydrodeoxycorticosterone and DHEA were found, but without any evidence of precursors that would have been expected in endocrine glands. To explain this, the authors suggest that there may be a peripheral source and/or an alternative pathway in the rat brain. In addition, and in the context of this chapter, both pregnenolone and progesterone were identified, together with 3α,5α-tetraphdroprogesterone and dihydroprogesterone. The finding of 3β,11β, 17α,20α-hydroxy derivatives of tetrahydroprogesterone may indicate some inactivation for this potent neuroactive steroid, while the presence of 20α-reduced pregnenolone, progestrone and 5α-dihydroprogesterone may suggest an alternative regulatory pathway for potent activity of tetrahydroprogesterone. Subsequent unequivocal identification and quantification were achieved by 3-ion SIM for the MO-TMS ether derivatives and 2-ion SIM for HFBs.

The usefulness of steroid profiling has also been exploited by the work of Meffre et al. (2007) who used this method to quantify numerous steroids in the brain and plasma of male and pseudopregnant female rats after they had been subjected to traumatic brain injury (TBI) by frontal cortex injury. The rationale for the experiments was twofold: (1) the fact that some circulating steroids are known to be important in recovery from TBI and (2) that pseudopregnant female rats recover better from TBI than do male rats. Therefore, in these experiments, plasma steroid levels were measured to study the effects of pseudopregnancy and TBI at brain regions within, adjacent to and distal to the site of the lesion 6 and 24 h after the injury. Plasma corticosteroid levels were measured and found to be high in all groups consistent with the acute stress caused by TBI. GC-MS profiling showed the presence in brain samples of the following steroids: pregnenolone, progesterone, 5α-dihydroprogesterone and isomeric tetrahydro derivatives. Several C_{19} steroids were quantified, including DHEA, 4-androstenedione, 5α-DHT, plus the C_{18} oestrogen, 17β-oestradiol. Higher levels of progesterone and its reduced metabolites mentioned above were recorded in brain and plasma samples of male rats and pseudopregnant females, compared with sham-operated males. Six hours after TBI,

male brain levels of pregnenolone, progesterone and its 5α-dihydro derivative increased, although testosterone concentrations decreased. However, in pseudopregnant female rat brains, the concentrations of 5α-dihydroprogesterone and 3α,5α-tetrahydroprogesterone increased. As plasma levels of the former metabolite were unaffected by TBI, the authors suggest that a localised activation of the 5α-reduction pathway of progesterone may occur in both male and pseudopregnant female brains. Two other conclusions were drawn from these experiments: (i) the increase in neuroactive steroid concentrations in male rat brains after TBI may be consistent with a neuro protective role and (ii) that the high circulating levels of progestagens may provide protection against TBI.

O'Dell et al. (2004) used GC-MS to measure the concentrations NASs in rat brain frontal cortex and in plasma 30–60 min after administering alcohol (2 g/kg i p) to the animals. The concentrations of pregnenolone, allopregnanolone (3α-hydroxy-5α-pregnan-20-one) and allotetrahydrodeoxycorticosterone (3α,21-dihydroxy-5α-pregnan-20-one) were determined. Thirty minutes after alcohol administration plasma and frontal cortex pregnenolone levels rose to a maximum. In addition, allopregnanolone levels rose to maximal value 60 min after alcohol administration. Thus, the first response appeared to be an increased activity of cholesterol side-chain cleavage to produce the pregnenolone.

In order to establish whether or not the 'classical' endocrine glands could also produce NASs, levels of these steroids and pregnenolone were measured in the frontal cortex and brain of adrenalectomised/gonadectomised rats, 30 min after alcohol administration, exactly as above. No significant increases in concentrations of pregnenolone, allopregnanolone or allotetrahydrodeoxycorticosterone were recorded. Taken together, these results confirm the well-known fact that cholesterol side-chain cleavage occurs in the adrenals/gonads, and also that these glands may be capable of production of NASs. More examples of the use of GC-MS in progesterone and its metabolites in brain are given in Table 7.3.

7.12 Synthetic Progestagens

7.12.1 Alphaxolone (3α-hydroxy-5α-pregnane-11,20-dione) and alphadolone acetate (3α-hydroxy-5α-pregnane-11, 20-dione-21 acetate)

These two neuroactive synthetic progestagens are used in combination as the anaesthetic "Althesin".

An improved gas chromatographic method for the determination of alphaxolone has been described by Sear et al. (1980). The method uses a nitrogen selective alkaline flame ionisation detector. Plasma samples were extracted with light petroleum and extracts derivatised as *O*-methyl oximes and then *O*-methyl oxime-3-acetates. GLC was carried out using a column of 3% OV 17 on Celite 545 (80–100 mesh) at

Table 7.3 Experiments illustrating the use of GC-MS methodology in detecting/quantifying progesterone and its reduced metabolites in rat brain and plasma

Experiment	Steroids Quantified	Extraction:Purification	Derivatisation	GC-MS Method	Quantities Measured; Validation Details	Reference
Detection/ measurement of C-20 steroids in rat brain	Preg. Prog. α-DH Prog., Allopregnan Epiallopregnan	Rat brain, homogenised; SPE (2 cartridges)	Used highly electron affinity reagent-NFMP hydrazine. Sensitivity 20-fold higher than with original steroids	EC-APCI-MS-MS	–	Higashi et al. (2003)
Effects of induced stress on NASs in rat brain	Preg, Prog, 5α-DH prog, allopregnan, epiallopregnan	–	NFMP hydrazine (as above)	EC-APCI-MS	Short term mental stress markedly increased prog and reduced derivs in rat brain	Higashi et al. (2005)
Profiles of free and conjugated steroids in plasma from women of fertile age	Preg, pregnan isomers, Oestradiol (16 women in follicular and luteal phase)	–	–	GC-MS	Sulphates > free steroids by 3:1. Results indicated prog > 5β-DH prog > 3α,5β-pregnan sulphate	Havlikova et al. (2006)

Effects of stress and alcohol administration on brain and plasma NASs	5α-reduced prog; isomeric pregnans	MeOH; acetic acid extraction SPE purification	Utilised permanently-changed HMP (60–150-fold increase in sensitivity relative to free steroids)	LC-ESI-MS-MS	Limits of method: 0.25 ng/g (brain), 0.25 ng/mL plasma; (20 mg brain used, 20 μL, plasma) C.V. were (intra) <8.2 and (inter) 6.0%	Higashi et al. (2007b)

preg., pregnenolone; prog., progesterone; pregnan., pregnanolone; DH prog., dihydroprogesterone; NFMP hydrazine, 2-nitro-4-fluoromethylphenyl hydrazine; HMP, 2-hydrazino-methyl pyrimidine; ECAPCI, electron-capture atmospheric pressure chemical ionization; LC-ESI-MS-MS, liquid chromatography–electron spray ionization-MS-MS; NASs, neuroactive steroids.

an initial temperature of 235°C, increasing by 1° per min. The method showed good precision and improved sensitivity and selectivity compared with a similar method using TMS derivatives.

A rapid and sensitive HPLC assay for alphaxolone and pregnanolone in rat plasma has been reported by Visser et al. (2000). The assay involved addition of the internal standard pregnenolone to the sample, protein precipitation, alkaline derivatisation with dansyl hydrazine and dichloromethane extraction of the steroids. Extracts were then chromatographed using isocratic reverse-phase HPLC on a 3 μm Microsphere C18 column with fluorescence detection using 332 nm excitation and 516 nm emission wavelengths.

7.12.2 Cyproterone Acetate (1,2α-methylene-6-chloro-pregna-4,6-diene-3,20 dione 17-acetate)

Originally developed as a progestagen, cyproterone acetate was found to have anti-androgenic properties. It now has major clinical applications, and acts by competition for the androgen receptor, blocking androgen synthesis and suppressing any compensatory rise in gonadotrophins.

A radioimmunoassay was developed by Nieuweboer and Lubke (1977) at Schering AG which used an antiserum to an 11α-hydroxy cyproterone acetate-11-hemisuccinate conjugate and a tracer prepared by coupling the same hapten to [^{125}I] iodohistamine or [^{3}H] tyrosine methyl ester. Although cross-reactivities of a range of related steroids were documented, serum concentrations for these were not given, making assessment of assay specificity difficult. In a later publication from the same company, Baumann et al. (1996) showed that the RIA gave 20–40% higher results compared to those obtained by GC-MS.

A fully automated LC-MS/MS assay for cyproterone acetate has been described by Christiaens et al. (2004). This utilised in-line solid phase extraction onto a pre-column. Use of a column switching system enabled the elution of the sample in back-flush mode, followed by reverse phase liquid chromatography and tandem mass spectrometry using electrospray ionisation in positive ion mode. Ion species of *m*/*z* 417.4 for cyproterone acetate and *m*/*z* 387.5 for the medroxyprogesterone acetate internal standard were selected to generate daughter ions of *m*/*z* 357.4 and 327.5, respectively. The method detection limit was 300 ng/mL cyproterone acetate.

The advantages of LC-MS for the simultaneous determination of a range of steroids in contraceptives and river water samples were demonstrated in a recent publication by Matejicek and Kuban (2007). A fast and sensitive HPLC-ion trap MS method was developed for ethynylestradiol, gestodene, levonorgestrel, cyproterone acetate and desogestrel, with an on-column detection limit for cyproterone acetate of 4 pg per injection of 1 μL.

7.12.3 Fluorogestone Acetate (11β,17α-dihydroxy-9α-fluoro-pregn 4-ene-3,20-dione17-acetate)

A synthetic progestagen with activity higher than progesterone itself, fluorogestone is applied vaginally to large animals to induce oestrus synchronisation.

A conventional double antibody RIA for fluorogestone was described by Gaston-Parry et al. (1988) using an antiserum raised in rabbits against a fluorogestone-3CMO-ovalbumin conjugate. Ewes treated with fluorogestone acetate (FGA) – impregnated intravaginal sponges showed plasma concentrations of 3.0 μg/L on day 1 and 1.5 μg/L on day 4 of treatment.

More recently Monnoyer et al. (2005) have developed an assay for FGA in sheep plasma using megestrol acetate (MGA) as an internal standard. Following liquid–liquid extraction of plasma (500 μL), extracts were analysed by reverse phase HPLC and detected by MS with a Turbo Ion Spray source using MRM mode. Precursor ions (M + H)(+) at *m/z* 407.2 and 385.1 for FGA and MGA respectively generated product ions at *m/z* 267.1/285.1 for FGA and *m/z* 267.1/224.0 for MGA. The assay working range for FGA was 0.2–5.0 μg/L with a detection limit of 0.2 μg/L.

7.12.4 Gestodene (17α-ethynyl-13-ethyl-17β-hydroxy-gona-4, 15-dien-3-one)

Gestodene is used in combination with ethynylestradiol in combined hormonal contraceptives.

An antiserum raised against gestodene-3CMO-BSA and [9,11-^{3}H]-gestodene tracer were used to develop a conventional RIA by Nieuweboer et al. (1989). The method used diethyl ether extraction and charcoal separation of bound counts, and showed no cross-reactivity with gestodene metabolites or ethynylestradiol.

Analysis of gestodene as one of a panel of progestagens has been described recently by Matejicek and Kuban (2007). This LC-MS method used a reverse phase Zorbax SB-Phenyl column with aqueous methanol as the mobile phase together with ion-trap MS to give a detection limit of 14 pg gestodene.

7.12.5 Medroxyprogesterone Acetate (17α-hydroxy-6α-methyl-pregn-4-ene-3,20-dione 17-acetate)

Medroxyprogesterone acetate (MPA) is used in depot form as a long-acting contraceptive, and in tablet or depot form as a treatment for endometrial cancer. The molecule also has glucocorticoid activity and can cause adrenal axis suppression and a “Cushingoid” syndrome.

A wide variety of analytical methods for MPA are available.

Published immunoassay methods include (1) EIA with chemiluminescence detection for analysis of human plasma (Kim et al., 1995), (2) a time-resolved FIA for the detection of MPA in pork tissues which compares well with LC-MS/MS results (Tieming et al., 2006) and (3) a capillary electrophoresis immunoassay with chemiluminescence detection (Peng et al., 2007).

GC-MS measurement of plasma MPA has been described by Dikkeschei et al. (1985). After addition of medroxyprogesterone propionate internal standard, plasma samples were extracted with C18 cartridges and the extracts derivatised and detected as 3-enol-trifluoroacetyl esters.

HPLC determination of MPA was reported by Uzu et al. (1992), using the fluorogenic reagent 4-(*N*,*N*-dimethylaminosulphonyl)-7-hydrazino-2,1,3-benzoxadiazole together with a peroxyoxalate chemiluminescence detection system.

A simple LC-MS/MS procedure for MPA in human plasma has been described by Kim and Kim (2001). Extraction of plasma samples with pentane, followed by LC-MS/MS in SRM mode allowed the detection of 0.05 ng/mL MPA.

Giannetti et al. (2005) used LC-MS/MS to determine MPA concentrations in pork tissues and serum following C18 solid phase extraction and elution with ethanol.

7.12.6 Mifepristone (17β-hydroxy-11β-[4-dimethylaminophenyl] -17α-[propynyl]-estra-4,9-dien-3-one)("RU486";Roussel Uclaf)

Mifepristone is an anti-progestagen which sensitises the myometrium to prostaglandin-induced contractions and softens and dilates the cervix. It is used in combination with the prostaglandin gemeprost for the termination of first-trimester pregnancy.

Plasma concentrations of mifeprestone are approximately 3 mg/L following a single 600 mg dose, with maximum plasma concentrations 0.7–1.5 h post dose (Lahteenmaki et al., 1987).The pharmacokinetics are non-linear due to binding of the drug to α_1 acid glycoprotein, and a long half-life of 14.5–17.5 h has been reported following a single dose (Brogden et al., 1993).

Mifepristone is metabolised to mono- and di-desmethyl derivatives and hydroxylated metabolites, and this fact has hampered the development of specific immunoassays.

A sensitive HPLC method with a detection limit of 6 ng/mL has been reported by Guo et al. (2006). Plasma samples were extracted using C18 solid phase cartridges, and extracts were then analysed by HPLC using a C18 column and a methanol:acetonitrile:water (50:25:25, v/v/v) mobile phase with UV detection at 302 nm.

7.12.7 Norethisterone (17α-ethynyl-17β-hydroxy-4-estren-3-one)

Norethisterone (NE) is widely used as a contraceptive agent, but it is also prescribed for the treatment of endometrial and breast cancer.

Although several RIAs were developed for norethisterone in the 1970s, they suffered from lack of specificity due to cross-reactivity with A-ring dihydro- and tetrahydro-metabolites. "Bridge" effects similar to those reported for progesterone RIAs were also found with NE assays using homologous 11-hemisuccinate-derived antisera and 11-hemisuccinate [^{125}I] radiolabels.

A direct EIA for NE in plasma and saliva has been developed by Turkes et al. (1982). This homologous EIA, with antiserum and enzyme label both derived from the 11-ligand, had adequate sensitivity, unlike the corresponding RIA using an [^{125}I] ligand.

A GC-MS assay for plasma NE has been published by Pommier et al. (1996). After addition of the testosterone acetate internal standard, plasma samples were extracted with ether:dichloromethane (3:2, v/v) at alkaline pH. The dried extracts were converted to pentafluoropropionyl derivatives and determined by GC-MS using a mass-selective detection at *m*/*z* 486 for NE and *m*/*z* 476 for the testosterone acetate internal standard. The working range of this method was 0.1–10 ng/mL.

7.12.8 Norgestrel/Levonorgestrel

Norgestrel is supplied as a racemic mixture of D and L forms of 13β-ethyl-17 α-ethynyl-17β-hydroxygon-4-en-3-one. Only the D-enantiomer is biologically active, and this is available as levonorgestrel.

Studies on RIA methods for D-norgestrel in the 1970s demonstrated that a homologous system using antiserum and label derived from D-norgestrel-3CMO gave the most specific assays (Cameron et al., 1975).

This same homogeneous assay configuration was used by Munro et al. (1996) to develop an EIA using rabbit polyclonal antiserum to a levonorgestrel-3CMO-BSA conjugate and a levonorgestrel-3CMO-horse radish peroxidise label.

LC-MS/MS methods for levonorgestrel have been published by Wu et al. (2000) and by Theron et al. (2004), and recently a semi-automated 96 well plate method using LC-MS/MS has been reported by Licea-Perez et al. (2007). This last method uses semi-automated liquid-liquid extraction, derivatisation with dansyl chloride and LC-MS/MS analysis using a small particle size column to achieve a run time of 2.7 min, permitting the analysis of up to 300 samples per day.

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Chapter 8
The Measurement of Estrogens

Geoff Holder, Hugh L.J. Makin and H. Leon Bradlow

8.1 Introduction

Biologists use the word 'estrogen' when referring to molecules which have the ability to induce uterine growth or vaginal cornification in the immature or ovariectomized rodent. The word estrogen was derived from two Greek words – *oistros* meaning frenzy and *gennein* – to beget. Chemists and biochemists, however, often restrict their use of this term to molecules that contain a characteristic 18-carbon steroid nucleus with an aromatic (phenolic) A-ring, both those that are biologically active estrogens and those without biologic activity but which are of intrinsic interest, such as the estrogen conjugates. This chapter is concerned only with these steroid compounds. The structure and inter-relationship of some common estrogens are given in Fig. 8.1. In addition to the biological estrogens, there are a wide variety of both natural and synthetic compounds which have estrogenic activity when measured by one or another parameter. While many of the assay procedures described in this review are applicable to these compounds, their application to non C18-steroids will not be discussed here. Methodology for these non-steroidal compounds can be found in reviews by Wang et al. (2002), Wu et al. (2004), Muir (2006), and Delmonte and Rader (2006). While not wishing to downgrade the importance of previous work in the estrogen field, the authors have taken a deliberate decision to exclude most publications prior to 1975, not because these do not

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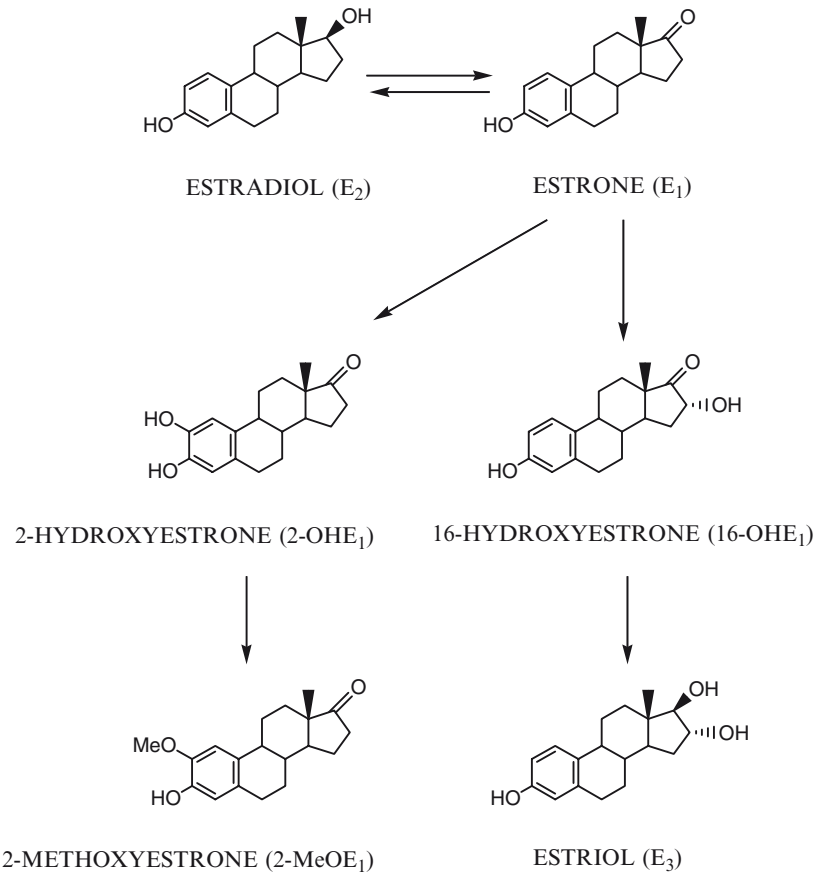


Fig. 8.1 Structures and metabolic inter-relation of some important estrogens. The estradiol molecule illustrated here is 17β-estradiol (estradiol-17β) but is referred to throughout this chapter as estradiol except where it is necessary to draw a distinction between estradiol and its isomer 17α-estradiol

have value but simply because space is not unlimited and readers of the present chapter might be expected to be seeking information about methodology which is less than 30 years old. Readers seeking pre-1975 information in this area can find it in Oakey and Holder (1995).

8.2 Biochemistry and Physiology

Although this chapter is about analysis of estrogens, it is felt that, in common with other chapters, it is appropriate to give a brief outline of the biochemistry and physiology of estrogens. This is not comprehensive but aims simply to outline the biosynthesis, catabolism, mechanism of action and the physiological role of estrogens. Readers in search of more details can refer to reviews on this subject (Gruber et al., 2002; Simpson, 2003).

8.2.1 Biosynthesis of Estrogens

Estrogens are ultimately derived from cholesterol by a biosynthetic sequence, part of which is illustrated in Fig. 8.2. All C18 estrogens are derived from C19 androgens

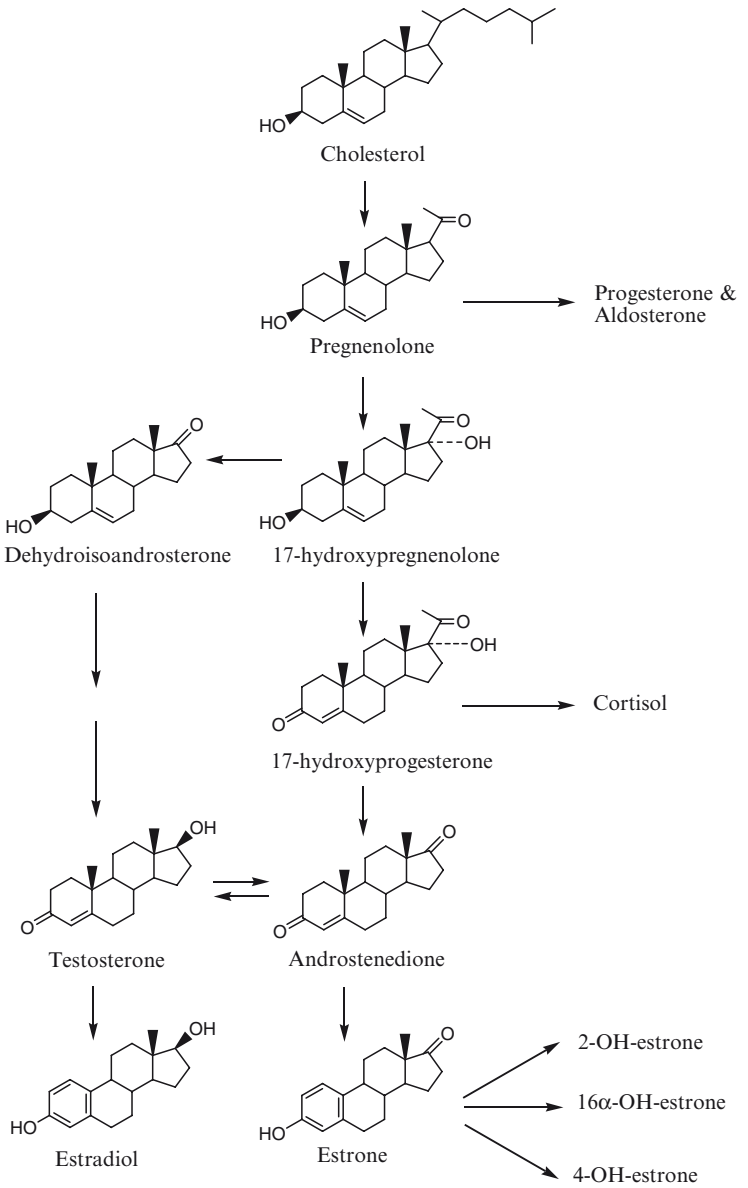


Fig. 8.2 Pathways of estrogen biosynthesis

by aromatization (Simpson et al., 2002), in particular androstenedione and testosterone, to yield estrone and estradiol. The process (Hong et al., 2007) requires a multienzyme cytochrome complex (CYP19) which carries out three hydroxylations, two at C-19 and one unknown, resulting in an aromatic ring A, formic acid and the loss of two molecules of water (Fig. 8.3). During pregnancy, 16α-hydroxydehydroepiandrosterone (16-OH-DHEA) derived from the fetus serves as an additional source

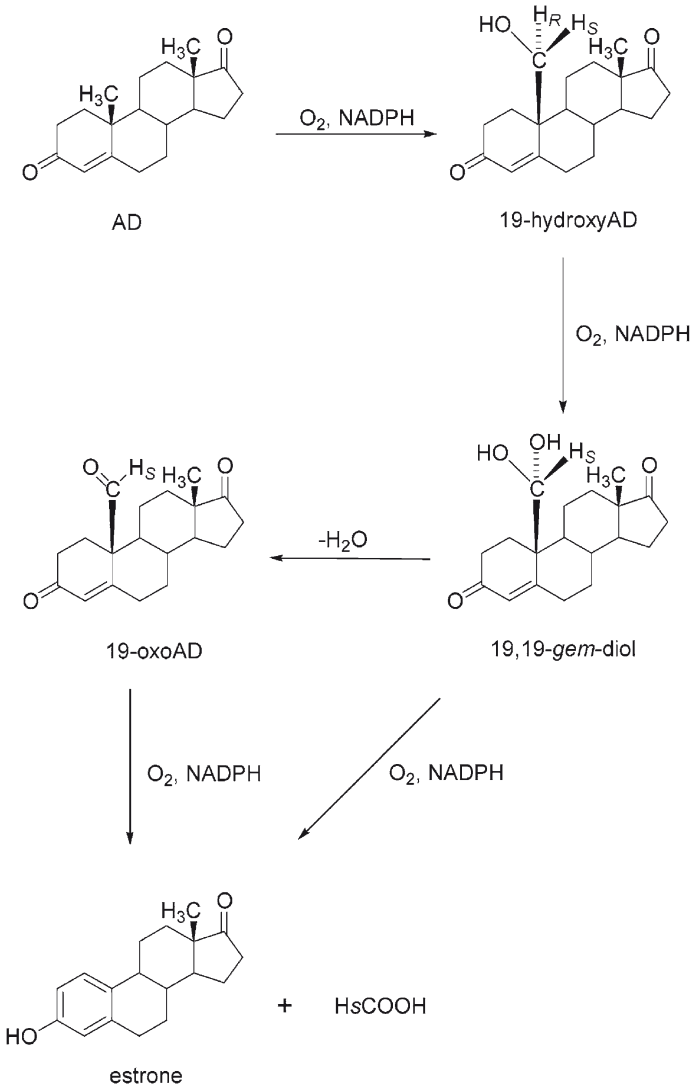


Fig. 8.3 Aromatization of androstenedione (AD) in human placenta (from Numazawa et al., 2005, with permission; copyright 2005 American Chemical Society). A more detailed mechanism of this reaction can be found in Hong et al. (2007)

of estriol. Estradiol has profound effects on the developing brain (McCarthy, 2008; McCarthy et al., 2008), including, paradoxically, *in utero* effects on brain masculinization (Schwarz and McCarthy, 2008). Production and metabolic actions of estrogens were reviewed in 2002 (Gruber et al., 2002).

Synthesis of estrogens in women occurs mainly in the ovaries in the premenopausal state and, in the postmenopausal state, aromatization in peripheral tissues, primarily by the adipose tissue, which plays an increasing role. In men, the gynecomastia which often accompanies obesity is also thought to arise from oestrogens formed in the adipose tissue. Lesser amounts are formed in target tissues such as the breast (Suzuki et al., 2007) and the endometrium (Olson et al., 2007) as well as in the brain. Extra-gonadal local production of estrogens is controlled by tissue-specific promoters of *CYP19*[1] expression and in breast cancer both *CYP19* expression and aromatase activity are increased (Simpson, 2003, Suzuki et al., 2008a, b) suggesting that aromatase in breast cancer is a key regulator of intratumoral estrogen concentration. Aromatase activity has also been demonstrated in other non-steroid-producing tissues (e.g. muscle, adipose and brain and Leydig cells). It has also been suggested that aromatization of testosterone in the brain plays a role in the androgen signaling process (Hahn and Fishman, 1985; Hahn et al., 1985; McEwen and Alves, 1999; Zhao et al., 2005). Trace amounts of estrogen synthesis have been reported in other tissues but do not seem to be biologically significant. Estradiol binds reversibly in the serum to sex hormone-binding globulin (SHBG) and to albumin with around 2–3% of the total being unbound or 'free' (Gruber et al., 2002). While binding to SHBG facilitates transport in the serum of the relatively hydrophobic estradiol molecule and limits the immediate availability to target tissues, SHBG itself has a membrane-mediated regulatory role in conjunction with ER-mediated estrogen response in breast cancer cells, involving cross-talk at the MAP kinase level (Fortunati and Catalano, 2006).

8.2.2 Metabolism of Estrogens

In contrast to the role of reductive mechanisms in the metabolism of androgens, corticosteroids, and progesterone, the metabolism of estradiol is entirely oxidative in nature – oxidation at C-17 and hydroxylation at peripheral sites. As Jellinck and Fishman (1984) have shown, the oxidation is an attack at C-2 either directly or by way of an arene oxide, with no evidence for a reactive intermediate. The most vulnerable peripheral site for hydroxylation is C-2 followed in order by C-1, C-16, C-4 and the least vulnerable sites at C-6, 7 and rarely at C-15 (Jellinck et al., 1984b). Reaction of specifically labeled 2-hydroxyestrone in the presence of charcoal and thiols was reported by Jellinck and Fishman (1984), while similar displacement of

[1]CYP19 in capitals denotes the enzyme whereas *CYP19* in italics denotes the gene – this convention is adopted throughout this chapter.

tritium from specifically-labelled 2-hydroxyestrone in the presence of peroxidase was shown by Jellinck and Bradlow (1990). All of these hydroxylations are catalyzed by specific P450 cytochrome enzymes with great site specificity (see Fig. 8.4). Among the commonest enzymes are CYP1A1 for C-2, CYP1B1 for C-4 and CYP3A4/5 for C-16 (Jellinck and Fishman, 1988). Polymorphic forms of these cytochromes frequently have very different reaction rates from the major form

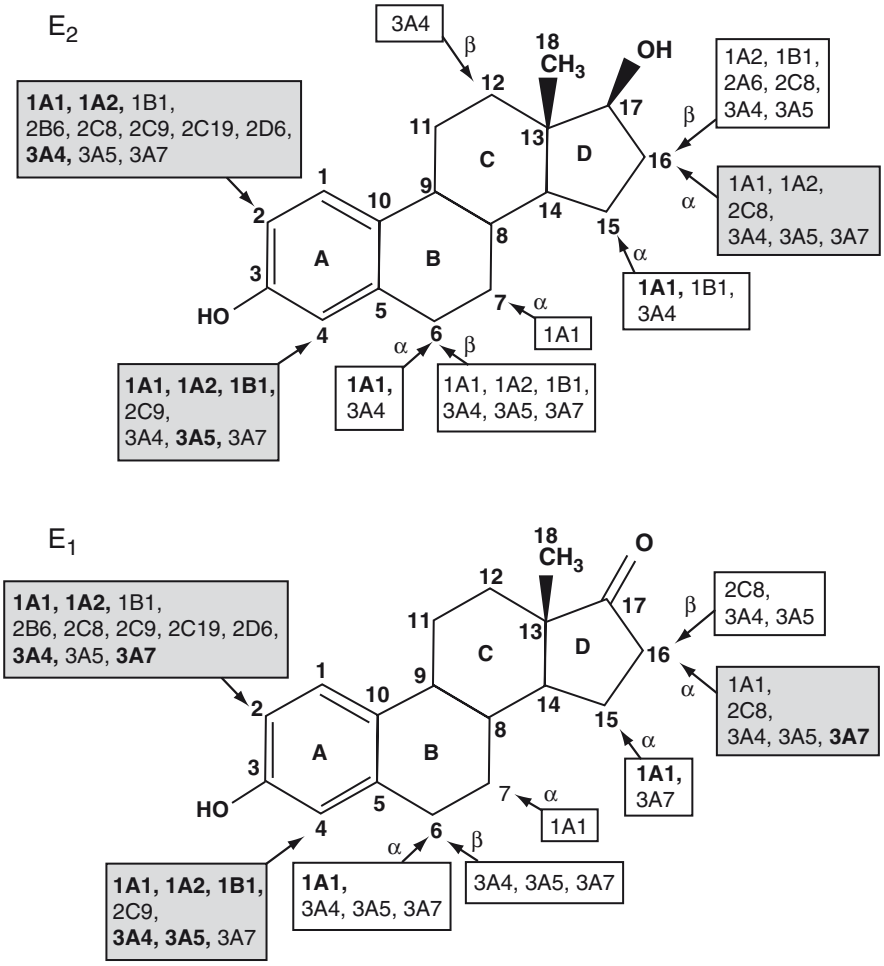


Fig. 8.4 A summary of the region-selective hydroxylation of estradiol (E2) and estrone (E1) by 15 human CYP isoforms. Each isoform was selectively expressed in insect cells transfected with a baculovirus expression system containing cDNA for each of the desired CYP isoforms (Lee et al., 2003). These are all *in vitro* studies and care must be taken before extrapolating to the *in vivo* situation (Lieberman, 2008). The shaded boxes highlight the CYPs catalysing the well-known major metabolic pathways (2-, 4- and 16α-hydroxylations) (from Zhu and Lee, 2005, with permission; copyright 2005 Elsevier)

found in most people. Thus, Taioli et al. (1999) have shown that *CYP1A1 mSp*-gene variant found in African American women results in an increased risk for breast cancer. Except for the C-17 oxidation, which is reversible, ending in a 70:30 estrone:estradiol (E1/E2) ratio (Fishman et al., 1960), the hydroxylations are all irreversible. Cytochrome P450-mediated metabolism of estrogens and its regulation has been reviewed by Tsuchiya et al. (2005).

The metabolites are further subjected to various reactions, the commonest being conjugation at C-3, forming glucuronides and sulfates, using UDPglucuronate and glucuronyl transferase (Guillemette et al., 2004; Pfeiffer et al., 2006) or 3′phosphoadenosine-5′-phosphosulfate (PAPS) and sulfotransferase (Suzuki et al., 2003, 2005; Utsunomiya et al., 2004) to give water-soluble conjugates which are readily excreted. Glucuronides are excreted via the kidney and sulfates are diverted in part via the bile and enter the gut. Some is excreted in the feces and the rest undergoes enterohepatic recirculation which slows the final total excretion of sulfates and gives rise to a slower blood decay curve. Because of urinary excretion, most of the estrogens consumed as birth control pills or hormone replacement therapy ultimately end up in the sewage system and finally into the outflow from sewage treatment plants. These compounds are present at a sufficient level to affect fish developing below sewer outfalls. Methods for measuring these compounds in sewage have been developed (*vide infra*).

In vivo methylation at C-2, C-4, or less frequently at C-3 leads to compounds which have very different activities. Thus, 2-methoxyestradiol is being studied as an anticancer drug (Lakhani et al., 2003, 2005, 2007; Han et al., 2005). The catechol estrogens can form semiquinones which react irreversibly with glutathione and other nucleophiles (Jellinck and Fishman, 1984). These semiquinones can also react with DNA to form adducts (Fig. 8.5) which are stable in the case of the 2,3-catechols but result in depurinating adducts with the 3,4-catechols which increase the rate of mutation (Cavalieri et al., 2002, 2006; Cavalieri and Rogan, 2006). 16α-OH-estrone can react via the Heyns rearrangement to bind irreversibly to histones and estrogen receptors (ERs) to yield long acting estrogen responses (Swaneck and Fishman, 1988; Lustig et al., 1989) (Fig. 8.6). Formation of glutathione conjugates of catechol estrogen quinones *in vivo* may act as a detoxification mechanism (Raftogianis et al., 2000).

8.2.3 Intracrine Production of Estradiol

Intracrine, also described as paracrine or autocrine, refers to the local production of estradiol in breast cancer and other tissues from peripheral estrone sulfate and androstenedione (Sasano et al., 2006, 2008; Suzuki et al., 2008a, b). For the production of the active estradiol, aromatase and/or steroid sulfatase are necessary followed by 17β-hydroxysteroid dehydrogenase. Figure 8.7 illustrates this process. In postmenopausal women, it is likely that peripheral circulating androstenedione is of adrenal origin whereas estrone sulfate probably arises in the adipose tissue, possibly from

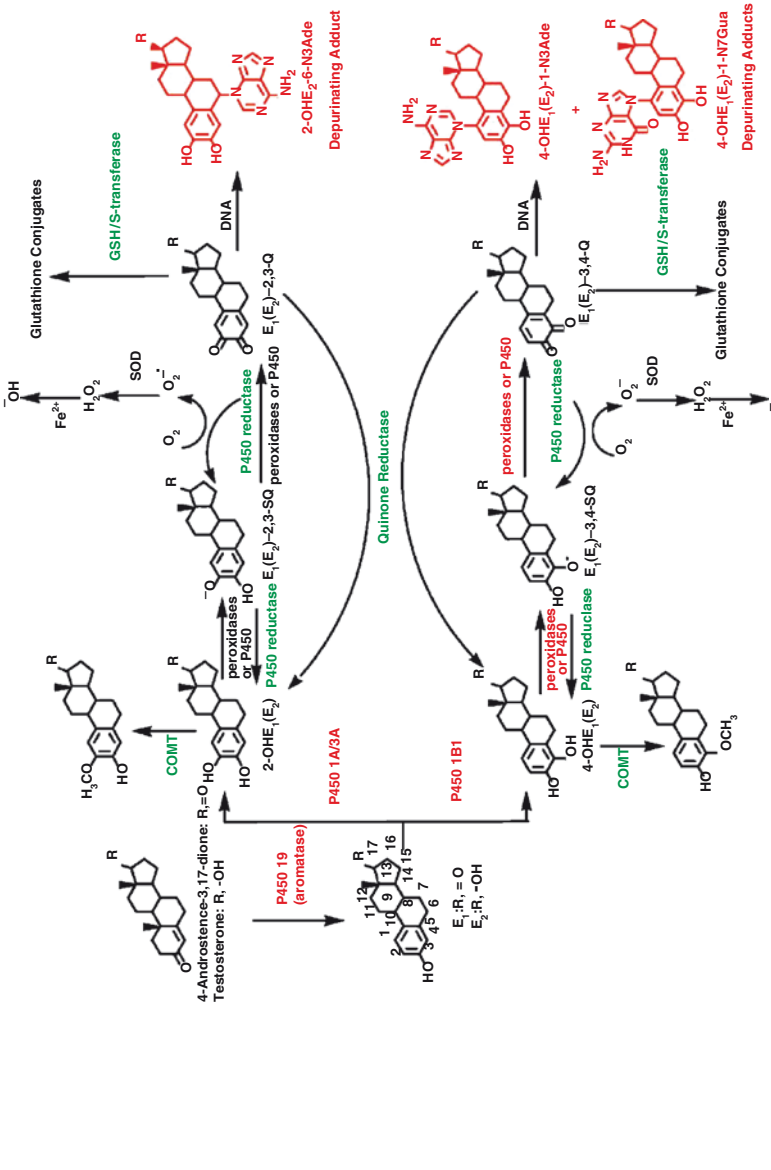


Fig. 8.5 Formation, metabolism and conjugation of catechol estrogens and production of DNA adducts. Activating enzymes and depurinating adducts are in red, and protective enzymes are in green (from Cavalieri et al., 2006, with permission; copyright 2006 American Chemical Society)

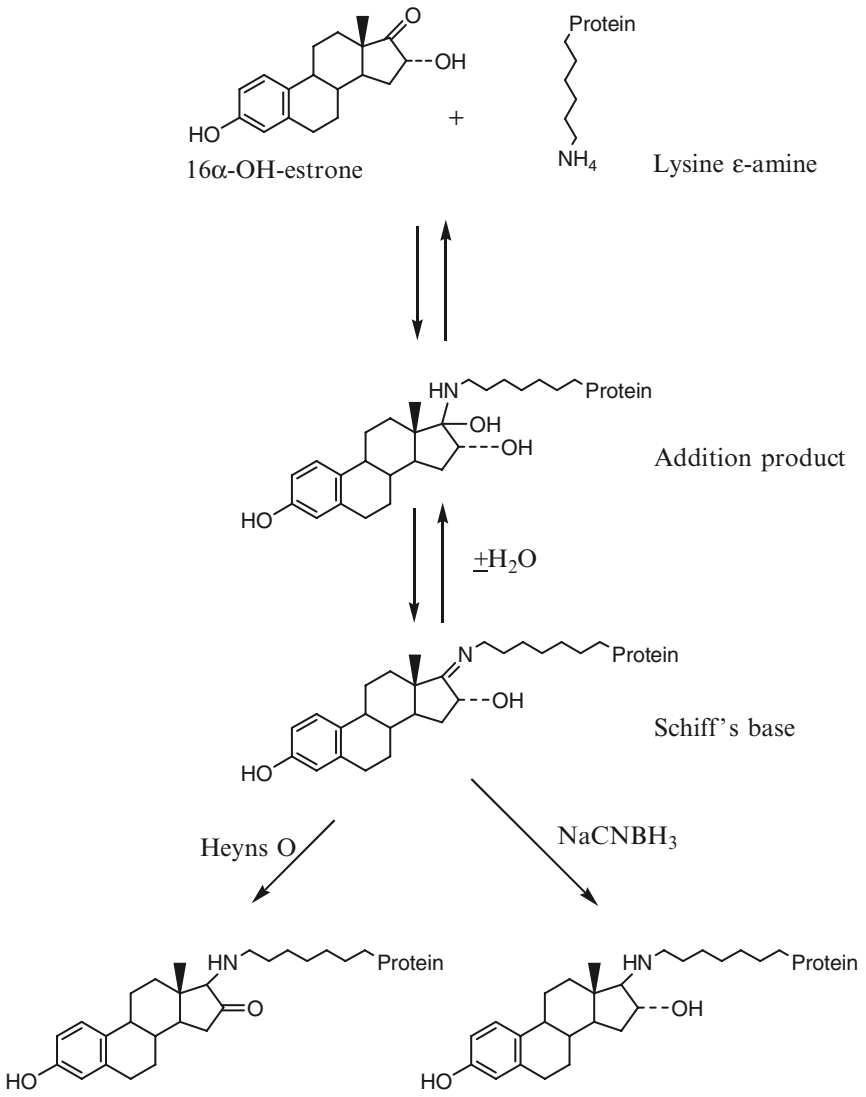


Fig. 8.6 Irreversible binding of 16α-hydroxyestrone to histone protein via the Heyns rearrangement

adrenal DHEA sulfate after hydrolysis of sulfate and re-sulfation after A-ring aromatization. Ovarian contribution to peripheral estrone sulfate is significant, and it falls significantly after menopause (Woods et al., 1989; Hankinson et al., 1998; Michaud et al., 1999; Dorgan et al., 2001; Lamar et al., 2003; Kaaks et al., 2005; Eliassen et al., 2006a, b). It is noteworthy that only the aromatization process is irreversible, suggesting that it is a possible key enzyme in the intracrine production of estradiol (Pasqualini and Chetrite, 2005; Suzuki et al., 2008a, b). As aromatase and estrogen sulfotransferase

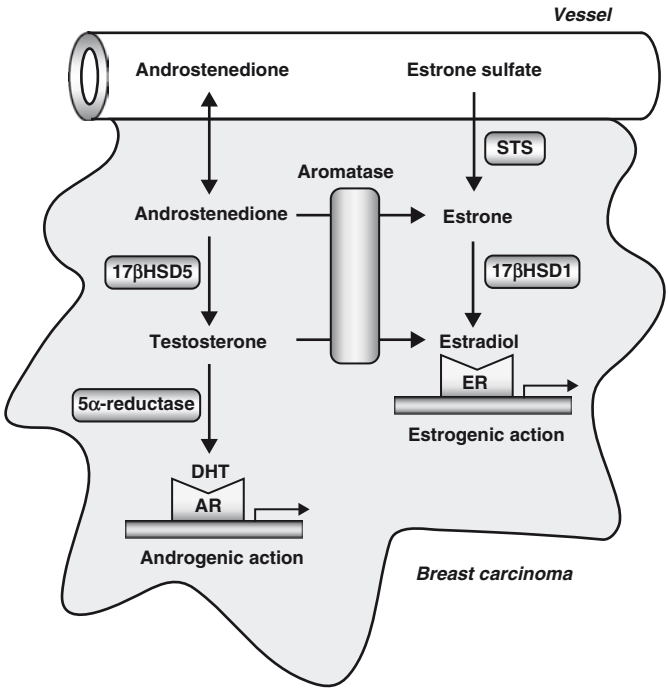


Fig. 8.7 Scheme representing the intratumoral production of sex steroids in breast carcinoma. High concentrations of circulating inactive steroids, such as androstenedione and estrone sulphate, are precursor substrates to the intratumoral production of estrogens and/or androgens in breast carcinoma. Biologically active sex steroids, such as estradiol and 5α-dihydrotestosterone (DHT), are produced and act on the breast carcinoma cells through estrogen (ER) and androgen (AR) receptors, respectively. STS: steroid sulfatase, 17βHSD: 17β-hydroxysteroid dehydrogenase (from Suzuki et al., 2008b, with permission; copyright 2008 Springer)

play an important role in the production of active estradiol in breast carcinomas, these enzymes are clearly the potential targets in the therapeutic treatment of these tumors (Stanway et al., 2007a; Herold and Blackwell, 2008; Lønning and Geisler, 2008a, b; Ponzone et al., 2008) in addition to, or as well as, the already widely used selective estrogen receptor modulators (Lee et al., 2008; Shelly et al., 2008).

8.2.4 Mechanism of Action of Estradiol

The main (ligand-dependent) pathway of action for estrogens is by binding to the estrogen receptors. The activated dimerized receptor(s) then binds to specific DNA sites (estrogen response elements [EREs]), directly, or assisted by a number of cofactors in a complex (Thanki et al., 1979; Jost et al., 1984; Matthews and Gustafsson, 2003; Gruber et al., 2004). There are also non-genomic and non-ligand pathways of ER activation. Figure 8.8 illustrates these various pathways. There are two ERs (ERα and ERβ) and, thus, both hetero and homodimers can be formed

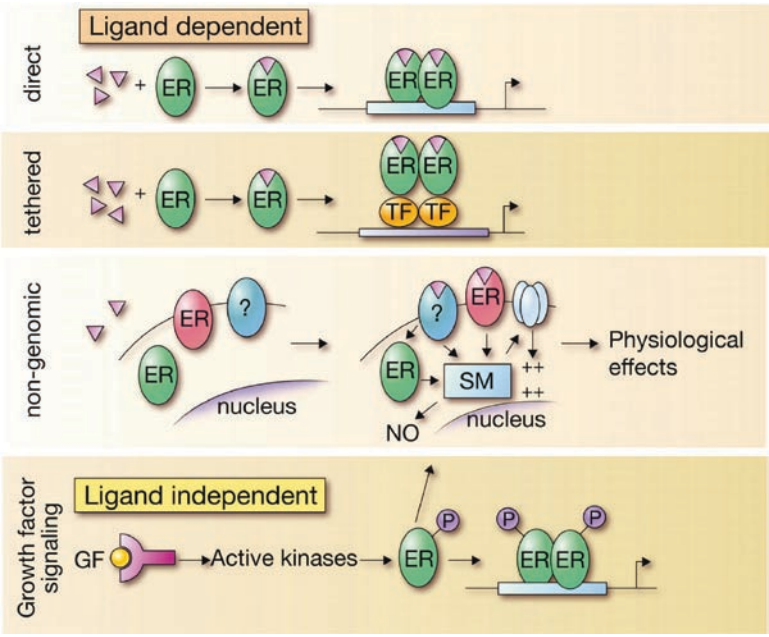


Fig. 8.8 Model representing the mechanistically distinct molecular pathways used in the regulatory actions of ERs. The classical (direct) pathway includes ligand activation and a direct DNA binding to estrogen response elements (ERE) before modulation of gene regulation. The tethered pathway includes protein-protein interaction with other transcription factors after ligand activation, and thereby gene regulation is affected by indirect DNA binding. A third mechanism, also called nongenomic with rapid effects, is not as well understood as the genomic mechanism but has been observed in many tissues. The ligand activates a receptor, possibly associated with the membrane; either it is a classical ER, an ER isoform, or a distinct receptor or, alternatively, a signal activates a classical ER located in the cytoplasm. After this rather unclear event, signaling cascades are initiated via second messengers (SM) that affect ion channels or increase nitric oxide levels in the cytoplasm, and this ultimately leads to a rapid physiological response without involving gene regulation. The ligand-independent pathway includes activation through other signaling pathways, like growth factor signaling. In this case, activated kinases phosphorylate ERs and thereby activate them to dimerize, bind DNA, and regulate genes (from Heldring et al., 2007, with permission; copyright 2007 *The American Physiological Society*)

(Dahlman-Wright et al., 2006; Heldring et al., 2007). The level of ERα in tumor tissue is used as a classsifier to distinguish receptor positive and receptor negative breast tumors which are treated by different therapies – ERβ may also have a role in predicting response to therapy (Murphy and Watson, 2006). ERβ receptors appear to play a role opposing the action of ERα receptors (Matthews and Gustafsson, 2003; Hanstein et al., 2004; Nilsson et al., 2004; Koehler et al., 2005; Dahlman-Wright et al., 2006; Zhao et al., 2008). The specific role of the receptors has been studied by means of knockout mice in which either or both of these receptors have been deleted (Couse and Korach, 1999; Warner and Gustafsson, 2004).

In addition to this pathway of action, binding of estradiol to membrane- bound receptors, particularly in caveoli (small invaginations in the plasma membrane),

activates G-proteins linked to these sites and initiates action by means of a cascade of phosphorylations (Russel et al., 2000; Simoncini et al., 2003). While nuclear action of estradiol takes a finite time for gene activation and protein synthesis to occur, membrane action occurs rapidly and responses are almost instantaneous (Song and Santen, 2006; Cheskis et al., 2007; Raz et al., 2008).

8.2.5 *Physiological Role of Estradiol*

Apart from the well known role of estradiol in normal female development and its role in ovulation and the maintenance of pregnancy, estradiol has many other functions – in both sexes (Rochira et al., 2005). Recent studies on the effects of mutations of the human *CYP19* gene (Zirilli et al., 2008) indicate the important role of estradiol in fetal and early post-natal development in both sexes – in boys until testosterone secretion is initiated to start masculinization. Females with aromatase deficiency are characterized by raised androgen levels and virilization and develop with eunuchoid habitus and failure to close the epiphyses, both of which are responsive to estradiol treatment. Males with aromatase deficiency have similar epiphyseal closure problems with suggestions of effects on glucose and lipid metabolism and reproductive function (Lanfranco et al., 2008). In the absence of a functional testosterone receptor, genetic males will develop as sterile females (Galani et al., 2008; Mueller, 2008). In a favorable direction, estradiol plays a role in stimulating early bone growth (Zirilli et al., 2008) and maintaining bone strength, and osteoporosis is a common postmenopausal disorder as estradiol levels decline. Estradiol metabolites also play a role; 16α-OHestrone increases bone strength while 2-OHestrone decreases it (Lotinun et al., 2001; Napoli et al., 2005; Armamento-Villareal et al., 2007; Napoli and Armamento-Villareal, 2007).

Estrogen is involved in spermatogenesis, and there is evidence that aromatization in the brain plays a role in signaling mechanisms – for example, aromatization of secreted testosterone in the hypothalamus plays a major role in the hypothalamic-pituitary regulation of testicular function (O'Donnell et al., 2001). Evidence also exists that estrogen plays a role in maintaining cognition and other events in the body (Yaffe et al., 1998; Lebrun et al., 2005; Luine, 2008) and estrogen has a role in skin tone by stimulating collagen formation (Son et al., 2005; Patriarca et al., 2007).

8.2.6 *Estrogen and Breast Cancer*

On the negative side, steroidal estrogens as a group and post-menopausal estrogen therapy have been classified as carcinogenic by the WHO International Agency for Research on Cancer (IARC, 1987, 1999). In a review, Liehr (2000) pointed out that estradiol itself is actually a weak carcinogen and that the metabolites possess all of the carcinogenic activity. The suggestion by IARC that estrogen hormone

replacement therapy is carcinogenic is, however, not universally accepted (Mueck and Seeger, 2007, 2008). Estradiol clearly plays a role in risk for breast cancer, and there is also evidence that it plays a role in prostate cancer and in human papilloma virus (HPV) related diseases. In these latter cases, differential formation of estrogen metabolites is involved. In breast cancer, increased formation of 4-hydroxyestradiol, a known carcinogen, plays a role by causing depurination of DNA – see Figs. 8.5 and 8.6 (Cavalieri et al., 2006, Cavalieri and Rogan, 2006; Bolton and Thatcher, 2008), that is followed by an error- prone repair leading to mutations.

Steroid metabolism in breast cancer has been reviewed recently (Foster, 2008). Other studies have shown that the ratio of 2-hydroxyestrogens to 16α-hydroxyestrone (2/16 ratio) is a measure of risk for breast cancer (Kabat et al., 1997, 2006; Ho et al., 1998; Meilahn et al., 1998; Muti et al., 2000; Fowke et al., 2003) – women with elevated 2/16 ratios (greater than the mean) are at lower risk for breast cancer and cancer *in situ*, although there is at least one study which did not support this conclusion (Ursin et al., 1999). Indeed Fowke et al. (2003) found a reversal (cross-over) of 2/16 ratio versus breast cancer risk in urine samples collected from women after initiation of treatment. A recent study (Eliassen et al., 2008) could find no association between the 2/16 ratio in non-HRT users with ER+/PR+ tumors but did find a positive association in ER-/PR-tumors. They suggest that this latter finding needs validation as their numbers were low. In addition, survival curves indicate that even in those women with breast cancer those with higher 2/16 ratios live longer. Breast cancer risk in Singapore Chinese women is significantly greater than the risk in both Afro-American and white women in the USA, but there appears to be no significant difference in the 2/16 ratio between these groups (Ursin et al., 2001) nor is there a significant difference between the ratio in premenopausal women with first degree family history and those without such history. The equivocal nature of the results is illustrated by the findings of the Danish prospective study (Wellejus et al., 2005) suggesting that high urinary 2-hydroxyestrogen metabolites are associated with enhanced risk of breast cancer in post menopausal women using HRT but not in women not on HRT, which does not seem to accord with the view that 2-hydroxylated metabolites are anti-estrogenic (Schneider et al., 1984). 2-methoxyestrone is under consideration as an anti-cancer agent (Lakhani et al., 2007) and 2-hydroxycatechol estrogens have been shown to have strong anti-aromatase activity and may be involved as part of an intracrine control mechanism of estrogen production in post-menopausal women (Neves et al., 2008). The anomalous association observed by Wellejus et al. (2005) between increased 2-hydroxylation and increased risk of breast cancer may of course simply be a reflection of increased catabolism of the estrogens in the HRT group (Berstein et al., 2000), may be an *in vivo* protective response to their carcinogenic potential or indeed may be due to some confounding factor in their subject group.

Because of the widespread use of aromatase inhibitors in the treatment of breast cancer (Smith and Dowsett, 2003), there is a developing need for methods to measure extremely low levels of estradiol to determine the degree of estradiol suppression (e.g. Lønning and Geisler, 2008a). A wide range of methods for making these

ultra-sensitive measurements will be discussed (*vide infra*) and newer methods continue to be developed, which are capable of measuring down to 1 pmol/L (0.27 ng/L). These include both biological and LC-MS/MS approaches.

8.2.7 Exercise and Breast Cancer Risk

Evidence for a beneficial role for exercise in decreasing breast cancer risk has been demonstrated in a number of recent studies. These studies and many others show a decreased level of circulating estradiol in women who train intensively and this has been correlated with decreased risk for breast cancer (Gilliland et al., 2001; Matthews et al., 2003, 2004; Bentz et al., 2005; Bardia et al., 2006; Lahman et al., 2007; McTiernan, 2008; Peplonska et al., 2008). Vigorous exercise or anorexia, which result in a loss of fat depots show a sharp increase in 2-hydroxylation (Snow et al., 1989; Fishman and Bradlow, 1997), which is known to play a protective role against breast cancer.

8.2.8 Effect of Diet on Estrogen Metabolism and Cancer Risk

Estrogen metabolism is sensitive to diet and drugs. Indole-3-carbinol, derived from cruciferous vegetables, which has been reported to have a protective effect against HPV-induced cervical cancer (Jin et al., 1999), selectively stimulates 2-hydroxylation in breast cancer cells (Tiwari et al., 1994) to a level sufficient to have a therapeutic benefit in laryngeal papillomatosis (Coll et al., 1997; Rosen et al., 1998) and cervical dysplasia (Bell et al., 2000). The reduction in cancer risk induced by cruciferous vegetables has recently been reviewed (Higdon et al., 2007). Indole-3-carbinol may also be beneficial in breast and prostate cancer by *inter alia* raising the 2/16 estrogen metabolite ratio. Other dietary substances increasing 2-hydroxylation include: increases in protein in the diet (Anderson et al., 1984), omega-3 fatty acids (Osborne et al., 1988), soy isoflavones (Xu et al., 1998, 2000), and flaxseed (Brooks et al., 2004) – possibly by effects on aromatase and 17β-hydroxysteroid dehydrogenase (Brooks and Thompson, 2005). 2-hydroxylation is increased by high levels of estradiol used for HRT (Berstein et al., 2000), and the urinary 2/16 ratio of estrogen metabolites is influenced by thyroid hormone (Fishman et al., 1965; Michnovicz and Galbraith, 1990; Lee et al., 2003a). 2-hydroxylation is lowered by a high carbohydrate diet (Anderson et al., 1984), omega-6 fatty acids (Osborne et al., 1988) and Cimetidine (Tagamet) (Michnovicz and Galbraith, 1991).

8.2.9 Estrogen Metabolism and Prostate Cancer

In prostate cancer, a similar shift in the 2/16 ratio is observed (Muti et al., 2002). Other evidence for a role for estrogen in prostate cancer has been described (Wang and Wong, 1998; Ho et al., 2006). Trials using 3,3′-diindolylmethane to increase

2-OH-E1 concentrations in patients with prostate cancer are underway, though this compound may have other effects in prostate cancer cells (Li et al., 2007a).

8.2.10 Human Papilloma Virus

HPV is intimately involved in the etiology of cervical cancer (Monk and Tewari, 2007). Proliferation of HPV is stimulated by 16α-hydroxyestrone. Since 16-hydroxylation is elevated significantly at the rim of the cervix (Bradlow et al., 2007 unpublished); any HPV entering the cervix would find a favorable milieu for growth. On the other hand, increased 2-hydroxylation inhibits HPV proliferation

Even in the beneficial role of promoting pregnancy (Rao, 2000), there is a small increase in estradiol-mediated breast cancer right after delivery which tapers down (Gallenberg and Loprinzi, 1989; Barthelmes et al., 2005) and is followed by a much larger protective effect of pregnancy, particularly before age of 25 years. Late pregnancy provides no protective effect and in addition increases the risk for breast cancer (Charles et al., 2004).

8.2.11 Estrogens and Contraception

In addition to estradiol and its metabolites there is considerable interest in the 17-alkylated estrogens used in birth control pills. These have the advantage that when given orally they are not rapidly inactivated as is the case for estradiol. Ring B unsaturated estrogens (equilin and equilinin) are derived from horse urine and widely used in hormone replacement therapy. The commercial product, Premarin, is actually a mixture of a number of compounds and the precise composition has never been revealed. Their metabolism is similar to that of estradiol, although different enzymes are involved (Bhavnani et al., 1989).

8.3 Analysis 1: *Purification*

Because of the interplay between all of the different estrogens and their catabolites involved in beneficial and harmful roles in the body, it is clear that measurement of estradiol alone, useful in many situations, is not always helpful and concentrations of other oestrogens can provide useful information. Estrogen measurements are important in clinical biochemistry practice for the diagnosis and management of particular human diseases, for assessment of the risk of perinatal death, for investigation of medical problems which arise in the postmenopausal years and for investigation and treatment of human infertility. Some requirements, but on a numerically smaller scale, occur in applications to veterinary practice. Research investigations involving studies of human subjects and with isolated organs, cells

and subcellular fractions may require estrogen estimations. An increasing awareness of the impact of synthetic and natural chemicals on the environment and the possible effects of these chemicals on human development has meant that detection and measurement of estrogens in environmental samples is also necessary. The possible adverse effect of such estrogens in water has lead to the increasing development of methods for the analysis of both free and conjugated estrogens in river, waste water, sewage sludge, and effluents. In view of these very different objectives, no all-encompassing guidance can be offered as to which method should be selected for a particular purpose. Each application will have both its own restrictions and the method selected must be appropriate for the fluid or material to be analysed. Analysis, taken in the widest sense, encompasses extraction of the analyte(s) from the sample, separation into individual components and their determination. This chapter will focus on the techniques for each of these phases in turn. NB to the best of our knowledge, no differences in estrogen concentration between serum and plasma have been described. While we have endeavoured to be precise when discussing/describing published methods, we have not felt the need to make great efforts to distinguish between serum and/or plasma as the analytical matrix.

8.3.1 Extraction

Many methods now avoid the extraction step, at least for the analysis of body fluids; some rely on the specificity claimed for a selected antibody to permit measurement of one estrogen in the presence of others, so avoiding a separation procedure – a claim that is not always correct and certainly needs testing before reliance is placed on results using the method. Certain methods employ the physicochemical properties of the estrogens to separate them and quantitate fractions by simple chemical or optical techniques. While direct (non-extraction) immunoassay methods are convenient and quick, it is clear that such procedures are generally not specific and particularly when the analyte is at low concentration, these methods are far from satisfactory (Stanczyk et al., 2003; Bay et al., 2004; Dowsett and Folkerd, 2005; Lee et al., 2006; Ankarberg-Lindgren and Norjavaara, 2008). The demands of routine clinical assays for plasma estrogens may often be satisfied by direct immunoassays, but the specificity of such assays is not usually adequate for research purposes (Toniolo and Lukanova, 2005; Stanczyk et al., 2007). Automated immunoassay methodology, which largely relies on non-extraction methodology, while giving excellent reproducibility, is also generally non-specific, in that agreement between different methodologies is often very poor, especially when concentrations are low (Taieb et al., 2002; Demers, 2008) and should be validated by comparison to results obtained by GC-MS (Coucke et al., 2007) or other similar technique. That such an approach has merit was demonstrated by Stanway et al. (2007b) who used GC-MS/MS to demonstrate a good correlation of results with those obtained using a ‘direct’ RIA for estrone

sulfate. With increasing use of modern aromatase inhibitors (e.g. anastrozole) in the treatment of breast cancer patients, there is a need to develop clinically applicable methods which are capable of detecting the degree of suppression of endogenous oestrogen, which may be >98%. This requires methods with detection limits for estradiol of <1.0 pmol/L (<0.27 ng/L). A recent highly sensitive ^{125}I-RIA method (Geisler et al., 2008) for measurement of plasma estrone, estradiol and estrone sulfate concentrations has been reported, requiring extensive extraction and purification prior to RIA. This method used ^{3}H-labeled internal standards, extraction with ether, LH20 separation of estrone and estradiol and conversion of estrone to estradiol using sodium borohydride. The aqueous layer remaining after ether extraction was vortexed (×2) with ethanol, centrifuged and treated with sulfatase enzyme. The free estrone was then purified on LH20 and converted to estradiol by borohydride reduction. Each of the three fractions was then assayed by an optimized RIA for estradiol. No details of the actual RIA are given – described as 'reported elsewhere' with no reference given, though details can of course be acquired from the authors. This approach does however emphasize the need to employ pre-immunoassay purification in order to improve sensitivity/specificity.

The extraction of estrogens from biological materials is dependent on the matrix involved. Sometimes, it is possible to extract the estrogens by a simple solvent extraction. Sometimes, it is necessary to hydrolyse conjugates prior to the extraction or the conjugates may be extracted whilst intact. The key requirement is to use an extracting solvent sufficiently polar to remove the conjugates, yet immiscible with the fluid in which they are contained. Lipid and protein as well as water may also be taken up and further purification is needed. Health and Safety regulations may now reduce the use of solvents like diethyl ether in some laboratories, because of the fire hazard and the explosive risk from ether peroxide, or benzene, because of its leukemogenic potential. Ethyl acetate or t-butyl methyl ether (e.g. Supko and Phillips, 1995; Shou et al., 2004) could be substituted as the extraction solvent. Solvents like chloroform, methylene chloride, or tetrahydrofuran are also discouraged on safety grounds but are still used. It must always be remembered that steroid estrogens are acidic and can be extracted with varying degrees of success into alkaline washes and after acidification extracted back into solvent – as most other steroids are neutral, such washing provides increased specificity (Mitchell and Davies 1954). This technique was used prior to immunoassay of estradiol and estrone in nipple aspirate fluid from premenopausal women (Chatterton et al., 2004).

Consideration may have to be given to the serum or plasma protein binding of the steroid by sex hormone binding globulin or albumin. Most solvent-extraction methods are highly effective in disrupting the protein binding, leading to a near quantitative recovery of estrogens in the organic solvent, especially if extraction is done from acidified matrix. In non-extraction immunoassay methods however, reagents must be added to disrupt the plasma protein binding and ensure that the plasma proteins do not upset the saturation analysis. Table 8.1 summarizes some

Table 8.1 Examples of extraction methodology for estrogens (1990–date)

Estrogen	Matrix	Extraction	Quantitation	Reference
E2 metabolism in MCF-7 breast tumour cells *in vitro*	Cell culture medium Cells	β-Glucase hydrolysis. ExtreLut QE column, eluting with CH_2Cl_2 and derivatization with BTSFA; extraction after sonication with CH_2Cl_2 Microsomes extracted with EtAc; derivatization with BSTFA	GC-EI(+)-MS	(Spink et al., 1990); (Hayes et al., 1996)
Equilin, Equilenin and metabolites of Equilin sulfate	Canine urine and plasma	Dilution with MeCN, β-glucuronidase MeCN centrifugation	LC-electrochemical detection	Chandrasekaran et al. (1995)
E2	Human serum	Extraction with diethyl ether	^{125}I-RIA	Klein et al. (1995)
E1, E2, E3 and guaiacol estrogens (gEs)	Wistar strain rat brains	E1 and gEs: extract homogenate with EtOH purify on IsoluteC18 catridge; derivatize as *O*-methyloximes and run on Silica gel minicolumn; TMSi ether E2 and E3: Homogn; extract with EtAc:CHCl3; purify on silica gel columns, TLC × 2, RP-HPLC TMSi ether	GC-MS/MS	Shimada et al. (1999)
[d]E2, Equilenin	Human urine	96-well SPE block	PE/Sciex API3000; LC-APCI(+)-MS/MS	Rule and Henion (1999)
E1, E2, E3 and Ethinyl estradiol	Activated sewage sludge and river water	Filtration through glass fibre paper, eluting with MeOH. SPE using graphitized carbon (Carbograph 4), eluting with CH_2Cl_2:MeOH (4:1)	Sciex API 2000 with Turbo Ion spray(−); LC-MS/MS (SRM)	Baronti et al. (2000)
E1, E2	Cancer tissue	Flow sheet in paper (Fig. 8.1). Homogenization; extract ether x3; Lipidex 500/HPLC separation into E1 and E2 fraction; E1 converted to E2 and E2 purified on LH20	RIA	Geisler et al. (2000) Hamalainen (1982), Lønning and Ekse (1995), Geisler et al. (2008)
E1, E2, E3 and ethinyl estradiol	River water and sediment	Freeze-dried sediment samples extracted with MeOH:acetone (1:1) and ultrasound. Dried re-dissolved in same solvent and subjected to Sep-Pak C18. This paper investigates other SPE systems for extraction of water	RP-LC-DAD-MS	Lopez de Alda and Barcelo (2000, 2001a); Topic reviewed in Lopez de Alda and Barcelo (2001b)

Ethinyl estradiol	Cattle hair	Wash with 10%aq. TWEEN; dry and finely cut with scissors; digest with 1 M NaOH at 40°; adjust to pH 8.4 with 0.2 M Ac buffer; dialyse with CH_2Cl_2 inside dialysis bag at 37° for 4 h; steroid in solvent; Evap and form TMSi ethers using MSTFA:TMIS:DTT (1000:5:5)	GC-MS/MS (Finnegan GCQ+ ion trap- EI(+))	Durant et al. (2002a); application in cattle in Durant et al. (2002b)
E1, E2, ethinyl estradiol and mestranol	Sewage sludge and sediments	Freeze dried extracted ×2 EtOH + acetone ×2 using ultrasound; centrifuge and combine extracts; sludge extracts purified by gel permeation chromatography on GPC-PTFE column; Silica gel column eluting with Hx:EtAc (65:35); Evap to dryness and vsediment taken up with 100 mL groundwater with 1.3 g/L tetrabutylammoniumbromide and poured through RP-C18 cartridges and analytes eluted with Acetone; HPLC purification. TMSI derivatives	GC-MS/MS	Ternes et al. (2002)
Ethinyl estradiol	Rhesus monkey plasma	Extracted with EtAc, derivatized with dansyl chloride and back extracted into Hx	API 4000 with Turbo Ion spray; LC-ESI(+) –MS/MS (SRM)	Anari et al. (2002)
Catechol estrogens and GSH-conjugates	Rat prostate tissue	Minced and ground to fine powder in liquid N_2 – suspended in 50 mM NH_4Ac pH 5; MeOH added to 60% and extracted with Hx to remove lipid; aqeuous phase diluted with 50 mM NH_4Ac, pH 4.4 to reach 30% MeOH and applied to a Certify II Sep-Pak	RP-HPLC with electrochemical detection (12-channel ESA CoulArray)	Cavalieri et al. (2002) Rogan et al. (2003)
E1, E2, E3; 16-OHE1; 2-OH- E1 and -E2; 4-OH-E1 and -E2; 2MeO-E1 and -E2; 4MeO-E1 and -E2	Breast tumor tissue	Dissect away fat; homogenize in TRIS buffer (0.1 M, pH 8.6):HClO4 (0.01N) (1:1) final pH 3.4; centrifuge 2,000 g 10 min; add 0.4 g acid alumina; CCEs absorbed by alumina were eluted with 0.2 M acetic acid; other estrogens extracted from supernatant with Ether; extracts combined	RP-HPLC using UV and electrochemical detection; identity by GC-MS as TMSi ethers using EI(+)	Castagnetta et al. (2002); GC-MS method – Castagnetta et al. (1992)
E1 and E2	Human urine	*Helix pomatia* hydrolysis. Sep-Pak C18 + NH_2 columns, eluting with MeOH	LC-RIA	Rinaldi et al. (2003)

(continued)

Table 8.1 (continued)

Estrogen	Matrix	Extraction	Quantitation	Reference
E1, E2, Ethinyl estradiol, 16α-hydroxyestrone	River sediment and Water samples	Microwave-assisted or ultrasonic extraction with MeOH, followed by purification on small Silica Gel minicolumns eluting with EtAc:Hx (4:6); Oasis HLB SPE cartridge, eluting with EtAc	GC-EI(+)-Ion Trap-MS using TMS derivatives	Liu et al. (2004 b) Liu et al. (2004 a)
E1 and E2	Bovine plasma	Extraction with diethyl ether, separation on LH-20, eluting with Bz:MeOH (85:15)	EIA	Takenouchi et al. (2004)
Ethinyl estradiol	Human plasma	Extracted with methyl-*t*-butyl ether, derivatized with dansyl chloride and back extracted into Hx	API 3000 with Turbo Ion spray; LC-ESI(+)-MS/MS	Shou et al. (2004)
E2	Human nipple aspirate	Extraction with EtAc:Hx (3:2). Evaporate to dryness, dissolve in iso-octane, extract with NaOH, neutralize with HCl and back extract with EtAc	IA	Chatterton et al. (2004)
E2	Human testicular fluid	Extraction with diethyl ether	Micromass Quattro LC-ESI(+)-MS/MS	Zhao et al. (2004)
7 ketolic estrogens	Human urine	β-gluc-ase and sulfatase hydrolysis. Apply to ExtreLut QE columns and elute with diethyl ether. Derivatize with *p*-toluenesulfonyl hydrazide	Finnegan LCQ-DECA; LC-ESI(+)-MS^n	Xu et al. (2004)
d_4-E2 and d_4-E1	Mouse plasma and brain samples	Extract with EtAc and derivatize with dansyl chloride	API 4000 with Turbo Ion spray; LC-ESI(+)-MS/MS (MRM)	Xia et al. (2004)
E1, E2, EE2	Water	Polypropylene hollow fibres coated with dihydroxylated polymethylmethacrylate; De-absorbed using CH_2Cl_2 with ultrasound; TMS formation on fibre with MSTFA	Shimadzu QP2010 GC-MS-?EI-SIM	Basheer et al. (2005)
Ethinyl estradiol and Norethindrone	Human plasma	Extract with methyl-*t*-butyl ether, derivatization with dansyl chloride	API 4000 with Turbo Ion spray; LC-ESI(+)-MS/MS (MRM)	Li et al. (2005)
E2	River water	Extracted by filtration through glass filter, E2 washed off with Acetate buffer pH 5:MeOH (1:9). On to C18 cartridge, eluted with CH_2Cl_2	Fluoro-IA	Matsumoto et al. (2005)

E1, E2 and other estrogenic compounds	Fish serum	Extraction with diethyl ether. Evap to dryness and re-dissolve in 100 g/L NaCl (pH 5), load onto Sep-Pak C18 and elute with ether	GC-EI(+)-MS as TME ethers	Yang et al. (2006b)
E2, Ethinyl estradiol	Sewage effluent	Solids filtered off; liquid through Strat-X cartridges, eluting with MeOH; separated on silica gel mini-columns, eluting with Hx:acetone (65:35)	ELISA	Hinteman et al. (2006)
E2	*In vitro* CYP19 activity	Protein removed by iso-propyl alcohol and centrifugation	API 3000; LC-APPI(-)-MS/MS using toluene as a dopant	Trosken et al. (2006)
E2	Human plasma/ serum	SPE on BondElut Certify column, elution with EtOH and derivatization	GC-EI(+)-MS/MS	Santen et al. (2007a); Wang et al. (2005)
E1, E2, E3 and E4	Human plasma	Deproteinized with MeOH, diluted to 10% MeOH and applied to LC-18 SPE cartridges, eluting with MeOH	LC-DAD	Zarzycki et al. (2006)
E1, E2, EE2	Sewage and river samples	Use of hemimicelles and admicelles of SDS and CTAB. SDS hemimicelle coated alumina gave best results	LC-DAD–fluorescence	Garcia-Prieto et al. (2006)
E1, E2 (α and β), E3 (free and conjugated)	Cow's milk	Incubation with *Helix Pomatia* at 37° overnight. Addition of MeOH:H_2O (2:1) added. Vortex mixed and centrifuged. Upper fat layer discarded. Aqueous layer extracted with Hx and then extracted ×2 with CH_2Cl_2. Solvent removed and residue dissolved in MeOH:H_2O (95:5), homogenized and passed through C18SPE column, eluting MeOH after washing. Derivatize with dansyl chloride	LC-MS/MS; API 3000-ESI(+) – MRM monitoring MH^+, monitoring product ion *m/z* 171 $(dansyl)^+$	Malekinejad et al. (2006)
Ethinyl estradiol	Human plasma	96 well extraction with methyl-*t*-butyl ether, SPE and derivatization with dansyl chloride	API 4000 using turbospray; LC-ESI(+)-MS/MS	Licea-Perez et al. (2007)
E1, E2 and equilenin	Human urine	Stir bar absorptive extraction and derivatization with acetic anhydride:pyridine (1:1)	GC-EI(+)-MS	Stopforth et al. (2007)

(continued)

Table 8.1 (continued)

Estrogen	Matrix	Extraction	Quantitation	Reference
10 estrogen metabolites	Human urine	Hydrolysis with β-glucuronidase, Sep-Pak C18 cartridges; ion-exchange chromatography QAE-Sephadex in acetate form; QAE-Sepadex columns separate into estrogens containing vicinal and non-vicinal *cis*-hydroxyls	GC-EI(+)-MS as TMS derivatives	Knust et al. (2007)
17α-ethinyl estradiol	Cattle hair	Hair ground in liquid N_2; Vortex-mixed with MeCN in sonic bath; centrifugation; centrifugal filter; derivatization using dansyl chloride	LC-MS/MS. Q-trap 2000 MS with Ion Source Turbospray (+) –MRM	Pedriera et al. (2007)
E2	Whole cow's milk	Homogenized milk samples extracted with EtAc ×2. MeOH added to precipitate triglycerides; E2 purified on LH-20 minicolumn	Commercial IA for E2	Pape-Zambito et al. (2007, 2008)
E2	Human serum	Extract with CH_2Cl_2; dry and derivatize with pyridine sulfonyl chloride	Sciex API 2000 and API 4000 Q TRAP LC-ESI(+)MS/MS	Xu and Spink (2008)
E1, α– and β-E2	Milk, eggs and meat	Milk and eggs hydrolysed with *Helix Pomatia*, extraction with diethyl ether; meat hydrolysed extracted with MeOH:Acetate buffer; Both purified on Chrom P SPE cartridge; androgens and estrogens separated by liquid/liquid partition using pentane; derivatized as TMSi ethers	GC-MS/MS using NCI with methane as the reagent gas. MRM	Courant et al. (2008). Courant et al. (2007a)
α– and β-E2, E1 and E3	Soil samples	Extraction with PLE (pressurized liquid extraction) using acetone –other solvents evaluated; concentrated and purified on C18 RP-SPE cartridges, TMSi derivatives formed	GC-MSD. EI(+) – MID	Beck et al. (2008)
E1, E2, EE2	Tap and sewage water in Sweden	Use of hollow-fibre microporous membranes (Q3/2 Accurel polypropylene)	HP5973 GC-MSD-EI(+)	Zorita et al. (2008)
E1, E2, E3, 2–OH–E1, 2-OH–E2, 16-OH-E1, 2-methoxy-E1	Human urine	De-proteinization with MeCN; Restricted Access Material (RAM) pre-column Shim-pack MAY1-ODS	Shimadzu LC-MS 2010A-ESI(−)	Liu et al. (2008)

E1, estrone; E2, estradiol; E3, estriol; E4, 15α-hydroxyestriol; EE2, ethynyl estradiol; CH_2Cl_2, methylene dichloride; EtAc, ethyl acetate; MeCN, acetonitrile(methyl cyanide); BSTFA, bis-trimethylsilyl-trifluoroacetamide; SPE, solid-phase extraction; Hx, hexane; Bz, benzene; NaOH, sodium hydroxide; NaCl, sodium chloride; HCl, hydrochloric acid; EtOH, ethanol; MeOH, methanol; xtraction, extraction.

recent methods used for the extraction of unconjugated estrogens and Table 8.2 gives a summary of modern extraction methods for estrogen conjugates. It should not however be assumed that earlier methods may not still be useful for particular applications and knowledgeable readers will find many examples of early extraction methodology being applied with success in recently published methods. Details of pre-1990 methods can be found from the estrogen chapter in the first edition of this book (Oakey and Holder, 1995).

Table 8.2 illustrates very well the value of LC methodology, which can accommodate estrogen conjugates without necessitating hydrolysis, which was required in the past when using gas chromatographic procedures and may still be used today (e.g. Geisler et al., 2008a; Lønning and Geisler, 2008a). Extraction from the aqueous media is simpler than from the tissue and if tissue estrogens are protein-bound, it may be necessary to add sodium hydroxide to the medium – a procedure used by Mitchell and Davies, (1954), which is a classic paper recommended to readers as an illustration of what separations were achieved more than 50 years ago. Moleculary imprinted polymers (reviewed by Pichon, 2007), in effect artificial antibodies, have been synthesized and shown to have affinity for EE2 and E2 (Dong et al., 2003) and can be used to separate 17α- and 17β-E2 (Szumski and Buszewski, 2004). Bisphenol has been extracted using MIPs but this approach has only recently been applied to estrogen measurement in environmental samples (Bravo et al., 2005; Watabe et al., 2006; Le Noir et al., 2007).

8.3.2 Separation

Although the extraction procedures may sometimes purify the estrogen or conjugate sufficiently for analysis, further separation may be necessary, depending upon the specificity of the final quantitation method, which may indeed include an element of separation (e.g. GC- or LC-MS/MS). This is particularly important in applications in which the end-point is based on the reaction of a functional group common to several estrogens (i.e. older assays using group-specific binding agents (e.g. rabbit uterus cytosol, SHBG) or less specific antisera). In those instances when an immunoassay is used as the end-point, the selectivity inherent in the best antisera might suggest that the need for a separation technique is redundant. However this must never be assumed without thorough testing, which must be done in the context of the use to which the assay will be put. Assays developed and evaluated for one use, for example in adults, must never be assumed to be equally applicable for other uses, for example in pediatric investigations, or using other matrices. Relying solely upon the specificity of a biological end-point is inherently risky. A simple separation procedure prior to assay can often give untold benefits and save the analyst from unnecessary embarrassment. With the increasing use of hyphenated LC- and GC-mass spectrometric methods, prior separation of estrogens by the classical methods is decreasing because both LC- and GC-methods provide high resolution which is

Table 8.2 Some modern methods of extraction of conjugated estrogens (see Table 8.1 for abbreviations used in this table)

Estrogen	Matrix	Extraction	Quantitation	Reference
E1 and E2 metabolism in accessory sex glands of male pig	Incubation media	Centrifuged, pellet extracted with MeOH; Sep-Pak C18 separation (unconjugated estrogens eluted with diethyl ether and conjugated eluted with MeOH); MeOH fraction solvolysed and Sep-Pak separation repeated; MeOH fractions from solvolysis hydrolysed with glucuronidase; a further Sep-Pak separation producing three fractions – free, sulfates and Glucuronides	Radioactivity in each fraction	Raeside et al. (1999)
5 estrogen sulfates	Human urine	96 well SPE and fractionation into mono- and di-sulfates	API 3000; LC-Turbospray(-) -MS –MRM	Zhang and Henion (1999)
E1 sulfate	Breast cancer tissue	See Table 8.1 in same reference; after ether extraction of free Es, EtOH was added to unextracted aqueous material, vortex and centrifuged; ethanol extract removed, dried and conjugate hydrolysed using sulfatase enzyme 48 h at 37°; ether extract and purification on LH20	RIA as estrone	Geisler et al. (2000)
Estrone sulfate	Male plasma	Extraction with cycloHx:EtAc(1:1) to remove unconjugated estrogens; aqeuous layer extracted with MeOH; separated on C18 Hypersil mini-columns, eluting E1-sulfate with appropriate volume of MeOH: water (25:75)	RIA and LC-MS/MS API 3000 LC-ESI(+)-MS/MS	Giton et al. (2002) – applied by Giton et al. (2008)

E3-3-Gluc and E3-16-Gluc	Human pregnancy urine	Urine filtered and loaded onto pre-column (*N*-vinylacetamide co-polymer Shodex MS Pak PK-2A), which absorbs the glucuronides; after washing the glucuronides are re-directed to the analytical column	LC-UV (254 nm) ThermoFinnigan LCQ LC-ESI(−)-MS/MS	Yang et al. (2003)
15 free and conjugated estrogens	River water samples	Filtered through glass fibre filter paper; SPE (Shodex Autoprep EDS1). Cartridges eluted with EtAc for free estrogens and with triethylamine:MeOH for conjugate fraction; free fraction further purified on a short Florosil column	Micromass Quattro Ultima LC-ESI(−)-MS/MS	Isobe et al. (2003)
E3-3G, E3-16G, E3-3S, E2-3G, E2-17G, E2-3S, E1-3G, E1-3S, E1, E2, E3	Human urine, waste water and treated and untreated sewage	Filtered where necessary; SPE on graphitized carbon (Carbograph 4) eluting with CH_2Cl_2:MeOH (4:1) for free steroids and then backflushing with CH_2Cl_2:MeOH (3:2) containing 10 mM sodium acetate for conjugates	Sciex API 2000 with TurboIon spray (-); LC-MS/MS (MRM)	D'Ascenzo et al. (2003)
E1, E2 and E3 plus their sulfate and glucuronide conjugates	Human serum	Extraction with $CHCl_3$; evaporation to dryness and taken up in LC elution solvent	Micellar electrokinetic chromatography (MEKC) using sodium cholate micelles. Detection by UV at 214 nm	Katayama et al. (2003)

(continued)

Table 8.2 (continued)

Estrogen	Matrix	Extraction	Quantitation	Reference
E1, E2 and ethinyl estradiol and sulfate and glucuronide conjugates	Treated and untreated sewage	Filtered through glasspaper, loaded onto Oasis HLB cartridges. Free estrogens eluted with EtAc and more polar estrogens, after washing, with 75%MeOH/water containing 2%NH4OH. Samples were further purified on weak anion exchange DEAE column and finally de-salted using Isolut C18 cartridges	Quattro LC with Z-Spray; LC-ESI(−)-MS/MS (MRM) for conjugates; Micromass LCT ToF MS monitoring negative ions for free estrogens	Reddy et al. (2005)
α-E2, β-E2, E1 and E3, ethinyl estradiol and sulfate and glucuronide conjugates	River sediments	Microwave assisted extraction with MeOH:water; loaded onto Waters Oasis-WAX or MAX cartridges; after washing estrogens of interest were eluted with 5%NH_4OH in water:THF (60:40)	Finnigan Ion trap MS (LCQ Advantage Max) using ESI(−)-MS/MS (SRM)	Matejicek et al. (2007)
E2 fatty acid esters	Adipose tissue and serum	Tissue homogenized (Ultra-Turrax); homogenate and serum extracted with Diethyl ether:EtAc (1:1); free and ester separated on LH20 column in small disposable pipettes; E2 assayed; esterified E2 released by alkaline hydrolysis and purified on LH20 column prior to assay	Time-resolved RIA	Badeau et al. (2007)
E1, E2, E3 and their sulfate and glucuronide conjugates	Human urine	Urine extracted using Oasis HLB cartridge, eluting with MeOH containing 2%NH_4OH; LC in RP and HILC (hydrophilic interaction liquid chromatography - increased ionization and improved resolution)	API 4000 Q TRAP with TurboIon spray; LC-ESI(−)-MS/MS	Qin et al. (2008a)

further enhanced by the selectivity of mass spectrometry. Recent publications include LC-MS/MS procedures which can be carried out on 200 μL serum with no pre-purification (e.g. Guo et al., 2008). Group separations prior to such assays may still however be useful.

Many of the historical methods used paper or thin-layer chromatography. It should be noted that particulate matter from both of these support phases, and material leached from them, sometimes presented serious problems of interference in the analytical methods, which in those days were usually based upon some form of saturation analysis, leading to non-specific blank problems, seldom encountered today with commercial immunoassays. Paper chromatography is no longer used as it is too cumbersome. Thin-layer chromatography still has wide applicability for steroid separations and there are many examples in the separation of androgens and estrogens where TLC has proved extremely valuable (e.g. Pahuja et al., 1995; Jellinck et al., 2001, 2005; Hobe et al., 2002; Gottfried-Blackmore et al., 2007, 2008). Zarzycki (2008) has described a simple temperature-controlled chamber for 2D HPTLC separation of estrone, estradiol, estriol and estetrol.

Although the use of pre-packed cartridges, particularly for silica and modified silica, is widespread (e.g. Labadie and Budzinski, 2005; Vulliet et al., 2007), home-made mini-columns, usually in Pasteur pipettes, of Sephadex LH-20 and Celite are still commonly used (see Table 8.3). Sephadex LH-20 is a cross-linked dextran polymer treated to alkylate most of the hydroxyl groups. When suspended in organic solvents, particularly alcohols, the gel swells. Separation can be achieved by washing the estrogens through the gel with a more organic solvent. In some solvents the gel floats but it may be successfully held down by a thin layer of washed silver sand. Sephadex LH-20 may not separate all estrogens into fractions that are discrete enough for analysis. Bolton and Rutherford (1976) for example showed that estradiol was not fully separated from 6-oxo-estradiol so this may cause problems in samples taken during pregnancy.

Molecular-sieve materials like Sephadex have been used to resolve mixtures of conjugated estrogens. Most applications have dealt with human pregnancy urine that contains relatively large quantities of conjugate. A review of isolation techniques using gel filtration was published by Ager and Oliver (1983) and Sjovall and Axelson (1984), whilst separation of individual estrogen glucuronides and sulfates was achieved by using diethylaminoethyl Sephadex (Musey et al., 1977; Fotsis, 1987; Fotsis and Adlercreutz, 1987).

Immunoabsorption at first sight offers a simple approach to purification, utilizing as it does the selectivity of antibodies. Gaskell and Brownsey (1983) first employed this approach using a solid-phase coupled antiserum for the extraction of 17β-estradiol from plasma, prior to GC-high resolution MS, although similar extraction methods (Glencross et al., 1981) had been described and used for ethinyl estradiol prior to immunoassay (Dyas et al., 1981). Such methods have not been used primarily because solid-phase antisera are expensive to produce in-house and are not available commercially. Thienpont et al. (1988) showed that for GC-MS analysis of estradiol in serum, reverse-phase C_{18} LC in addition to the usual extraction and Sephadex LH-20 purification gave results which were

comparable to those using immuno-extraction. Monoclonal antibodies for estrone and estradiol were chemically linked to controlled pore glass particles which when packed into a column acted as a specific immunoabsorption system for extraction of estrone, estradiol but not ethinyl estradiol from wastewater prior to LC-ESI-MS (Ferguson et al., 2001). Monoclonal antibodies to 4-hydroxy-E1(E2)-2N-acetylcysteine were produced and immobilized on an agarose bead column, which was used to 'capture' and pre-concentrate the analyte of interest from human urine samples (Markushin et al., 2005). A polyclonal antibody with affinity for estrone, estradiol and estriol was linked to Sepharose 4B and used to extract these three estrogens from pregnancy serum and urine prior to quantitation using micellar electrokinetic chromatography (MEKC) (Su et al., 2005). Early studies found that the removal of the analyte bound to immunoaffinity pre-columns was not without its difficulties (Farjam et al., 1991). There has been no subsequent report confirming this.

Some modern methods involving column chromatographic separations are listed in Table 8.3.

Electrophoresis has been used for separation of estrogens, particularly in the pharmaceutical industry and interested readers are referred to Chapter 12 that includes a section dealing with this topic. There is no doubt that micellar electrokinetic chromatography is an efficient way of separating estrogens (e.g. Chan et al., 1995; Ji et al., 1995) and a limited number of analytical methods for estrogen analysis in the biological field have been described for the measurement (e.g. 16 estrogens and their glucuronide and sulfate conjugates in human serum using MEKC and UV detection [Katayama et al., 2003] and measurement of estrone, estradiol and estriol in pregnant women's urine [Su et al., 2005]). MEKC has developed considerably over the past 10 years and it is now available on microchips (MCMEKC – reviewed by Kitagawa and Otsuka, 2008). However, only one application of MCMEKC in the estrogen field is quoted in this review (Collier et al., 2005). While MEKC may offer separatory advantages over GC and/or LC, the difficulty of interfacing with MS and MS/MS and thus its reliance on less sensitive methods of quantitation, mitigates against its use at the present time.

8.4 Analysis 2: Quantitation

Many of the early methods for estrogen quantitation are now obsolete, particularly those using low specificity ligands in competitive binding assays which required extensive time-consuming purification of the analyte. Nevertheless low specificity ligands can be used with LC separation to yield specific assays (Katayama and Taniguchi, 1993). The majority of assays of individual estrogens of clinical or biological significance where large numbers of assays are required still use some form of ligand binding assay, usually with an antibody – immunoassay. Many different labels have been used in this way such as ^{3}H, ^{125}I, enzymes, fluorescence, chemiluminescence (Wisdom, 1976; Doerr, 1976; Schuurs and van Weemen, 1977; Bosch

Table 8.3 Some column chromatographic separations of estrogens prior to quantitation (1999 onwards)

Estrogen analyte	Method of separation	Method of quantitation	Reference
E1 and E2 in tissue of coral, *Montipora verrucosa*	After extraction with diethyl ether, E1 and E2 were separated and purified using Celite columns eluting with EtAc in iso-octane	In house RIA	Tarrant et al. (1999)
E1, E2, E3 in urine	β-glucuronidase, SPE and Celite chromatography (method 1) or hydrolysis, then ether extraction (E1 and E2) and EtAc extraction (E3) and purification on Celite column (method 2)	In house RIA	Falk et al. (1999)
E1, E2 and E1-sulfate in human breast cyst fluid	After extraction with ether, free estrogens partitioned between MeOH:Hx; MeOH fraction separated on Sephadex LH-20; aqueous fraction after ether extraction subjected to sulphatase, ether extraction and LH-20 separation	GC-MS of TMSi ethers	Raju et al. (2000)
E2 fatty acid esters in serum and follicular fluid	Sephadex LH-20, Lipidex 5000, followed by Sephadex LH-20	Time-resolved FIA (DELFIA, Wallac)	Vihma et al. (2001); Vihma et al. (2003a, b) and Badeau et al. (2007)
E1 and E2 in serum	Esoterix Lab assay; after extraction with Hx:EtAc(4:1), washed with dilute base and purified on Sephadex LH-20 mini-columns	Esoterix RIA	Dorgan et al. (2002)
E2 fatty acid esters in lipoproteins	After EtAc extraction free E2 and esterified E2 were separated on LH-20. Esters were separated on silica gel TLC	Radioactive counting	Hockerstedt et al. (2002)
E1 & E1-sulfate in male plasma	After extraction with cyclohexane:EtAc, samples were re-constituted in MeOH:H_2O and purified on C18 Hypersil C18 minicolumns	RIA	Giton et al. (2002)

(continued)

Table 8.3 (continued)

Estrogen analyte	Method of separation	Method of quantitation	Reference
	Purification on Celite columns Fiet et al., (1994)	RIA	
E1, E2 and E1 conjugates in urine from *Macaca mulatta*	After hydrolysis with β-glucuronidase and extraction with diethyl ether; estrogens were separated on Celite columns, eluting with EtAc in iso-octane	In-house RIA	Shideler et al. (2003)
E1, E2α and β, 2-MeO-E1, 4-MeO-E2, 2-OH-E1, 2-OH-3-MeO-E2, 16-OH-E1, 2-MeO-E2, E3, 16-epi-E3 in urine	Use of Serdolit XAD-2 resin. Gluc-ase/ sulfatase. K carbonate added and extracted with EtAc; dried and TMS ethers made	GC-EI-MSD monitoring M+	Lee et al. (2003b)
Wide variety of phytoestrogens, lignans, estrogens and catechol estrogens in urine	Use of DEAE and QAE ion-exchangers in Cl^-, Ac^-, Bor-, Carb- and OH^- forms and Lipidex 5000 columns for purification	GC-MS as TMSi ether derivatives	Adlercreutz et al. (2004)
E1 and E2 in bovine plasma	After extraction with ether, purification on Sephadex LH-20 micro-columns	EIA	Takenouchi et al. (2004)
E1 and E2 glucuronides in sewage	After SPE using Oasis HLB cartridges, further purification using weak anion-exchange DEAE columns was used	LC-MS/MS and LC-ToF-MS	Reddy et al. (2005)
E2, E3, EE2 in lake and tap water and sewage	SPE using a monolith poly(acryamide-vinylpyridine-*N,N′*-methylene bisacrylamide) polymer syntheized inside a polyether ether ketone (PEEK) tube	LC with DAD and fluorescence detection	Wen et al. (2006); Fan et al. (2005)

(continued)

Table 8.3 (continued)

Estrogen analyte	Method of separation	Method of quantitation	Reference
Estrogen metabolites in urine	Enzyme hydrolysis and SPE on SepPak C18, purification on QAE-Sephadex cartridges in acetate form. Further separation on QAE-Sephadex cartridges in borate form into catechol and non-catechol estrogens	GC-MS using SIM as TMSi ethers	Knust et al. (2007)
E1, E1-S and E2 in male serum	Solvent extraction, Celite chromatography using ethylene glucol as stationary phase, eluting with 40%EtAc in iso-octane; extraction, silica gel chromatography, derivatization; C18 SPE	RIA; GC-MS; LC-MS/MS	Hsing et al. (2007)
Estrogenic activity in sediment extracts	Freeeze-dried sediment was extracted with Hx:Ac(3:1) and CH_2Cl_2:Ac (3:1) using Soxhlet and Accelerated Solvent Extraction; purified on polystyrene-diphenylbenzene GPC columns	GC-ECD or GC-NCI-MS or GC-PCI-MS; tested for estrogenicity using *in vitro* ER-CALUX reporter gene assay	Houtman et al. (2007)
E1, E2, E3, EE2 in environmental water samples	Use of stir bar extraction using polysiloxane/β-cyclodextrin coating	LC-UV detection	Hu et al. (2007)
E1 and17β-E2 in milk	EtAc extraction followed by Sephadex LH-20 to separate E2 from cholesterol and E1	RIA	Pape-Zambito, Magliaro and Kensinger (2007, 2008)
E1, E2 and E1-sulfate in plasma	Ether extraction. E1 and E2 separated by Sephadex LH-20. E1-sulfate hydrolysed, E1 converted to E2, purified on LH-20 columns	RIA	Geisler et al. (2008) modified from Geisler et al. (2000).

et al., 1978). Separation of free and bound analytes uses dextran-coated charcoal, second antibody precipitation, solid-phase (heterogeneous) assay or some form of manipulation that avoids the separation step (homogeneous assays), for example, proximity scintillation assay or delayed fluorescence. An important and increasing number of assays are performed by LC-MS and GC-MS (reviewed by Giese, 2003). Indeed GC-MS is often referred to as the 'gold standard' for steroid assays. Increased throughput that has been developed for these MS techniques, particularly LC-MS methods, makes them increasingly suitable for routine assays of multiple samples with a range of estrogens in each sample using stable isotope labelled estrogen standards to correct for losses during purification (e.g. Sepkovic et al., 1994). Even with the advent of these new MS methods, immunoassay is still widely used for many purposes.

8.4.1 Immunoassay

The development of immunoassays for steroids started with the pioneering studies of Abraham (1969) and Midgley et al. (1969). Following their reports there was an explosion of new developments in the field. Since the early methods are no longer in use and have been supplanted by continual improvement, the period before 1975 will not be included in this chapter. For those interested in this earlier period, information can be found in Oakey and Holder (1995).

The variety of techniques and endpoints possible in immunoassays has been reviewed by Gosling (1990) and Giese (2003). It is noticeable that an estimation of steroid hormones and in particular of estrogens, in many but not all biological samples remains a formidable task. The combination of the small non-immunogenic nature of these molecules, the presence of metabolites of very similar structure and their low concentrations in many biological fluids still presents a serious challenge to the analyst.

The binding techniques in use are basically of the competitive type, in which label and analyte compete for a restricted number of binding sites on the binder. Although antisera have been obtained which recognize different sites on the steroid molecule, two-site binding assays of the non-competitive immunoradiometric type have not yet been developed. It may be that sites on the steroid molecule are too close to allow simultaneous binding of two large antibody molecules. In other words, when the steroid is bound to, say, the capture antibody, steric hindrance may restrict binding of the other, labeled antibody to the steroid. In the ELISA assay the analyte and the compound coupled to phosphatase compete for binding to the surface binder on the bottom of the wells on the ELISA plate. After the plate is washed with buffer *p*-nitrophenylphosphate is added and the color development is measured spectrophotometrically (Klug et al., 1994). Although some progress was made toward the development of a non-competitive two-site binding assay (idiometric) for estradiol (Barnard and Kohen, 1990; Barnard et al., 1991) there has been no serious interest in this

method for technical reasons, although a number of assays using this approach have been described, for serum estradiol (Mares et al., 1995) and urine estrone-3-glucuronide (Barnard et al., 1995).

A novel technique for separation and determination of natural and synthetic estrogens by the use of a new microemulsion has been described by Tripodi et al. (2006). Microemulsions are dispersed systems with nanometer sized droplets of an immiscible liquid that are stabilized by surfactant and co-surfactant molecules. The authors separated and measured estriol and estradiol valerate extracted from pharmaceutical formulations in a tablet form using a microemulsion method after verifying the specificity, accuracy and reliability.

Antisera with the ability to bind estrogens can be produced by immunizing animals with estrogens covalently linked to large proteins, referred to as estrogen–protein conjugates. The feasibility of this approach was demonstrated by Abraham (1969) and by Midgley et al. (1969). Antisera raised in this way either directly or through monoclonal antibody technology are now common binding reagents in many assays. There are, however, certain limitations regarding the specificity for steroids of antisera obtained in this way. In considering the specificity of estrogen assays, it is perhaps worth noting that the degree of specificity required is rather exceptional. A binding site for estrogen on the antibody, for example, may be required to distinguish between 17β-estradiol and 17α-estradiol which differ only in the spatial arrangement of a single hydroxyl group, or between estradiol and estrone, which differ only by the presence of a hydroxyl group or an oxo group at C-17. These requirements, which are commonly seen in the steroid field, appear to be much more stringent than those for polypeptide hormones in which the epitopes are considerably larger.

Immunization of whole animals with estrogen–protein conjugates produces (polyclonal) antisera that appear to contain populations of different species of antibodies each with a range of specificities and affinities for the hapten (estrogen), structurally related estrogens, the chemical bridge between hapten and protein, and the protein itself. These populations appear to vary with time between immunization and collection (Abraham, 1974) and, of course, between different animals even of the same species. Consequently, each bleed of serum must be considered as a separate and somewhat different entity. It is often stated that the specificity of antisera to haptens and related steroids is dependent to a large degree on the position in the hapten at which the bridge to the immunogenic protein is formed as well as the structure of the bridge. It may also affect the choice of bridge structure used for the tracer in estradiol assays (Tiefenauer et al., 1988).

The use of such polyclonal antisera has practical consequences as the specificity of these antisera can be changed by alterations in analytical technique. For example, incubation time (Vining et al., 1981), order of addition of labeled and non-radioactive steroids (Pratt and Woldring, 1976), use of solid-phase separation rather than dextran-coated charcoal or double-antibody technique, or avoidance of a separation step, may all alter specificity. Presumably, this is brought about through changes in the relative importance in the binding reactions of different

members of the variety of antibody species in the population. Thus, once an analytical system has been defined and validated, rigorous adherence to the selected protocol is mandatory in order to maintain specificity, accuracy and sensitivity and this requires storage of large quantities of polyclonal antisera. With the introduction of monoclonal antibodies for estrogen immunoassay Kim et al. (1982); Kohen et al. (1983); Ghosh (1988); Mandal and Ali (1988); O'Connor et al. (2003), which contain only one species of antibody, this feature should no longer present a problem. The 2-hydroxyestrone monoclonal antibody with the link at C-17 developed by Klug et al. (1994) shows no cross-reactivity to almost all steroids but recognizes 2-OH-E1 and 2-OH-E2 equally. The 16-OH-steroid monoclonal antibody is highly specific against steroids differing in ring D but does not distinguish between 16-OH-E1 and 16-OH-E1- 3-glucuronide (Bradlow et al., 1998) – this method has been validated by Falk et al. (2000). For a more detailed account of the structural aspects of the specificity of antibodies to steroids, readers are referred to Franek (1987). Miller and Valdes (1991) also discuss methods to minimize cross-reactivity.

Despite some confusion in the trade literature, 'monoclonal' does not necessarily imply monospecific in the sense of recognition of a single species of ligand. The binding site on a monoclonal antibody may accommodate a variety of structurally related steroids. There is no clear evidence that monoclonal assays are more specific than those using polyclonal antisera. What clones may offer is a continuous supply of antiserum of known affinity and specificity, which has obvious commercial advantages and causes fewer problems for the user. Antisera, whether monoclonal or polyclonal, designed to bind unconjugated estrogen, may often bind the corresponding glucuronide or sulfate as well. This is particularly so if the glucuronic acid or sulfuric acid residue is attached to the functional group in the natural steroid which has also been used to link the hapten to the protein (Kellie et al., 1972; Bradlow et al., 1998). This problem is evident too in direct assays (Diver, 1992).

General experience shows that, despite best efforts to select the optimum position for conjugation of estrogen to immunogenic protein, the antisera evoked are inevitably capable of binding more than one estrogen. Therefore, in order to ensure the greatest analytical specificity, it is often necessary to include in the method a step by which the required estrogen is isolated before immunoassay. In some ways, the high degree of specificity shown by antisera has rendered unnecessary the incorporation of the various chromatographic techniques available for the resolution of individual estrogens before less specific competitive binding assays. However, for analytical work of the highest quality, inclusion of a solvent extraction (Ankarberg-Lindgren and Norjavaara, 2008), solid-phase cartridge extraction (Martinetti et al., 1997) or a chromatographic step (Giton et al., 2002; Shideler et al., 2003; Lee et al., 2006) enhances the assurance of specificity of the assay and improves sensitivity, although few of the current routine assays, automated or manual, use such a protocol. Some column chromatographic purification procedures prior to immunoassay are listed in Table 8.3. Non-extracted (the so-called direct) immunoassays for estrogens, which may be of value in adults are

usually not useful for estrogen assay in young children where estrogen levels are low (Bay et al., 2004; Toniolo and Lukanova, 2005; Albrecht and Styne, 2007). The same problem arises when attempted to measure the low levels of estrogen in patients receiving aromatase inhibitors (Lønning and Geisler, 2008a, b) or in improving the value of estradiol measurements in the assessment of breast cancer risk or fracture risk (Santen et al., 2008). LC-MS/MS or cell-based recombinant assay systems (*vide infra*) are becoming valuable in this area (Wang et al., 2005; Santen et al., 2007a, 2008).

The more manipulations incorporated into an immunoassay protocol, the greater the chance of manipulative loss. In assays that involve chromatography, it is possible to introduce a known quantity of a radioactive analyte of high specific radioactivity into the fluid to be analyzed. Manipulative loss can then be assessed by measuring recovery of the radioactive isotope in a portion of the sample at the stage immediately before radioimmunoassay, i.e. after extraction, chromatography, evaporation of solvent and reconstitution in assay buffer. The quantity of analyte determined by radioimmunoassay can then be corrected for this manipulative loss. Some adjustment may be required to the amount of label used for radioimmunoassay or a correction applied. The radioactive isotope used to monitor recovery in this way is invariably tritium as tritium labeled steroids are available with high specific activity. Steroids referred to as labeled with ^{125}I usually have the iodine in a histamine or tyrosine residue attached to the steroid itself. The chemical behavior of these iodinated derivatives does not mimic closely the behavior of natural estrogen, which rules out their use to monitor loss. ^{14}C label has an inherent specific radioactivity too low for use as a monitor. When mass spectrometry assays are used the use of stable isotope labeled recovery standards using deuteriated or even ^{13}C- labeled analyte(s) makes for easy quantitatition. Blackwell et al. (2003) have also developed an improved assay suitable for use at home to detect the time of ovulation. Table 8.4 lists some commercial immunoassays available today and information about suppliers.

8.4.1.1 Novel Applications

The non-competitive assays of the type described by Barnard and Kohen (1990) have not yet been exploited but alternative end-points have enabled some estrogen assays to be simplified. An enzyme-linked immunosorbent assay (ELISA) was used by De Lauzon et al. (1992) to predict ovulation in women. The antigen was immobilized as a spot on a nitrocellulose membrane. The spot was immersed in urine in the presence of an antibody that recognized free estrogens and some of the 17-glucuronide and sulfate conjugates. A peroxidase-labeled second antibody allowed a colorimetric discrimination to be made between basal estrogen concentrations and the pre-ovulatory surge. This type of assay has the potential for testing at the home of patients or application in areas where there is limited access to sophisticated instruments or resources. Similar work by Lasley et al. (1991) showed that blood estradiol and urine estrone conjugates in paired samples gave similar

Table 8.4 Some available commercial immunoassays for estrogens in biological fluids

Estrogen	Type of immunoassay[a]	Source and address	Website	Information/comment
E2 and E3	EIA	Cambridge Bioscientific, CB5 8LA, UK	www.bioscience.co.uk	Antibody supplied by Affinity BioReagants (bought by Thermo Fisher Scientific in July 2008)
E2, E3	EIA [SE] ELISA [SE]	Cayman Chemical, Michigan, 48108 USA	www.caymanchem.com	Can download .pdf file on method from site
E1, E2 and E3	EIA [SE] ELISA [SE]	Oxford Biomedical Research, Maryland, USA	www.oxfordbiomed.com	Product spec sheets available
E1, E2 and E3	EIA [SE]	IDS Ltd, Tyne and Wear, UK NE35 9PD	www.idsltd.com Assay kits supplied by Cayman Chemicals	Product inserts as .pdf files
E2 and unconjugated E3	RIA	Immunotech (Beckman Coulter) Praha, Czech Republic	www.immunotech.cz	Inserts available as .pdf files
E2	Homogenous time-resolved fluorescence IA	Cisbio Assays, 30204 Bagnols-sur-Ceze, France	www.htrf.com	Method details available as .pdf files
E1, E1-sulfate, E2, E3	RIA RIA/ELISA	DSL, Webster, Texas 77598-4217, USA	www.dslabs.com	Not much information on website
E1, E2 and E3	EIA, ELISA and RIA	BioSource Europe SA, Nivelles 1400 Belgium	www.biosource-diagnostics.com	Summary of method + .pdf files of package inserts available
E2 and Ethinyl E2	EIA (RIDASCREEN) [SE]	R-Biopharm AG. 64293 Darmstadt, Germany	www.r-biopharm.com	Little info on website
2-hydroxy-E1 and 16-OH-E1 in urine	EIA (monoclonal Abs) ESTRAMET	Immunacare PA 19422, USA	www.immunacare.com	Method and kit inserts available
E2	RIA (coated tube) RIA (double Ab)	DiaSorin Sp.A 13040 Saluggia, Italy	www.diasorin.com	No package inserts available on website
E2	Double antibody-IA using Eu-labeled E2. Time resolved fluorescence	Perkin-Elmer Inc., MA 02451, USA	www.perkinelmer.com	Same protocol as automated AutoDELFI system

[a]These are all 'Direct' assays, though protocols of assays marked [SE] make provision for solvent extraction before assay if required.

patterns through the menstrual cycle. Other workers have used enzyme labels to provide rapid though insensitive quantitative values for estradiol in multiple serum samples (Bouve et al., 1992).

Alternative labels have provided very sensitive end-points for estrogens. Nicolas et al. (1983) achieved femtomolar (0.27 pg/L) sensitivity for estrone and estradiol with a bioluminescent assay. Time-resolved fluorescence with rare-earth chelate labels has also been used (Barnard et al., 1989). Indeed, this label has been used as a basis for both automated (AutoDELFIA) and manual (DELFIA) immunoassay systems for estradiol. Choosing appropriate rare-earth chelates with differing emission spectra has facilitated the simultaneous measurement of more than one ligand (Ito et al., 1999).

Most immunoassays are carried out on serum/plasma, which of course require separation of the red cells and/or protein. A homogenous immunoassay for estradiol in whole blood involving upconversion fluorescence resonance energy transfer (FRET) has been described (Kuningas et al., 2006, 2007). Although published in 2006/7, the authors appear not to have utilized this method in any further clinical studies, though further discussion of improved methodology has been published (Kokko et al., 2007, 2008).

8.4.1.2 Automated Immunoassays

The use of non-isotopic labels and coupling of antisera to suitable matrices, together with modern precision engineering and computers, has made the automation of immunoassays a viable proposition (Wheeler, 1992, 2001). Automation, while valuable in laboratories with high throughput requirements, necessarily generates large volumes of data, which require careful scrutiny to reduce to a minimum the risk of unrecognized anomalous results.

These assays suffer from the problems associated with automation, such as dead volumes and electromechanical failures. Most machines are ‘closed’ systems – they can only be used with the reagents and tubes or cuvettes from the supplier. Virtually all machines measure only one estrogen, estradiol, at present. Commercial considerations determine the choice of analytes available. Some manufacturers are prepared to offer assistance with the development of novel analytes, especially on the open systems. Most of these systems offer the ability to measure other analytes, frequently on the same sample. They offer walk-away automation and standard curves that are stable for long periods, removing the need to run complete curves with every assay. In return, the customer is expected accept any problems of specificity or lack of sensitivity. Indeed, several analyzers have sensitivity for the measurement of estradiol that makes it difficult to use for pediatric specimens or in post-menopausal women. The user is frequently required to accept the validity of the standard curve that is run internally in a microprocessor or generated at the factory and adjusted in each run. This may not be valid under all circumstances.

8.4.1.3 Interference in Immunoassays

Modern immunoassays are susceptible to interference though not to the same extent as in the past – indeed it is now considered to be a rare event but even so, it must not be ignored (Marks, 2002). The days of non-specific interference from lipid and from materials eluted during the course of purification prior to immunoassay are long gone to be replaced by other problems, for which the analyst must be alert. It is not entirely clear what 'interference' means in this context, but we have taken it to include any factor which leads to inaccurate or imprecise output. It therefore includes, other estrogens (e.g. Cao et al., 2004), steroids or indeed drugs (e.g. Tejada et al., 1998; Sinicco et al., 2000) which may cross react with the antibody (ies) (i.e. lack of antibody specificity), presence of specific interfering compounds which are declared by the manufacturers of commercial immunoassays (e.g. AutoDELFIA indicate that plasma containing EDTA or citrate is unsuitable as these compounds form chelates with europium), variations by users in the protocol which may affect the results and hetero- or auto-phile antibody interference (reviewed generally by Levinson and Miller, 2002). There has been a case report of a monoclonal gammopathy interfering with an estradiol assay during the monitoring of fertility treatment (Kairemo et al., 1999). While such interference is now relatively rare and, even when it does occur is rarely likely be significant (Anckaert et al., 2006), many more assays are being carried out and thus there may be, in absolute terms, more erroneous results, which may give rise to serious errors. Proper quality assurance measures must be applied to all immunoassay methods, whatever their source. Usually in-house immunoassays are validated before use but claims from suppliers/manufacturers of immunoassay kits for estrogens, like any other steroids, of exceptional sensitivity or specificity must be tested rigorously. The data provided must be scrutinized and the source of the data identified. Claims may often be shown to be correct when investigated but care must be taken to establish the nature and source of the samples from which the data was derived. Validating claims of accuracy and specificity should always employ comparison of immunoassay results with results from accepted reference methods or use biological material validated by such a reference method. It is accepted today that such reference methodology should involve mass spectrometry (GC-MS or LC-MS or -MS/MS). Comparisons of one immunoassay against another are really not acceptable as a proper validation, although such comparisons do provide assurance of continuity and/or comparability. However carefully quality assurance is carried out, it does not provide complete assurance against single anomalous results occurring within a batch of assays – recognition that this has occurred requires vigilance by the analyst. In endocrinology, this falls to the clinical endocrinologist who must guard against anomalous or unexpected results and maintain a high index of suspicion (Jones and Honour, 2006) – does the result fit with the clinical situation of the individual patient? Some references to validation of automated systems are listed in Table 8.5. Sections 8.6 gives some external quality assurance information on some estrogen assays and Chapter 13 also provides data about the external quality assessment of steroid assays.

Table 8.5 Some automated systems for serum/plasma estradiol measurement (references given are, wherever possible, to full evaluations of these methods, often by comparison of results with other immunoassays or mass spectrometric methods). Information about these assays usually comes from manufacturers

Instrument	Principle	Manufacturer	Reference
ACCESS	Antibody-coated paramagnetic microparticles with alkaline phosphatase catalysed chemiluminescent end-point	Beckman-Coulter, CA92835-3100, USA, www.beckmancoulter.com	Yang et al. (2004)
ADVIA CENTAUR (Classic, -XP and -CP	Antibody-coated paramagnetic microparticles with chemiluminescent (acridinium ester) end-point	Siemens Healthcare Diagnostics Inc., NY 10591-5097, ILL 60015-0778, USA, www.diagnostics.siemens.com	Yang et al. (2004), Massart et al. (2006),
ACS-180	No longer sold but existing systems supported. Same principle as Centaur systems		Taieb et al. (2003a),
IMMULITE, IMMULITE-2000 and 2500	Antibody-coated beads with alkaline phosphatase catalysed chemiluminescence end-point		Dancoine et al. (1997), Rodriguez-Espinosa et al. (1998), Tello and Hernandez (2000), Elmlinger et al. (2002), Yang et al. (2004), Soldin et al. (2005), Arslan et al. (2006)
ARCHITECT i2000	Antibody-coated paramagnetic microparticles with chemiluminescent end-point (CMIA)	Abbott Laboratories, ILL 60064-3500, USA www.abbottdiagnostics.com	Taieb et al. (2003b, 2007), Sluss et al. (2008)
AxSYM	Antibody coated microparticles with alkaline phosphatase generated 4-methyl-umbelliferone fluorimetric end-point	Architect and AxSYM package inserts etc. available as .pdf files at www.abbottdiagnostics.co.uk	Reinsberg and Jost (2000), Cao et al. (2004), Dighe et al. (2005)
AutoDELFIA	Competition between Eu-labeled E2 and serum E2 for polyclonal E2 antibody. 2nd antibody bound to coated microtitre plates. Time-resolved Eu-fluorescence end-point	Perkin-Elmer Inc. MA 02451, USA www.perkinelmer.com	Yang et al. (2004), Scalas et al. (2007), Ankarberg-Lindgren and Norjavaara (2008)

(continued)

Table 8.5 (continued)

Instrument	Principle	Manufacturer	Reference
ELECSYS or Cobas e	Competition between serum E2 and Ruthenium labeled E2 complex for biotinylated E2 antibody. The immunocomplex binds via streptavidin to the magnetic microparticles which are the captured magnetically on the surface of the electrode. Application of voltage induces chemiluminescence	Roche Diagnostics GmbH, D-68298 www.roche.com	Yang et al. (2004). Anckaert et al. (2002) for comparison of ELECSYS E2 II with VIDAS E2
VITROS ECi	Competition between serum E2 and an E2-horseradish peroxidase E2 conjugate for heterogenous biotinylated antibodies. The antigen/antibody complex is captured by streptavidin on the wells. Washing removes unbound. End point is a luminescent reaction catalysed by the bound HRP oxidizing luminol derivative, the light being increased by the electron transfer agent (substituted acetanilide)	Ortho-clinical Diagnostics Inc., NY 14626, USA www.orthoclinical.com	Lee et al. (2006)
VIDAS Mini, 30 or 60	Antibody-coated reagent strip (SPR) – competition between estradiol in serum and alkaline phosphate-estradiol conjugate. Fluorescence end-point (4-methyl-umbelliferone) [ELFA]	bioMerieux Inc. NC 27712, USA www.biomerieux-usa.com	Anckaert et al. (2002). Vidas E2 compared with ELECSYS 2010 E2 II
AIA-360	Antibody coated onto magnetic beads – competition between estradiol in serum and alkaline phosphate-estradiol conjugate. Fluorescence end-point (4-methyl-umbelliferone)	Tosoh Bioscience Inc., CA 94080, USA www.tosohbioscience.com	No literature found
LIAISON – Estradiol	Competitive immunoluminometric assay using Ab-coated magnetic microparticles	DiaSorin SpA, 13040 Saluggia, Italy www.diasorin.com	No literature found on website

With the development of non-extraction (direct) methods for steroids aimed at improving precision, eliminating the use of volatile, inflammable solvents and making automation feasible, came the problem of interference from endogenous binding proteins (e.g. Key and Moore, 1988; Masters and Hahnel, 1989; Micallef et al., 1995). It is also necessary to produce calibration standards in a medium that mimics the biological fluid being analysed. Any fluid used for calibration is unlikely to be exactly identical in terms of binding proteins with the sample, particularly as biological and pathological variations mean that no two samples are alike. In practice this problem has been overcome or minimized by incorporating into the assay protocol agents such as danazol, 5α-DHT or mesterolone (e.g. DeBoever et al., 1990) to block or denature the endogenous binding proteins. There is an additional difficulty in these direct systems, which arises with the use of the ligand bearing a large label, e.g. ^{125}I. In this case, Masters and Hahnel (1989) have demonstrated that the labeled ligand may bind preferentially to binding reagent added in the form of an antibody, rather than to the endogenous binding globulin. In contrast, non-labeled analyte will be distributed more equally between added antibody and endogenous binding globulin. If the quantities of endogenous binding proteins in unknown and calibration standards are different, then accurate comparisons cannot be made. A choice of a more suitable antibody that eliminates or minimizes this effect must be made. 'Direct' assays also give rise to possible interference from other steroids (i.e. lack of specificity or sensitivity) and, while such assays for estriol in pregnancy or estradiol for fertility monitoring are convenient and quick, they work because the levels of interfering steroids are sufficiently low to be ignored. Care must be taken in utilizing such immunoassays for other clinical situations where the concentration of analyte is much lower and interfering estrogens become highly significant. There are countless examples of this problem (*vide supra*). Thus, for analytical work of the highest quality, inclusion of an extraction or separation step enhances the assurance of the specificity of the assay and by removal of low levels of interference, improves sensitivity. Examples are discussed above and purification procedures used today for this purpose are summarized in Table 8.3.

8.4.2 Gas–Liquid Chromatography (GC) and GC-MS

Gas-liquid chromatography (GC) has been widely applied to the measurement of selected estrogens in urine, blood, amniotic fluid and as environmental contaminants (e.g. Croley et al., 2000; Lopez de Alda and Barcelo, 2001b; Petrovic et al., 2002, 2004; Diaz-Cruz et al., 2003; Hajkova et al., 2007; Stuart, 2007). Use of GC in analysis of estrogens in environmental samples will be discussed in more detail in Section 8.5. Before the development of sensitive and specific immunoassays, GC with electron capture (EC) or flame ionization (FI) detectors offered one of the most sensitive methods of analysis. However, extensive purification procedures were required before introduction of the sample to the column, particularly with

electron capture detection systems, which were susceptible to poisoning and were not without radioactive hazard – disposal of ^{63}Ni sources pose a problem. Over the last 20 years, there has been very little use of GC except when coupled to mass spectrometry (GC-MS). A PubMed search over the last 20 years identified only three descriptions of use of GC without MS for estrogen analysis – GC-EC to measure 2- and 4-hydroxylase activities in microsomal preparations but, in addition, mass spectrometry was used for identification of the catechol estrogens formed (Roy et al., 1991), using thermionic and FI detectors to measure estrone and estradiol in ovarian follicular fluid (Vanluchene et al., 1990 – similar results were obtained using an LC-UV method – Vanluchene et al., 1991) and in measuring formation of 2- and 4-hydroxyestradiols in rat liver microsomes *in vivo* with minimum pre-purification, derivatizing as *t*-butyldimethylsilyl ethers (Pinnella et al., 2001). There would seem today to be no reason for using GC coupled to any detection system other than MS – one example of the failure of EC detection is given by Kuch and Ballschmiter (2001), who attempted to use GC-EC for the measurement of estrogens in environmental water samples but found that the background noise was too high and thus moved to GC-NCI-MS. GC, although destructive to many steroid analytes and especially their glucuronide and sulfate conjugates, provides high selectivity because of the use of capillary columns (internal diameter 0.2 mm) with the stationary phase fixed to the inner capillary wall and provides high resolving power. GC analysis of estrogen conjugates requires prior hydrolysis, usually by sulfatases and β-glucuronidase systems. The unconjugated steroids are re-isolated by further chromatography through Sep-Pak and Lipidex columns. Derivatives (methyl oximes and trimethyl silyl ethers) are prepared and then submitted to capillary GLC. An internal standard is added early in the procedure, and a set of calibration standards is analyzed in the same batch for reference purposes. Steroids are identified by retention time and by on-line mass spectrometry. Optimization software is available to assist in choosing the best conditions for separation (Jayatilaka and Poole, 1993). Urine estrogens were measured by Pfaffenberger and Horning (1975), Fotsis and Adlercreutz (1987) and Fotsis (1987). GC using flame ionization detection is still used in some clinical laboratories for the production of steroid 'profiles' but these appear to have little application to estrogens (Taylor, 2006) and would be improved if the FID was replaced with MS. GC-MS applied generally to steroids was reviewed by Wolthers and Kraan (1999), Shimada et al. (2001) and GC-MS analysis was considered by Giese (2003).

Although GC-MS was initially applied to the measurement of 11 related estrogens (in effect a profile) in a number of different body fluids more than 30 years ago (Adlercreutz et al., 1974), subsequent methods concentrated on specific estrogens of clinical interest – for example, estradiol (Siekmann, 1984; Thienpoint et al., 1988; Dehennin, 1989a) and estriol (Dehennin, 1989b). The advantage of MS is that it can confirm the structural identity of the compound being measured as well as quantitation (i.e. it provides significant increased specificity). GC-mass spectrometers are now widely available for bench-top use and are simple to operate. They can be used to process significant numbers of samples, but for the best resolution and specificity, they still require derivatization before analysis. While electron impact ionization,

monitoring positive ions, is widely used, sensitivity can be improved by judicious derivatization and use of other means of ionization and use of negative ions (e.g. Xiao and McCalley, 2000; Courant et al., 2007b; Santen et al., 2007a). For steroids the use of trimethylsilyl ethers has been widespread and a number of silylation reagents have been evaluated by Ding and Chiang (2003) who also provide electron impact mass spectra for nine of the most common estrogens and interpretation of the fragmentation seen in these spectra. Shareef et al. (2004) reported problems when using common derivitizing reagents, *N*,*O*-bis(trimethylsily) acetamide (BSTFA) and the tertbutyldimethylsilyl analog, which converted ethinyl estradiol into estrone during silylation. A subsequent publication (Shareef et al., 2006) showed that this unwanted conversion was a function of the reaction conditions (e.g. temperature and solvent in which the silylation was carried out). This reinforces the need to examine carefully all reagents and solvents used for derivatization to ensure that under the conditions used the compounds of interest are indeed converted to the expected derivatives.

Straightforward GC-MS using EI(+) and single-ion monitoring has detection limits of 1–10 ng/mL, depending on the estrogen being measured but can be improved by use of negative ion CI. Xiao and McCalley (2000) used GC-NCI-MS to measure the concentrations of a number of estrogens in human urine and having studied the efficacy of a number of derivatives (trifluoroacetyl-, heptafluorobutyryl-, pentadecafluorooctanyl- and perfluorotolyl-) in their system, concluded that pentafluorobenzoyl derivatives gave the best results, claiming that this was the first report of the use of these derivatives in this system for estrogens, which seemed to be correct. Using a signal to noise ratio of 3 as a measure of the limit of detection, they estimated that using 10 mL of urine the LOD was 100 ng/L. This method, modified to use tandem MS/MS, was recommended by Santen et al. (2007a) as the best method for measuring plasma estradiol for monitoring aromatase inhibitor treatment, quoting an achievable sensitivity of 0.125 ng/L when using 4 mL of plasma for analysis, although this figure was extrapolated from a LOD of 0.6 ng/L using 1 mL of serum (Wang et al., 2005). A very similar method was described by Courant et al. (2007b). This is also a GC-MS/MS or GC-HRMS method for use in plasma from prebutertal children intended to monitor effects of endocrine disrupters. This method used a Zebron ZB-5MS column connected to a Quattro MicroGC triple quadrupole with negative-ion CI using methane as the reagent gas and [3,4-$^{13}C_2$]-estradiol as an internal standard. GC-HRMS used a JEOL SX102A electromagnetic (i.e. dual focussing) mass analyzer, 2 mL serum samples, beta-glucuronidase overnight at 52°C. Samples extracted with 2 × 10 mL ether. Evaporated to dryness, re-suspended in MeOH: water (0.5:9.5 and applied to a ChromP SPE (500 mg) column, wash with *n*-Hx and elute with Hx:diethyl ether (7:3). Dryness and re-dissolved in dichloromethane: Hx (2:3) and added to a SiOH SPE column, washed with Hx:EtAc(60:40) and eluted with Hx:EtAc(85:15). Samples were then separated by HPLC on an (EC-Nucleosil 100-5 N $(CH_3)_2$-propyl column, collecting the appropriate fraction, which was then derivatized as perfluorobenzoyl derivatives using PFBBr in acetonitrile at 60°C for 40 min. Reagent removed and further TMSi derivatization using BSTFA at 60°C for 40 min. The method, developed for routine application, to measure estrone and 17α/β-estradiol used GC-NCI-MS/MS, monitor-

ing the $[M\text{-}PFB]^-$ ion in MS/MS but very little fragmentation of the parent ion occurred even at 40 eV collision energies. LOD (1 ng/L) and LOQ (2 ng/L) using 2 mL of plasma were reported. Figure 8.9 illustrates the sensitivity of this method comparing the product ion mass fragmentography from 500 fg of 17α- and 17β-estradiol as 3,17-di-TMS derivatives with the response from the 3-PFB-17-TMS derivatives. This demonstrates a 2.5-fold increase in S/N ratio by simply altering the derivative used. This method was used for measurement of *inter alia*, estrone, estradiol and estriol in milk, egg and meat samples (Courant et al., 2008). The GC resolution of 17α/17β-estradiol shown in Fig. 8.9, should be compared with the same separation by reverse-phase LC (Fig. 8.11) – GC achieving complete resolution, whereas LC does not.

Zacharia et al. (2004) used tetrafluorophenyl derivatization to measure estradiol as well as catecholestradiols and methoxyestradiols in rat plasma. This method was otherwise similar to the Sepkovic method (Sepkovic et al., 1994) using GC-MS, rather than GC-MS/MS or HRMS. As a result the sensitivity for estradiol appeared to be around 0.25 ng/mL, which is less sensitive by more than two orders of magnitude than that claimed by Wang et al. (2005) and Santen et al. (2007a).

Adlercreutz et al. (2004) have described an ID–GC-MS-SIM which can simultaneously measure both estrogens and phytoestrogens in a small urine sample. The procedure uses deuteriated analogs of all the estrogen analytes. This is an up-dating of the original method described by Fotsis and Adlercreutz (1987) and demonstrates, over the intervening 17 years, a four- to five-fold increase in sensitivity, primarily due to improvements in GC-MS systems and greatly improved data handling facilities.

While, at first sight, GC-MS and GC-MS/MS might appear to offer greater sensitivity than LC-MS/MS, simply because ionization methodology is usually more efficient, in fact the sensitivities are very comparable. The advantages of the various mass spectrometric methods for estrogen analysis has been reviewed but the disadvantage of derivatization required for GC procedures tipped the balance toward LC-MS methodology (Diaz-Cruz et al., 2003). However in the last 5 years that advantage has been whittled away with an increasing realization that efficiency of ionization by the 'soft' techniques used in LC-MS can be improved by analyte derivatization (e.g. Higashi et al., 2006).

Proper validation of all assay methodology is required before reliance can be placed on results generated by such a methodology and mass spectrometry is no exception. The insertion of 'MS' after a GC or LC method guarantees nothing. The mere assertion that a GC-MS method is 'fully validated' does not automatically confer reliability on results produced. Readers are advised to scrutinize carefully ALL methodology used to generate results and, where relevant, require quality assurance data. Estradiol levels in men (Mellstrom et al., 2008) and reproducibility of sex steroid assays by RIA and GC-MS (Hsing et al., 2007) both interesting and potentially valuable papers, rely on a GC-MS assay which is claimed to be 'fully validated' (Mellstrom et al., 2008) but the references given to support this claim do not in fact

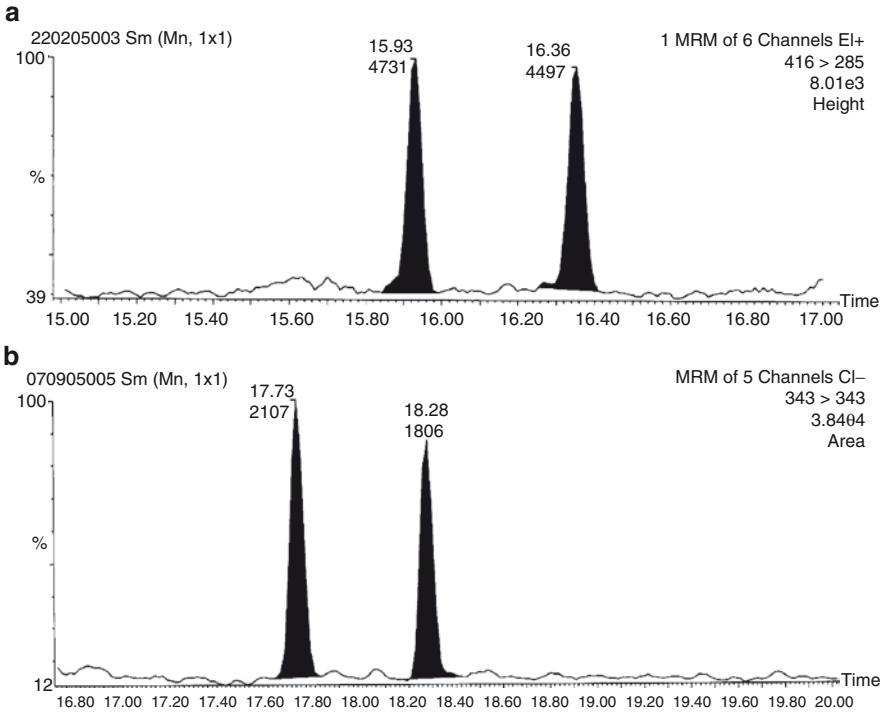


Fig. 8.9 GC-MS/MS of 17α- and 17β-estradiol – improvement of sensitivity by derivatization. (a) Diagnostic ion chromatograms observed for 500 fg of (a) 17α- and 17β-estradiol (3,17-diTMS derivatives, GC-EI-MS/MS, 416 > 285, S/N = 10) and (b) 17α- and 17β-estradiol (3-PFB-17-TMS derivatives, GC-NCI-MS/MS, 343 > 343, S/N = 25). 17α-estradiol derivatives elute before 17b-estradiol derivatives (from Courant et al., 2007b, with permission; copyright 2007 Elsevier B.V.)

do so. It may well be that this particular GC-MS assay has been fully validated but the data supporting this claim should be available for scrutiny.

8.4.3 Liquid Chromatography (LC) and LC-MS

High performance liquid chromatography (HPLC but now referred to simply as LC) can provide rapid, discrete fractions from a sample that, coupled with a suitable detector or analytical end-point, can be used as a highly specific measurement tool. The use of LC in the steroid field which includes estrogens was comprehensively reviewed by Makin and Heftmann (1988) and Ager and Oliver (1984) and was dealt with in the previous edition of this book (Oakey and Holder, 1995).

There have however been considerable developments in this area since then, particularly in the coupling of LC systems to mass spectrometry. Today most estrogen assays undertaken by LC methodology use MS or, preferably, MS/MS, for quantitation and identification. However there have still been some non-LC-MS methods described. A sensitive method for the estimation of seven estrogens (estrone, estradiol, estriol, estetrol, ethynylestradiol, 2-hydroxyestradiol and 4-hydroxyestradiol) in plasma (LOD 1–2 ng/L) was described by Katayama and Taniguchi (1993) using pre-column derivatization with 2-(4-carboxyphenyl)-5,6-dimethylbenzimidazole and fluorimetric detection and has been developed further by Delvoux et al. (2007) for the assessment of metabolic conversion of estrogens in lysates of placenta and endometrium. Other pre-column derivatization methods have been used (e.g. pyrene sulfonyl chloride followed by using fluorescence and laser-induced fluorescence detection [DeSilva et al., 1996], derivatization in urine with 1-ethyl-3-(3-dimethylaminopropyl carbodiimide) followed by fluorescence detection (Iwata et al., 1997) and formation of a Europium chelating label (5-(4″-chlorosulfo-1′,1″-diphenyl-4′-yl)-1,1,1,2,2-pentafluoro-3,5-pentanedione) followed by time resolved fluorescence (Matsumoto et al. (2002)). Other detection methods have been used – fluorescence without derivatization (Yoon et al., 2003; Reinen et al., 2008). UV detection is still used to monitor eluates but not as a primary detection/quantitation method, though diode-array detectors (DAD) still are valuable (Clifton et al., 2007) and, as these detectors are not destructive, it is often useful to use them in series with MS as they can provide an added and useful degree of specificity (Lopez de Alda and Barcelo, 2000, 2001a). Of the non-MS detection systems used for LC, electrochemical detection in various forms is still popular Fernandez et al. (1993); Suzuki et al. (1993); Devanesan et al. (2001); Penalver et al. (2002); Yamada et al. (2002); Yue et al. (2003); Mishra and Joy (2006). Use of a diamond rather than a glassy carbon electrode improved performance and involved less maintenance but fluorescence detection was still 25× more sensitive (LOD (S/N = 5) for estradiol in urine for EC was 10 ng/L and for fluorescence was 0.4 ng/L – Katayama et al., 2007).

LC on a reverse-phase ODS column coupled to UV and a radioactive detector was used to separate metabolites of estradiol and estrone produced *in vitro* by 15 different recombinant CYP isoforms. Structural elucidation was confirmed using GC-MS using GC-EI-MSD of TMS ether derivatives (Lee et al., 2003c). LC has been used as a means of improving sensitivity and specificity of immunoassay (e.g. Giesler et al., 2000) and has been linked on-line to ‘estrogen receptor bioaffinity detection’ using fluorescence polarization (Reinen et al., 2008).

The resolving power of LC itself may often be insufficient to provide adequate resolution but necessary specificity may be achieved by coupling to MS, as the specificity of the ions produced may compensate for poor chromatographic resolution. Additional specificity is obtained by using MS/MS. Figure 8.10 illustrates the separation of four major estrogens (estrone, 17β-estradiol, estriol and 16-hydroxy-estrone) using the LC-MS/MS method of Guo et al. (2008), where mass

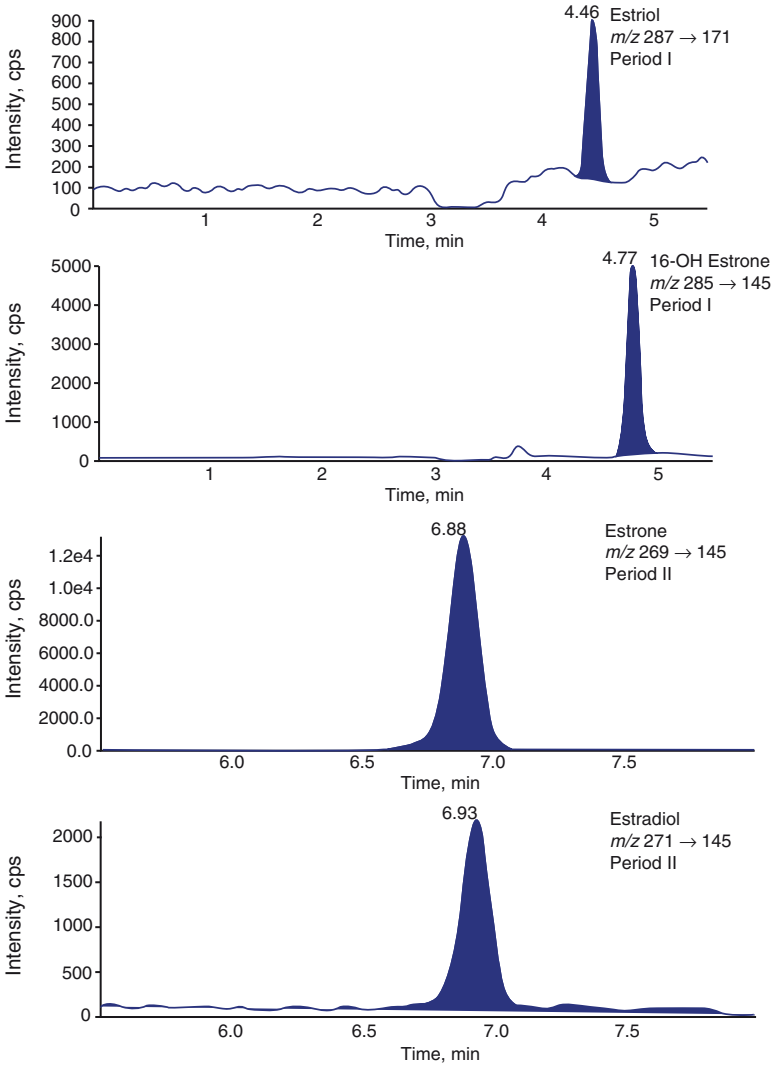


Fig. 8.10 LC-MS/MS of estrogens. The intensity of daughter ions detected after MRM (reactions monitored are shown immediately below the name of each estrogen – parent ion/daughter ion). The estrogens were underivatized and after negative ion electrospray ionization and MRM, the profiles of estrogen steroids corresponding to 100 pg/mL in standard solution (4% [w/w] bovine albumin) were obtained. Chromatograms were obtained using two periods. Period I: 0 → 5.50 min; Period II: 5.50 → 8.00 min (from Guo et al., 2008, with permission; copyright © 2008 Elsevier B.V.)

spectrometry has been used to separate estrone and estradiol which were not resolved in the chromatography. This method is listed in Table 8.7 below and also discussed in the text.

Interestingly the advantages of MS/MS have been utilized, linked to separation by capillary electrophoresis, for the separation of intact underivatized conjugates of estriol (E3-3G, E3-16G and E3-3S) and has been applied to the measurement of these estriol conjugates in amniotic fluid (Cho et al., 2006).

An interesting observation was made by Xu et al. (2002) who described a LC-MS/MS method for the measurement of 2-hydroxy- and 4-hydroxy-estrone using deuteriated standards, with two of the deuteriums on the C16 position. As this is a carbon α to the C17 oxo group, it is a position where deuterium can be lost, possibly due to enolization of the C17-oxo group. In fact, acid catalysed deuterium exchange is always a possibility, particularly as the deuterium atoms are usually introduced by the same exchange reaction. An improved and extended version of this assay, which also measures non-ketolic estrogens, was reported by Xu et al. (2005). This procedure can assay 15 different metabolites using 6 different deuteriated compounds as internal standards. The risk of deuterium loss observed in the earlier Xu et al., (2002) procedure was reduced by using deuterated internal standards which were less likely to lose deuterium label during derivatization (not using C-16 labeled 17-oxo estrogens as internal standards – e.g. 2,4,16,16-d_4-estradiol was used as the standard for both estradiol and estrone). As the risk of deuterium loss during the analysis is always present, use of ^{13}C-labeled internal standards would be preferable. So far as we are aware, no estrogen assay using such standards has yet been described – although improved precision of an LC-MS/MS assay for phytoestrogens has been ascribed to the use of such ^{13}C labeled standards (Grace et al., 2003).

Examination of the LODs of the methods listed in Table 8.7 demonstrates in general considerable improvement in sensitivity by derivatization prior to MS, which improves the ionization process. The lowest sensitivities (LODs of 0.5 ng/L), using such methods were described by Yamashita et al. (2007b) and Kushnir et al. (2008). Other LODs for these methods ranged from 1 to 10 ng/L, though the method reported by Twaddle et al. (2003), which did not use any derivatization, achieved a LOD of 10 ng/L. Most surprizingly of all, the method, which relies upon simple acetonitrile extraction, centrifugation and dilution with water before injection directly onto the LC column (Guo et al., 2008) reported a LOD of 2 ng/mL. On the other hand, Yang WC et al. (2008) reported LODs before (143,000 ng/L) and after derivatization (440 ng/L) much higher than other methods.

There have been a number of reviews on the use of LC-MS/MS methods for estrogen analysis which are of interest (Noppe et al., 2008 – estrogens in food; Albrecht and Styne, 2008 – in pediatric practice; Giese, 2003 – LC- and GC-MS). Other LC-MS/MS reviews of general steroid interest include Shimada et al. (2001), Higashi and Shimada (2004), Vogeser and Parhofer (2007), Wang and Griffiths (2008).

8.5 Analysis of Individual Estrogens and Metabolites

Many, if not all of the methods for the analysis of the individual estrogens, selected for discussion in this section, will have been listed in one of the tables or text in the previous sections but readers might find it helpful to be able to direct their attention to specific estrogens without trawling through the whole chapter, though it is hoped that a comprehensive Index will enable readers rapidly to identify areas of interest in this book. Selection at this point of particular estrogens or omissions of others implies nothing more than the personal views of the authors as to what estrogens might be of interest to colleagues. We hope that readers will agree with our choice and apologize to those who do not.

8.5.1 17β-Estradiol

Estradiol is the major active female sex hormone produced in premenopausal women, primarily in the ovaries, and the concentration in plasma rises just before the LH surge in the normal menstrual cycle and again mid-way through the luteal phase. Post-menopausally, ovarian production ceases and peripheral tissues become the source of the low levels of estradiol circulating in serum/plasma (<20 ng/L – Kushnir et al., 2008). Raised plasma estradiol concentration has been shown to be a risk factor for breast cancer in both pre- (Eliassen et al., 2006a, b; Hankinson and Eliassen, 2007; Eliassen and Hankinson, 2008) and post-menopausal women (Folkerd et al., 2006; Hankinson and Eliassen, 2007; Santen et al., 2007b). Clearly the plasma estradiol concentrations vary widely in the pre-menopausal woman (up to 300 ng/L reported by Kushnir et al. (2008) using an LC-MS/MS method) but it can be measured in samples collected on days 3–5 (follicular) and then 7–9 days before their next menstrual period (luteal phase). Measured by commercial immunoassay, plasma estradiol concentrations in control subjects at these times were 22–88 ng/L (follicular) and 69–192 ng/L (Eliassen et al., 2006a, b). If plasma estradiol concentrations are found to be raised, suppressive treatment may be indicated (Kendall and Dowsett, 2006). Monitoring of plasma estradiol response to this treatment is required and sensitive and specific methodology is necessary to fulfill this requirement. Immunoassay methodology used for this purpose have been shown to have serious problems (Dowsett and Folkerd, 2005; Santen et al., 2008), particularly in postmenopausal women. Wide variations in estradiol results between different immunoassays and laboratories have been reported (Wang et al., 2005; Lee et al., 2006). Estradiol levels are suggested as a marker for 10 year risk of fracture in post-menopausal women (Goderie-Plomp et al., 2004; Santen et al., 2008) and in men (Mellstrom et al., 2008) as well as being a potentially useful biomarker for benign prostatic hypertrophy (Hammarsten et al., 2009). Studies on estradiol in environmental samples are not discussed in this section as they are dealt with in Section 8.5.9.

Accurate measurement of plasma estradiol in normal children and children with disorders of puberty and sexual development is essential for proper and rapid diagnosis and treatment. Most hospital/clinical laboratories use commercial immunoassays, often using automated systems which have been validated for estradiol measurement in adults. Currently these systems seem to lack specificity and sensitivity for use in pediatric laboratory investigations (Rahhal et al., 2008). These authors express the view that GC-MS, often proposed as the gold standard of reference methodology, while sufficiently accurate and sensitive is too cumbersome and like Albrecht and Styne (2007) suggest that LC-MS/MS is likely to become the method of choice in pediatric investigations.

The difficulties with immunoassays have already been discussed in Section 8.4.1 and it must be emphasized again that an immunoassay designed specifically for particular application and properly validated over the expected concentration range can give accurate and useful results. Geisler et al. (2008) have developed an immunoassay for plasma estradiol, estrone and estrone sulfate, designed for use at low concentrations and after use of aromatase inhibitors but it is not clear exactly how the claimed sensitivity and specificity is achieved and validation by comparison with GC- or LC-MS/MS results was not carried out. Proper standardization of new methods against reference methodology or material already certified by such methodology (Stanczyk et al., 2007) is essential as are stability studies to ensure that any change in values on storage (e.g. Jones et al., 2007) are real rather than attributable to the assay specificity. These problems with immunoassays are not confined to estrogens and similar difficulties have been encountered with testosterone assay. Herold and Fitzgerald (2003) pointed out that when immunoassays miss target values by 200–500% as described by Taieb et al. (2003c) for testosterone, it becomes more accurate simply to guess the result within a narrow range – the guess would never be more than 300% inaccurate and would be faster and cheaper without the necessity to even take blood! The same point applies to estrogen analysis, particularly if assays designed for use where plasma estradiol concentrations are high – for example monitoring ovulation, assisted reproduction or ovarian reserve (Aboulghar, 2003; Taieb et al., 2003b, d; Coccia and Rizzello, 2008; Kwan et al., 2008) are uncritically applied to studies where estradiol levels are more than an order of magnitude lower (Taieb et al., 2002). Wang et al. (2005) have reviewed plasma estradiol values obtained by a number of methods, primarily RIA, in post-menopausal women. Since that time other human serum estradiol assays have been reported – that is, a chemiluminescence IA using immunoaffinity (Wang et al., 2006), temperature-dependent inclusion chromatography (Zarzycki et al., 2006), a screen-printed carbon electrochemical immunosensor (Pemberton et al., 2005) and reverse-phase LC coupled to on-line Era receptor affinity determined by fluorescence polarization (Reinen et al., 2008).

There are many methods available for estradiol estimation in body fluids and in environmental samples – an area of increasing interest. Some, hopefully the majority, of these methods have been discussed in previous sections and there are tables gathering together information about the use of immunoassay, for both commercial

and automated systems (Tables 8.4 and 8.5), GC-MS (Table 8.6), LC-MS (Table 8.7) and both LC- and GC-MS in the analysis of environmental samples (Table 8.11). Table 8.8 lists some of the recent LC- and GC-MS methods for estradiol in serum/plasma together with their quoted sensitivities.

The sensitivities quoted above should be compared with those immunoassays for estradiol of 10–15 ng/L but most direct immunoassays are inaccurate and imprecise at concentrations below 100 ng/L (Yang et al., 2004; Sluss et al., 2008).

There is still a wide choice of methods available and the analyst must decide the most appropriate method for the particular task. In a routine clinical laboratory where there is a requirement for the measurement of estradiol in large and continuing numbers, perhaps as a means for monitoring ovulation in pre-menopausal women, it is likely that some kind of automated immunoassay system would be suitable (see Table 8.5). Where estradiol concentrations are low such as in children or in postmenopausal women, particularly if on estradiol-suppressive treatment, immunoassay systems are likely to be of doubtful value and LC- and GC-MS/MS technology becomes important. Modern methods of measuring estradiol in human serum/plasma, particularly in the research laboratory or in subjects who have low plasma/serum estradiol levels, use LC- or GC-MS or –MS/MS, although immunoassay procedures may still be used in clinical environments. The continued use of such immunoassays is subject to a number of caveats – in young children (Bay et al., 2004; Albrecht and Styne, 2007; Ankarberg-Lindgren, 2008; Rahhal et al., 2008), in postmenopausal women (Rinaldi et al., 2001, 2002; Stanczyk, 2002; Lee et al., 2006; Santen et al., 2007a, 2008), need for standardization (Stanczyk et al., 2007) and other general comments – in particular the need for some sort of purification before an assay (Taieb et al., 2002; Stanczyk et al., 2003; Toniolo and Lukanova, 2005; Demers, 2008). Commercial immunoassays are summarized in Tables 8.4 and 8.5 and many of the other available immunoassays are discussed in Section 8.4.1.

Estrogens circulate in plasma bound to plasma proteins, particularly to SHBG and indeed estrogens stimulate the production of SHBG, the concentration of which in female plasma is thus higher than in males. The unbound or 'biologically available' estradiol in plasma is around 1–3% of the total. It would therefore be useful to measure the unbound concentration of estrogens and in the 1970s several methods for determining concentrations of unbound estradiol were published (see Table 8.9).

These methods, which are cumbersome and difficult to operate successfully, appear no longer to be used for estrogen analysis. There is however one paper (Pazol et al., 2004), which surveys methods for measuring free and bound estradiol in female macaques and compares results obtained by ammonium sulfate precipitation and ultrafiltration. Equilibrium dialysis has been used in a study of protein binding of 2-methoxyestradiol (Lakhani et al., 2006). It is also possible, knowing the concentrations of albumin, SHBG, testosterone and estradiol, to determine 'free' levels of steroid by calculation (Sodergard et al., 1982) and this method has been used recently by Eliassen et al. (2006a) and a similar calculation has been used by Hammarsten et al. (2009). Since the late 1990s, interest appears to have moved to the measurement of salivary estrogens on the basis that saliva is an ultrafiltrate reflecting the plasma/serum unbound steroid concentration. It is thus inevitable that

Table 8.6 Some GC-MS methods for estrogen estimation

Estrogen(s) analysed	Methodology used	Reference
Plasma E2β	Extract with micro-cellulose coupled E2-antibody. Derivatize with BSTFA/TMCS.GC-EI(+) -SIM of M^+. Int std = [$^2H_3$]-E2	Gaskell and Brownsey (1983)
14 endogenous estrogens in urine	Protection of C=O groups by formation of *N-O*-ethyloximes, Sep-Pak C18 extraction. *H. pomatia* hydrolysis purification Sephadex-QAE-Ac- form – borate form used for catechol estrogens, followed by free base DEAE-Sephadex and Lipidex 5000. Derivatized as TMS ethers. GC-EI(+) – SIM	Fotsis and Adlercreutz (1987); Ziegler et al. (1997), Falk et al. (1999, 2000); updated in Adlercreutz et al. (2004) and by Knust et al. (2007)
Serum E2β	Extract with CH_2Cl_2 – compared HPLC with immunoaffinity extraction. Derivatize as heptafluorobutyrates. GC-EI(+)-SIM of M^+	Thienpont et al. (1988); Thienpont and DeLeenheer (1998)
2-OH-E2 and 4-OH-E2 formed from E2 in microsomes – estimating OH-ase activities	Derivatize as heptafluorobutyrates. GC-CI –(-)	Roy et al. (1991)
E2-fatty acid esters in human tissues and blood	Extraction with MeOH, alumina chromatography, saponification, SPE on C18 and trimethylsilylation GC-EI(+) and SIM of M^+	Larner et al. (1992)
E1, E2, E3, 2-OHE1-3-OMe and 4-OH-E1-3-OMe in rat brains	Homogenize in isotonic saline. E1 and catechol estrogens -deproteinize with EtOH. Centrifuge and apply supernatant to Isolute C18 cartridge. Elute with EtOH after washing. Form *O*-methyl oximes. Purify on Silica Gel column. Run as OMO and after TMS formation as OMO-TMS derivatives; E2 and E3 extracted with EtAc:CHCl3 (6:1) and Hx:EtAc(10:1) respectively. Apply to Silica gel column. Preparative TLC and HPLC. GC-ESI(+) – MRM	Shimada et al. (1999)
2-OH- and 4-OH- E2 in rat liver microsomes	SPE using isolute phenyl end-capped cartridges, eluting with acetone. Formation of HFB esters. GC-with electron capture detection	Pinnella et al. (2001)
E1 and E2β in hair	Alkaline digestion of hair samples. Treatment with isobutylchloroformate. Isobutylcarbonyl derivatives extracted with Hx and remaining OH groups derivatized as chlorodifluoroacetates. GC-ESI(+) – SIM	Choi et al. (2000)
Studies of CYP1B1 pharmacogenetics. Formation of 2-OH-, 4-OH- and 16-OH-E2 from E2	Extracted with $CHCl_3$. TMS derivatives formed. GC-ESI(+)-MID, IS = [2,4,16,16-$^2H_4$]E2	Hanna et al. (2000); Belous et al. (2007)

E1, E2, E3, 2-OH-E1, 4-OH-E1, 2-OH-E2 and EthinylE2 in human urine	Urine at pH 5 subjected to Gluc-ase/aryl sulfatase hydrolysis. Extraction with Diethyl ether. Pentafluorobenzoyl derivatives formed. GC-NCI monitoring M^- ions. IS = [2,4-d_2]E2	Xiao and McCalley (2000)
Catechol estrogen metabolism *in vitro* by recombinant COMT	Extraction with CH_2Cl_2. Evaporate to dryness and derivatize as TMS ethers. GC-EI-MSD MID IS= [d_4]-E2	Dawling et al. (2001); Dawling et al. (2004)
EthinylE2 in cattle hair	Hair was washed with 10% Tween80. After washing with water, the hair was cut and digested with NaOH. Extraction with CH_2Cl_2 using dialysis bag. TMS derivatization. GC-Finnegan GCQplus ion-trap MS. EI(+) ionization MS/MS. IS = [d_3]testosterone	Durant et al. (2002a, b)
Identification of estrogens, hydroxyestrogens and methoxy derivatives in normal and malignant breast tissue	TMS derivatives formed – using DTT so enols of carbonyl groups will be made. GC-MSD. IS = equilenin	Castagnetta et al. (2002) using GC-MS method described by Castagnetta et al. (1992)
E2-17α and β + E1 in serum	Extraction with ether/Hx and purification using C18 cartridge. Derivatization as 3-[3,5-bis(trifluoromethyl)benzoyl] – 17-trifluoroacetates. GC-EI-MSD. IS= d_2 analogs of E2α, E2β and E1	Hobe et al. (2002)
E1 and E2 in serum	Extraction with CH_2Cl_2:iso-octane, dried and applied to Sep-Pak cartridge; derivatized with pentafluopropionic anhydride; GC-EI-MSD. IS = [16,16,17-$^2H_3$]E2 and [3,4-$^2H_2$]E1	Dorgan et al. (2002)
E2 in serum	Extraction with ether. Evap to dryness and re-constituted in 2-propanol:MeOH – separated on solid-phase XTRX type AXS column. Derivatized as heptafluorobutyrates. GC-ESI-MSD – monitoring M+ IS = [d_3]-E2	Wu et al. (2002); Yang et al. (2004); Sluss et al. (2007)
E2, catechol- and methoxy-estradiols in rat plasma	Serum protein pptd with acetone. Evap to dryness and taken up in MeCN. Derivatized as pentafluoropropionates. GC-NCI-MID. IS = 2-fluoroE2	Zacharia et al. (2004)
17α- and 17β-E2 and E1 in plasma from children	β-Gluc-ase added. Incubation overnight. Centrifuged and extracted with ether (×2). Evap to dryness and re-suspend in MeOH and apply to Chrom P SPE column. Elute with Hx:ether. Dry and re-suspend in CH_2Cl_2 and apply to SPE-SiOH column. Elute with Hx:EtAc. Evap to dryness and re-suspend in Hx:IPA and run on HPLC. Samples collected and derivatized as pentafluorobenzoyl derivatives. GC-NCI-MS/MS or GC-HRMS. IS = [3,4-$^{13}C_2$]-E2	Courant et al. (2007b)

(continued)

Table 8.6 (continued)

Estrogen(s) analysed	Methodology used	Reference
E2-17β and E1 in urine	*Helix pomatia* hydrolysis. Stir-bar sorptive extraction. Acetates formed using acetic anhydride:pyridine. Thermal desorption- GC-EI(+)-MS, monitoring base peak + 2/3 other major ions. IS = equilenin	Stopforth et al. (2007)
E2-17α and -17β in bovine serum and urine	Serum (1 mL) hydrolysed with E.coli β-gluc-ase. Extracted after addition of buffer with Hx containing 5%perfluorobenzoyl chloride. Purified on Na_2SO_4 minicolumn and re-constituted in MSTFA, forming mixed 3-PFB, 17-*O*-TMS derivatives. GC-NCI-MS/MS. Urine analysed as well. IS = [$^2H_4$]E2-17β	Biddle et al. (2007)
E2 in serum	Brief description in both Santen et al. (2007a) and Wang et al. (2005); 1 mL plasma/serum extracted on BondElut Certify SP cartridges, eluting with EtAc. Derivatized with PFB chloride and MSTFA forming mixed 3-PFB, 17-*O*-TMS derivatives. GC-NCI-MS/MS. Use of *O*-(2,3,4,5,6,-PFB)-hydroxylamine hydrochloride though not clear what the purpose of this was as E2 contains no oxo groups	Santen et al. (2007a) Wang et al. (2005) – method published in Abstract form by Sundaram et al. (2003) GC-MS/MS developed and done by Taylor Technology, Inc., Princeton, NJ 08540, USA
E1 and E2 in male serum	Solvent extraction, silica gel chromatography, derivatization, extraction. GC-MS-SIM. No details or reference to methodology	Hsing et al. (2007)

Table 8.7 LC-MS methods for estrogens (2000–date). Many LC-MS methods have been developed for use in detecting and measuring environmental estrogens. These methods are described in Section 8.5.11 (Table 8.11)

Estrogen	Derivatization	Column, solvent etc.	Ionization/detection	Reference
E1, E2, 2-OH-E1 and -E2, 4-OH-E1 and -E2 and guaiacol estrogens formed *in vitro* in rat brain	None	YMC-Pack ProC18 or J-Sphere ODS-H80 MeOH: NH_4formate(1:1)	Finnigan MAT LCQ ion-trap. ESI(−) or APCI(+) MID and MS/MS	Mitamura et al. (2000a)
	Formation of acetates			Mitamura et al. (2000b)
EE2 in rhesus monkey plasma	Dansyl chloride derivatives	Phenomenex Synergi 4μ Max-RP. Fast gradient of MeCN/HCOOH/H_2O	PE Sciex API 4000 TurboIon source (+). MRM monitoring *m/z* 171 in MS3	Anari et al. (2002)
Adducts of catechol estrogens (E1 and E2)	None	Ultrabase C18 RP column. Gradient elution MeOH/H_2O/Acetic acid.	Finnigan LCQ ion trap MS – ESI(+). MS^n experiments.	Jouanin et al. (2002); Van Aerden et al. (1998)
E1 and E2 formed *in vitro* from DHEA-S in MCF-7 cells	None	Symmetry C18 column, eluting with 1%formic acid:MeOH (3:7)	Waters ZMD mass spectrometer with ESI source (+ and – ions). MRM	Le Bail et al. (2002)
2-OH- and 4-OH-E1 in human urine	Pre-column formation of *p*-toluenesulfonyl hydrazones	Phenomenex Luna C18 using MeOH/formic acid(0.1%) gradient	Finnigan LCQ DECA ion trap with both ESI(+) for derivatized and APCI(+) for non-derivatized; using deuteriated standards; SIM monitoring MH^+ ion	Xu et al. (2002); cf Xu et al. (2005)
E1, E2, E3, EE2, mestranol, E2-3G; E1-3S, E2-17Ac in environmental samples	Trimethylsilyl ether derivatives using BSTFA	Purospher RP-18e	Single and triple quadrupole instruments with negative/positive ESI and APCI; comparison of results	Diaz-Cruz et al. (2003)
Serum EE2 in rats	None	Waters Xterra RP-18, eluting with MeCN/water	Micromass Quattro Ultima triple quadrupole MS/MS. ESI(−). Collecting [M-H]- in MS1	Twaddle et al. (2003)

(continued)

Table 8.7 (continued)

Estrogen	Derivatization	Column, solvent etc.	Ionization/detection	Reference
DNA adducts of 4-OHE1 and 4-OHE2 in breast tissue	None	Nano-LC. Gradient elution with MeOH/0.2% Acetic acid. Hypersil C18 (C8) BDS columns	Micromass Quattro II triple quad. Z-spray ion source and ESI(+) NanoFlow probe. MRM – MH^+ in MS1 and daughter ion in MS2	Embrechts et al. (2003)
Urine E3 3- and 16-G in pregnancy	None	Capcell Pak C18 as analytical column + Shodex MS Pak PK2A as pre-column for column-switching; eluting with MeCN/1% TEA at 40°	Thermo-Finnigan Advantage with orthogonal ESI(−); MRM – $[M\text{-}H]^-$ in MS1. $[M\text{-}OG]^-$ in MS2	Yang et al. (2003)
16-oxo-E2, 2-OH-, 4-OH-, 16α-OH-, 2-MeO-, 3-MeO-E1 and E1	Pre-column formation of *p*-toluenesulfonyl hydrazones	Phenomenex Luna C18, eluting with MeOH/1% formic acid. – isocratic and gradient.	Finnigan LCQ DECA ion-trap quadrupole MS. ESI(+). MH^+ MRM	Xu et al. (2004)
E2 in testicular fluid.	None	Waters X-Terra column packed with ODS. Isocratic 1%formic acid/MeCN	Micromass Quattro LC triple quadrupole – ESI(+). For E2 $[MH\text{-}H_2O]^+$ selected for MRM	Zhao et al. (2004)
E2-17β, E1 in mouse plasma and brain tissue	Derivatized with dansyl chloride	Phenomenex Synergi Max-RP eluting with MeCN/0.05% formic acid	SCIEX API-4000 with TurboIon Spray (+) – MRM, monitoring *N*-naphylene moiety (*m/z* 171)	Xia et al. (2004)
Polar and non-polar metabolites of E2-17β formed *in vitro* in human liver microsomes	Dansyl derivatives and trimethsilyl ethers	LC used to separate radioactive peaks. Identity confirmed by GC- and LC-MS Phenomenex Ultracarb 5 ODS, eluting with MeOH/0.1% formic acid	Micromass Quattro LC triple quadrupole MS/MS. ESI(+)	Lee and Zhu (2004); Lee et al. (2001), Lee and Zhu (2004) and review by Zhu and Lee (2005)

EE2 in human plasma	Dansyl derivative	Phenomenex Luna C18 column, eluted with 1%formic acid/MeOH	API 3000 with TurboIonSpray; (+) – MRM, monitoring *N*-naphylene moiety (*m/z* 171)	Shou et al. (2004)
E1 and E2 in human plasma	Derivatization with dansyl chloride	Phenomenex Synergi 4μMax-RP column, eluting with MeCN/0.1% formic acid	API 3000 using APCI (+). MRM collecting [M+H]+ in MS1 and monitoring *m/z* 171 in MS2	Nelson et al. (2004)
15 endogenous estrogens in human urine	Dansyl chloride derivatization	Phenomenex Synergi Hydro-RP column, eluting with gradient of MeOH/1% formic acid at 40°	Finnigan TSQ Quantum-AM triple quadrupole. ESI(+). MRM, selecting MH+ in MS1, monitoring N-naphylene moiety (*m/z* 171)	Xu et al. (2005); cf Xu et al. (2004); Xu et al. (2007)
Equilin sulfate and delta8,9-dehydroestrone sulfate in Premarin tablets	None	19 LC columns with various eluting solvents evaluated	Agilent single quadrupole 1100 LC connected to MS via DAD. ESI(−) SIM of $[M-H]^-$ ions	Reepmeyer et al. (2005)
EE2 and norethindrone in human plasma	Dansyl chloride derivatization	Genesis RP-18 column, eluting with MeCN/1% formic acid	Sciex API 4000 with TurboIonSpray (+). MRM, collecting MH^+ in MS1 and monitoring *m/z* 171 for EE2	Li et al. (2005)
E2-17β in human serum – reference method	Dansyl chloride derivatization	Eclipse XDB-C18 column, eluting with MeCN/0.1%acetic acid –gradient elution	Sciex API 4000 with ESI(+). MRM, collecting MH^+ in MS1 and monitoring *m/z* 171	Tai and Welch (2005)
E3 glucuronides (E3-3G, E3-16αG, E3-17βG)	Derivatization with 2-chloro-1-methylpyridinium iodide, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide, 2-picoylamine	Phenomenex Synergi Polar RP, or Zorbax Eclipse XDB-C18 eluting with MeOH/0.1% acetic acid	Three MS used: TSQ7000 Triple quad), Ion trap (LCQ) and a Micromass Q-ToFI (for accurate fragment mass measurements). Derivatization gave characteristic fragmentation allowing all three conjugates to be distinguished	Lampinen-Salomonsson et al. (2006)

(continued)

Table 8.7 (continued)

Estrogen	Derivatization	Column, solvent etc.	Ionization/detection	Reference
E1, E2	Studied various derivatizing reagents: nitrobenzoyl chloride, 2,4-dinitrofluorobenzene, 4-nitrobenzene_sulfonyl_ chloride, 4-nitrobenzyl bromide	YMC-Pack Pro C18 RS column, eluting with MeOH/water in various mixtures at 40°	Thermoquest LCQ using APCI(-). MRM.	Higashi et al. (2006)
Assay of UGT1A1 activity – E2 glucuronide	None	Shim-pack CLC-ODS, eluting with MeOH/ MeCN/0.15 mM perchloric acid	Sciex API 3000 triple quadrupole. TurboIonSpray – ESI(−). MRM. $[M-H]^-$ in MS1 and looking for *m*/*z* 271 in MS3 (loss of gluc moiety)	Wada et al. (2006)
E1 fatty acid esters formed after incubation with follicular fluid and plasma	None	Hamilton PRP-1, eluting with gradient of MeOH/10 mM ammonium acetate/ $CHCl_3$	Micromass Quattro Micro triple quadrupole MS. Positive ion mode- MRM selecting $[M-NH_4]^+$ in MS1. Daughter ion is fatty acid fragment	Miilunpohja et al. (2006)
E2 measurement as a means of assessing CYP19 inhibition	None	Symmetry Shield C8 column, eluting with water/MeOH using toluene as dopant	API 3000 triple quadrupole with APPI (-) ionization. MRM selecting $[M-H]^-$ in MS1.	Trosken et al. (2006)
E1, E2	Derivatization with isonicotinoyl azide and formation of methylated NA	YMC-pack Pro C18 RS, eluting with MeOH/10 mM NH_4formate at 40°	API-2000 triple quadrupole. ESI(+). MRM	Higashi et al. (2007)
E1, E2 in pregnancy serum	Derivatization with 1-(2,4-dinitro-5-fluorophenyl)-4-methyl –piperazine and methylation	YMC Pack C8 column, eluting with MeCN/ MeOH/ 10 mM ammonium formate	API 3000 triple quadrupole MS/ MS. – ESI(+), collecting M^+ in MS1 and monitoring $[M-47]^+$ for E1 and E2	Nishio et al. (2007)

EE2 in human plasma	Derivatization with dansyl chloride	Semi-automated using 96-well plates. ACQUITY UPLC BEH C18 column eluting with gradient of MeCN/1% formic acid in water at 40°	Sciex API 4000 triple quadrupole with TurboIonSpray (+). MRM	Licea-Perez et al. (2007)
E1, E2	Formation of picolinoyl esters	Cadenza CD-C18, eluting with gradient of MeCN/MeOH/0.1% formic acid at 40°	Finnigan TSQ triple quadrupole and/or API-5000, both using ESI(+). MRM	Yamashita et al. (2007a, b)
E1 and six metabolites: 16-OH-, 2-methoxy-, 4-methoxy-, 2-OH-3-methyl-, 2-OH- and 4-OH-E1		BDS Hypersil C18 column, eluting with gradient of MeCN/water	Sciex API 3000 triple quadrupole MS/MS equipped with Turbo ESI and APCI – both in + and – mode. MRM	Hsu et al. (2007)
E1, 17α- and 17β-E2, E3 and EE2 in cattle hair	Derivatization with dansyl chloride	Phenomenex Synergi Fusion-RP, eluting with MeCN/0.1% formic acid at 40°	Sciex API 2000 triple quadrupole MS/MS with TurboSpray – ESI(+). MRM	Pedreira et al. (2007)
4-OH-E2-N7-Gua and 4-OH-E2-N3-Gua adducts formed *in vitro* by recombinant CYP1B1	None	Phenomenex Jupiter 5μ C18, eluting with MeCN/water/ formic acid	ThermoFinnigan TSQ Quantum triple quadrupole. ESISRM/MRM	Belous et al. (2007)
Serum E1 and E2	Derivatization with dansyl chloride	Phenomenex Gemini Phenyl column, eluting with gradient MeOH/formic acid, 10 mM at 30°	Sciex API-4000 triple quadrupole MS/MS using TurboIonSpray (+). MRM	Kushnir et al. (2008)
Serum E1, E2, E3 and 16-OH-E1	None – direct injection of MeCN extracted serum	Supelco LC-8-DB, eluting with MeOH/water	Sciex API-5000 triple quadrupole coupled with ESI(−). MRM collecting $[M-H]^-$ in MS1	Guo et al. (2008)

(continued)

Table 8.7 (continued)

Estrogen	Derivatization	Column, solvent etc.	Ionization/detection	Reference
17α- and 17β-E2 in bovine serum	None	Phenomenex Gemini, eluting with MeCN/NH_4OH	API 3000 with APCI(-). MRM	Ferretti et al. (2008)
E conjugates (E1-3S, E1-3G, E2-3S, E2-3G, E3-3S, E3-3G, E3-16G) in human urine	None	Hydrophilic interaction LC using TSKgel Amide-80 and RP LC using Phenomenex Luna C18, eluting with MeCN/water/ammonium acetate	Hybrid quadrupole/Ion trap 4000Q TRAP MS. TurboIon ESI(−). MRM	Qin et al. (2008a)
40 estrogens and adducts in human urine	None	ACQUITY UPLC BEH C18 column with gradient MeCN/0.1% formic acid	Waters Quattro triple quadrupole MS/MS using ESI (+ and – mode). MRM	Gaikwad et al. (2008)
E2 in human serum	Pyridine-3-sulfonyl derivatives. Other derivatives examined	Phenomenex Lunar 3μ Phenyl-Hexyl column, eluting with MeCN/water/0.1% formic acid	Sciex API 2000 and API 4000 Q trap MS systems. TurboIon sources (+ ion mode in both systems). MRM, collecting MH^+ ion in MS1	Xu and Spink (2008)
16 estrogens	Underivatized and derivatized with *N*-methyl-nicotinic acid-hydroxysuccinimide ester and dansylation	Agilent XDB-C18 column, eluting with 10 mM ammonium acetate/MeCN	UniqueTM LC-ToF MS ESI(+) and APCI(-). SIM	Yang , WC et al. (2008)
Estrogens in human peritoneal fluid	Dansyl chloride derivatization	Capillary LC Phenomenex 4μ Synergi Hydro-RP, eluting with MeOH/0.1% formic acid at 40°	Finnigan TSQ Quantum Discovery MAX MS. ESI(+)	Xu et al. (2008); Xu et al. (2005, 2006)

E1, E2, E3 in urine	None	Agilent Zorbax Eclipse XDB-C18 column, eluting with MeCN/0.1% NH_3	Agilent 6410 triple quadrupole MSD. ESI(−) for estrogens	Alvarez-Sanchez et al. (2008)
E1, E2, E3 as part of a panel of 23 steroids in chimpanzee urine	None	Gemini C18 column, eluting with water/MeCN/0.1% formic acid at 30°	Micromass Quattro Premier XE triple quadrupole MS with a Z-spray ESI(+). $[M\text{-}H\text{-}H_2O]^+$ is usually precursor ion in MS1. Estrogens also give $[M\text{-}H]^-$ ions with lower background when using ESI(−)	Hauser et al. (2008)
17α- and 17β-E2 and E1	None	Chromolith Performance RP-18, eluting with MeCN/water	Esquire 2000 ion trap MS. ESI(−)	Cheng and Tsai (2008)

Table 8.8 Sensitivity of measurement of 17β-estradiol in serum or plasma by some modern LC- or GC-MS or MS/MS methods

Matrix used	Volume used	Methodology[a]	Sensitivity[b]	Reference
GC-MS – MS/MS				
Human serum	1 mL	Derivatized with PFB chloride, PFB-hydroxylamine and MSTFA; negative ion CI	LOD = 0.63 ng/L	(Santen et al., 2007, Lee et al., 2006)
Human serum	0.75 mL	Derivatization but not specified	Sensitivity not defined but = 2 ng/L	Hsing et al. (2007)
Pediatric serum	2 mL	3-Pentafluorobenzyl ether -17-TMS. Negative CI using methane	LOD = 1 ng/L; LOQ = 2ng/L	Courant et al. (2007b)
LC-MS/MS				
Human plasma	0.5 mL	Dansyl chloride-APCI positive ionization	LOD = 2.8 ng/L	Nelson et al. (2004)
Mouse plasma	50 μL	Dansyl chloride- positive ionization	LOQ = 50 ng/L	Xia et al. (2004)
Human serum	3–5 mL	Dansyl chloride derivatization – positive ESI	1 ng/L	Tai and Welch (2005)
Human serum	10–20 μL	Methylated 1-(2,4-dinitro-5-fluorophenyl)-4-methylpiperazine derivative. Positive ESI	Not clear but calculated as LOD = 12.2 ng/L	Nishio et al. (2007)
Human serum	0.5 mL	Dansyl chloride and – positive ESI	LOQ = 8 ng/L	Xu et al. (2007)
Pediatric serum	2 mL	3-Pentafluorobenzyl ether -17-TMS. Negative CI using methane	LOD = 1 ng/L; LOQ = 2 ng/L	Courant et al. (2007b)
Human serum	0.25–1 mL	Derivatization with picolinoyl chloride. ESI in positive mode	LOQ = 0.5 ng/L	Yamashita et al. (2007)
Human serum	200 μL 400 μL	Electrospray in negative ion mode. No derivatization	LOD = 2 ng/L; LOD = 1 ng/L	Guo et al. (2008)
Human serum	2 mL	Derivatization with *N*-methyl nicotinic acid *N*-hydroxysuccinimide ester. Positive ionization	LOD quoted as 440ng/L[c]	Yang, WC et al. (2008)

[a]Methodology for all entries in this table are given in Table 8.7 and are not repeated here.
[b]Sensitivity is a difficult term but where available limit of detection (LOD) is given. LOD is defined as having a signal to noise ratio (S/N) = 3, whereas limit of quantitation (LOQ) has an S/N = 10 Armbruster et al. (1994).
[c]This is very odd as concentrations of estrogens in the range 80–530 ng/L are quoted.
PFB, pentafluorobenzyl; MSTFA, *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide.

Table 8.9 Some early methods for the estimation of non-protein bound 17β-estradiol in serum/plasma

Estrogen	Principle	Values found (% of total)	References
Estradiol	Equilibrium dialysis	1.68% adult women; 2.15% adult men	Kley et al. (1977)
Estradiol	Equilibrium dialysis	Estradiol 2.42 pmol/L (0.66 ng/L)	Speight et al. (1979)
Estradiol	Steady state gel filtration	1–2.5% adult women; 0.9–2.2% adult men	Fisher et al. (1974)
Estradiol	Steady state gel filtration	0.84–2.71% pregnant women	Anderson et al. (1985)
Estradiol	Centrifugal ultrafiltration	2.42% adult men; 1.57% adult women (follicular phase); 0.96% pregnant women (third trimester)	Hammond et al. (1980)
Estriol	Centrifugal ultrafiltration	13.1–18.9% pregnant women (third trimester)	Moutsatsou and Oakey (1986)
Estradiol	Equilibrium dialysis	2.21% throughout menstrual cycle	Wu et al. (1976)
'Bio-estradiol'	Ammonium sulfate precipitation	MINOS study of serum hormone levels (Szulc et al, 2004)	Tremblay and Dube (1974) – for testosterone

salivary estradiol concentrations will be at lower concentration than estradiol in serum or plasma and thus the analytical challenge will be greater. Use of saliva in this context is not of course confined to estrogens and is widely used for other steroids (reviewed by Lewis, 2006) and hormones and there has been a recent review of the current status of salivary hormone assays (Groschl, 2008). Readers interested in this area might find it instructive to consult several articles relating to similar problems encountered while attempting to measure 'free' testosterone (Rosner, 1997, 2001; Vermeulen et al., 1999; Miller et al., 2004) and a position statement by the US Endocrine Society (Rosner et al., 2007). The problems described for testosterone assay are paralleled by those encountered with estradiol and attention must be paid to the problems referred to for testosterone as well as 'pre-analytical issues' (Raff and Sluss, 2008).

A search on PubMed, using the search term 'estradiol AND saliva', of papers published in the last 10 years produced a list of 100. Of these 100, 29 were accessible and appeared to be immediately relevant to the measurement of salivary estradiol and the methodology reported was therefore examined more carefully. All of the assays used were immunoassays and the majority of the publications used a commercial radioimmunoassay, which came from a single source (DSL, Webster, Texas – www.dslabs.com – see Table 8.4). This assay had been modified but the modifications related solely to a re-adjustment of the proportions of the components of the recipe (Jasienska et al., 2004). A 'rigorous evaluation' of a radiommunoassay for salivary and blood spot estradiol, using DSL components, is described by Shirtcliff et al. (2000) but there is no validation using mass spectrometry. There is very little infor-

mation on the DSL website about this assay but it is clear that the particular product (cat number DSL 39100), widely used in reported assays, is (2008) in the process of being withdrawn. A further search of PubMed, looking for publications containing a description of use of MS for validation of any of the immunoassays used for salivary estradiol measurement, revealed a single publication (Gaskell et al., 1980) where mass spectrometry had been applied to steroid analysis in saliva – nearly 30 years ago! The declared purpose of this 1980 study was 'to guide the development of routine immunoassay procedures which may subsequently be validated by comparison with reference GC-MS methods'. An admirable intention but, it seems, unfulfilled as there appear to be no published validations using comparison with mass spectrometry of any of the immunoassays used to measure salivary estradiol. Using this DSL methodology and the collection protocol published by Lipson and Ellison (1989), Jasienska et al. (2004) found salivary estradiol concentrations of 2.72 ng/L rising to 13.6 ng/L at ovulation. Gann et al. (2006) measured estradiol in nipple aspirate fluid, serum and saliva using the same methodology. Marrs et al. (2007) examining 24 healthy pregnant women found salivary estradiol concentrations at 37 weeks gestation of 15.9 ± 13.8 (SD) ng/L – these samples were mailed to a commercial laboratory in Phoenix, Arizona and assayed by 'enzyme immunosorbent assays'. A similar approach was adopted by Gandara et al. (2007). An enzyme immunoassay using a monoclonal antibody has been described (Tamate et al., 1997). A ^{3}H-radioimmunoassay 'in routine use in our laboratory', with a limit of assay sensitivity set at 2.15 ng/L, has also been used (Berg and Wynne-Edwards, 2001) to determine median salivary estradiol levels in men of 2.82 ng/L. At present, the situation seems to be entirely unsatisfactory and from the point of view of a steroid analyst, it appears that there is an uncritical use of unvalidated immunoassays with the consequence that reported results are of doubtful validity. For this reason, there seems to be little point in either quoting or reviewing the publications on this subject, which interested readers can acquire for themselves by a simple PubMed search. Both recent reviews in this area seem to support this view and suggest that there is a need to establish proper reference ranges and ensure that proper collection protocols be established and used (Lewis, 2006) reiterated by Groschl (2008) emphasizing that 'specific and standardized analytical tools are required……strict protocols for collection procedures … are mandatory'. In a study (Tivis et al., 2005), using the DSL salivary estradiol assay, designed to establish the best method to monitor estradiol concentrations in post-menopausal women, it was concluded that 'reliable estradiol measurements need to be based on serum samples, not saliva samples in this population'.

This is an illustrative area for those interested in steroid analysis and it is perhaps worth following one analytical thread. In 1999, an 'evaluation of a new assay for salivary estradiol' was published (Lu et al., 1999). This was a radioimmunoassay using antiserum and ^{125}I-labeled tracer, perhaps the two most important components of the assay mixture, supplied by DSL (Webster, California). Cross reactivities of the antiserum were quoted, though it is not clear where these data came from. Recovery and dilution studies were carried out giving satisfactory results. In the intervening 9 years, six interesting papers, which used *inter alia* this salivary estra-

diol assay have been published (Gann et al., 2001; Chatterton et al., 2004, 2005, 2006; Núñez-de la Mora, 2007, 2008). In all of these papers, the methodology used for salivary estradiol assay is referenced as Lu et al. (1999), yet this assay was 'validated' nearly 10 years ago and its output has never been compared to results obtained by reference GC- or LC-MS or MS/MS methodology, widely accepted as the 'gold standard' for validation (Stanczyk et al., 2003; Toniolo and Lukanova, 2005; Coucke et al., 2007; Demers, 2008) or standard material validated by GC- or LC-MS (Stanczyk et al., 2007). The lapse of time does not confer specificity on an assay rather it increases the need for constant and rigorous re-evaluation of the methodology used.

The development of immunoassay methods for the measurement of estradiol and other estrogens have been reviewed (Oakey and Holder, 1995; Giese, 2003). Appelblad and Irgum (2002) provide an excellent and comprehensive review, covering the period from around 1990, of the analysis of neuroactive steroids in biological matrices. While this considers all steroids which can be described as neuroactive, the steroid with the most references in the literature covered in this review is estradiol. This review thus deals with estradiol but is also useful in that puts it into context with applications other than clinical.

Most assays have a target analyte and specificity is estimated by the degree to which the assay measures the analyte and nothing else. There are however situations where the analyte may be unknown or specific analytes may be unsuspected in the matrix under consideration. In this situation, alternative assays, which focus on the biological activity of the compounds of interest, should be used. In the case of estrogens, 'estrogenicity' is the prime physiological activity of interest. Utilizing the knowledge of how estrogens exert their biological effects (see Fig. 8.8) – primarily through the estrogen receptors, some genetic manipulation of convenient cells can be carried out, producing cells which can detect 'estrogenic' compounds. Klein et al. (1994) described a yeast cell line which was transfected with ER, and β-galactosidase, or luciferase (RCUB). Following incubation with the extract of interest, interaction with the ER and binding to the ERE etc., the degree of estrogenicity was indicated by colorimetric or fluorescent means. The detection limit was 0.02 ng/L, which is superior to that of RIA or ELISA. Klein et al. (1995) used this yeast assay to measure residual estradiol in a breast cancer patient receiving Letrazole and showed that it was less than that indicated by RIA measurements. Coldham et al. (1997) reviewed the yeast assays and suggested that they were twice as sensitive as an MCF-7 assay and five times an uterotropic assay. They applied it to measuring the estrogenic potency of a variety of compounds with varying activity. Similar recombinant cell methodology has been published (Klein et al., 1994; Paris et al., 2002) and used by Wang et al. (2005) for estrogen measurement in postmenopausal female serum, comparing results with those obtained by GC-MS/MS and RIA.

More recently Bovee et al. (2004) developed a faster version of this assay, using Yeast Enhanced Green fluorescent Protein (YEGFP) as the reporter. This version can be carried out in 96 well plates in 4 h. This method is sensitive, rapid, and convenient with good reproducibility. Subsequently many investigators turned to mammalian cell lines, Balaguer et al. (1996) developing a HeLa cell assay using

Gal4-HEGO and 17 m5-G-Luc as the reporter elements. A tenfold increase in color was observed with 1 nM estradiol and 5 ng/L was the detection limit. The same group further refined this assay (Paris et al., 2002) using HeLa cells transfected with ER-α and an estradiol promotor linked to Luc. The assay responded to estradiol and compounds with estrogenic activity. It is suitable for prepubescent children. Wang et al., (2005) using this RCUB assay measured residual estradiol levels in postmenopausal women with better sensitivity than RIA or ELISA. The results were comparable to GC-MS values.

Seronie-Vivien et al. (2004) developed an overall estrogenic assay using a mammalian cell line linked to an ERα construct and a luciferase construct. This assay measured total estrogenic activity and had functional sensitivity of 10 pmol/L (2.72 ng/L). Other groups have described a more stable cell line that stably expresses an estrogen-responsive Luciferase reporter for the detection of ER agonists and antagonists. Wilson et al. (2004) described a T47D line which only needed to be transfected with a ERE promoter-luciferase reporter gene construct. Legler et al. (1999) have described a different T47g line in which PEREtata-Luc was transfected to give a stable highly sensitive cell line with a detection limit of 0.5 pmol/L (0.14 ng/L). It was inhibited by anti-estrogens and pseudo-estrogens gave potent responses. Fine et al. (2006) described a more stable gel-based yeast preparation for measuring low levels of estradiol.

A reference method using GC-MS has been published (Thienpont et al., 1988) and this method has been used to produce certified 17β-estradiol reference materials under the auspices of the European Union Bureau of Reference (Thienpont et al., 1997).

8.5.2 17α-Estradiol

Nearly 30–40 years ago there were reports of the presence of 17α-estradiol in peripheral plasma of several species, particularly ruminants. These results relied upon early immunoassays (Robertson et al., 1972; Robertson and Smeaton, 1973). There are also some reports of the presence of 17α-hydroxy estrogens in human urine published in the 1960s (e.g. Luukkainen and Adlercreutz, 1965). Very little interest had been shown in these estradiol epimers since then, although work by Adlercreutz et al. (1974), using GC-MS methodology and considerable pre-purification prior to GC-MS, indicated that 17α-estradiol was present in human bile, urine from one non-pregnant woman and in a pool of 20 mL late-pregnancy plasma obtained from ten women. A study by Hobe et al. (2002) investigated the metabolism of administered 17α-estradiol, following the increase in concentration of free and conjugated 17α-estradiol in serum after administration of 4 mg ^{3}H-17α-estradiol, using for quantitation GC-MS with negative ion CI after pre-column derivatization with 3,5-bis(trifluoromethyl)benzoyl chloride. Levels of 17α-estradiol down to 3 ng/L were reported. An interesting study, using LC-MS/MS demonstrated that 17α-estradiol can, like 17β-estradiol, be converted into catechol estrogens and that these can

form depurinating adducts (Rizzati et al., 2005). Very recently, an LC-MS/MS method (Ferretti et al., 2008) for measuring 17α- and 17β-estradiol in bovine plasma has been described using negative ion ESI and MRM focussing on $[M-H]^-$ ion in MS1. It has been developed for regulatory purposes under the auspices of the European Union in order to monitor illegal treatment of cattle with exogenous growth promoting substances. The epimeric estradiols can be measured in the range 0.03–1.0 μg/L.

Use of modern GC- (e.g. Hanselman et al., 2006) and LC-tandem mass spectrometry methods (e.g. Vulliet et al., 2008) has demonstrated the presence of both 17α- and 17β-estradiol in environmental samples – often with concentrations of 17α-estradiol being equal to or greater than 17β-estradiol. Details of methodologies used for these analyses are given in Table 8.11. As 17α- and 17β-estradiol have identical mass fragmentation, specificity of measurement relies on complete resolution of these two isomers during the chromatographic process. In the LC-MS method for analysis of steroids in environmental water described by Vulliet et al. (2008), which uses no derivatization of the estrogens prior to chromatography, the resolution of 17α/17β is almost identical to that achieved by Matejicek and Kuban (2008), who used derivatization prior to chromatography (see Fig. 8.11). Limits of detection of these two methods are very similar (0.01–0.03 and 0.08 ng/L, respectively), so it is not entirely clear what advantage derivatization confers.

Toran-Allerand et al. (2005) have used LC-MS/MS (Nelson et al., 2004) to demonstrate the presence of high endogenous levels of 17α-estradiol in mouse brain and hypothesize that it is produced therein and acts as part of an autocrine system in concert with a specific receptor ER-X. Dykens et al. (2005) have reported the results of a Phase 1 clinical study using 17α-estradiol as a neuro-protecting agent and reviewed the rationale behind this suggestion. Simpkins and Dykens (2008) propose a mitochondrial mechanism of neuroprotection, pointing out that 17α-estradiol is equally as neuroprotective as 17β-estradiol but 200-fold less active as a 'feminizing' hormone. If these ideas prove to be correct and 17α-estradiol does have valuable therapeutic effects in neurodegenerative diseases, analytical methods of increased sensitivity for this steroid in human body fluids will be required.

8.5.3 Estrone and Estrone Conjugates

Measurements of plasma/serum estrone have attracted less attention than estradiol perhaps because there is little rationale for measuring this estrogen in isolation from others. Estrone while lower in concentration in serum than estradiol, in premenopausal women, is higher in men, postmenopausal women, and many non-human species. Detailed data on serum levels of estradiol and estrone are given by Kushnir et al. (2008). Initial attempts at measuring estrone using tissue cytosol binding assays were generally unsatisfactory and required extensive purification of estrone prior to assay (e.g.Tulchinsky and Korenman, 1970). Early attempts to use antibodies and develop immunoassays improved results, but problems still remained

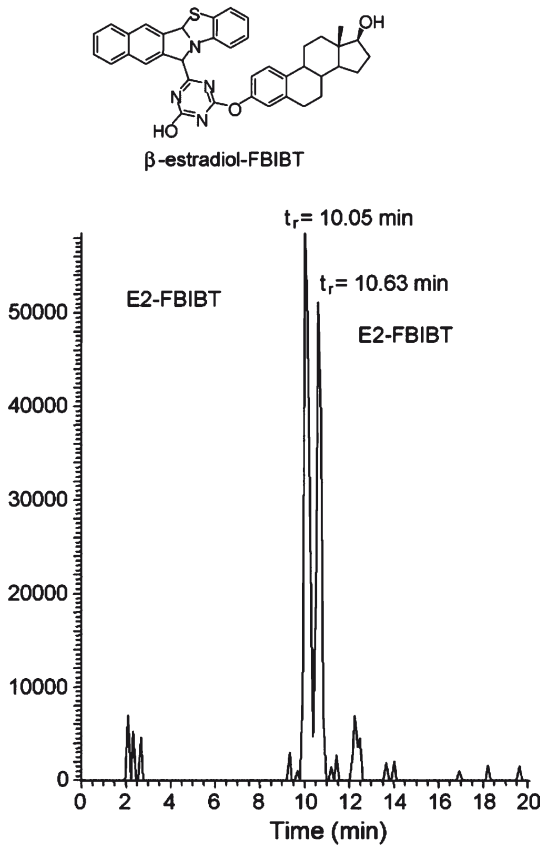


Fig. 8.11 Separation of FBIBT derivatives of 17β- (first peak) and 17α- (2nd peak) estradiol by LC-MS/MS (1 ng/mL of each added to river water). Separations shown were achieved on a Luna Phenyl–hexyl column (50 × 2 mm, 3 mm particle size), eluted at 25° with a linear gradient of MeCN/0.1% HCOOH. Better α/β resolution was achieved using aqueous CH_3COOH but a loss of around 18% in intensity of MS/MS signal was observed. Structure of FBIBT-β-E2 derivative shown at top of figure. MS/MS was carried out in a Finnigan LCQ ion-trap MS, using ESI(+). Parent ion was [M+H]+ *m/z* 639 and daughter ions were *m/z* 596 and 367 (from Matejicek and Kuban, 2008, with permission; copyright 2008 Elsevier B.V.)

(Wright et al., 1978c; McGarrigle and Lachelin, 1983). Reduction of the purified estrone to 17β-estradiol and analyzing the reduced compound has given better results. Estrone has been measured in saliva by enzyme immunoassay after ether extraction, using estrone-peroxidase conjugate and antibody coated wells (Folan et al., 1989), derived from an EIA method for estrone in serum which used Sephadex LH-20 columns after diethyl ether extraction (Folan et al., 1988). An immunoassay of estrone in female serum has been described using capillary electrophoresis to separate free and bound and laser-induced fluorescence (LOD (S/N = 3) which was 19.6 ng/L and a normal range in ten samples of 119–222 ng/L was

quoted – Su et al., 2003). Serum estrone in female pools was measured by RIA (carried out by Esoterix – a commercial organization in California) and by GC-MS derivatized as pentafluoropropionates (Wudy et al., 1992) and results compared (Dorgan et al., 2002). A similar study but using LC-MS/MS has been carried out by Hsing et al. (2007). The development of a fully automated immunosensor using laser activated total internal reflection fluorescence – with a reported LOD below 0.2 ng/L – has been reported for use in on-site monitoring of environmental samples (Tschmelak et al., 2004). There is a brief description of radioimmunoassay for estrone and estrone sulfate in a study of plasma estrogen levels and the prediction of a risk of breast cancer (Eliassen et al., 2006b), while serum estrone sulfate has been used to monitor response to treatment in breast cancer patients. After comparing results from a 'direct' immunoassay (DSL, Webster, TX, USA) and GC-MS/MS, the latter methodology was preferred (Stanway et al., 2007b). Estrone sulfate has been measured in serum, saliva and nipple aspirate fluid from healthy premenopausal women (Gann et al., 2006). Estrone has been measured in saliva by enzyme immunoassay during parturition at 37 weeks gestation (Marrs et al., 2007) and salivary estrone sulfate has been measured in the pregnant sow by RIA (Ohtaki et al., 1997).

Estrone-3-sulfate and 3-glucuronide assays are also considered in this section as estrone sulfate is quantitatively the most important estrogen in human serum and a potential precursor or estrone and estradiol in breast tissue (Chatterton et al., 2003). Estrone glucuronide has been measured in human urine by RIA and EIA throughout the menstrual cycle with the intention of devizing a simple on-site home monitoring system for women to determine the time of their fertile window (Cooke et al., 2007). With similar intentions in the veterinary world, a dipstick using particle capture immunoassay (Henderson and Stewart, 2000, 2002) has been developed allowing serum estrone sulfate in mares to be measured as a quick pregnancy test. A direct RIA (i.e. no pre-treatment of serum/plasma before assay) for estrone sulfate in serum/plasma has also been described, which gave good correlation with an 'established method' which involved hydrolysis of the sulfate and measurement as estrone but has not been validated against any mass spectrometric procedure (Ranadive et al., 1998). The sensitivity of this method was reported as a detection limit of 9 ng/L and it has been used in a study of changes in serum estrone sulfate levels in post-menopausal women as a result of treatment with estradiol orally and via a transdermal patch (Slater et al., 2001). A series of papers describe radioimmunoassay of estrone sulfate in plasma (Lønning and Ekse, 1995; Lønning et al., 1997; Geisler et al., 2008), and in breast tissue (Geisler et al., 2000), all of which use the same basic methodology – extraction of unconjugated estrogens with ether, sulfatase hydrolysis, extraction of liberated estrone, borohydride reduction and RIA of estradiol. Reported sensitivity of the assays used in 1995 and 1997 was 0.73 ng/L whereas by 2008, it was 0.19 ng/L – a near fivefold improvement. The RIA used in all these papers appears to be identical as it is described throughout as 'reported elsewhere'.

Immunoassays for any type of steroid require rigorous appraisal/validation before use and constant vigilance during use (continuing quality assurance) in order to ensure quality output (i.e. data on which the customer can rely) – a constantly

recurring refrain in this chapter. An example of such validation is the radioimmunoassay for estrone sulfate in male plasma developed by Giton et al. (2002) and used in an investigation into the possible role of serum estrone sulfate as a predictive marker for prostate cancer aggressiveness (Giton et al., 2008). The RIA used in this investigation used an antiserum which recognized estrone sulfate, and thus hydrolysis was not necessary. Cross reaction from DHEA sulfate was removed by rapid chromatography on Hypersil C18 mini-columns. A full evaluation was carried out including comparison of results from 16 plasma samples assayed by LC-MS/MS. 0.5 mL of plasma was used and the 'least detectable dose' was 0.082 nmol/L (29 ng/L). Useful data on methods for estradiol sulfate from 1971 onwards are given in this paper as well as normal estrone sulfate values recorded previously – in this publication, normal values for men between 22–84 years were 1.82 ± 1.15 nmol/L (mean ± SD, $n = 90$).

For a study of metabolic inter-conversion of estrone and estradiol in placental and endometrial tissue, a previously published LC method (Katayama and Taniguchi, 1993) using reverse-phase LC, derivatization of estrogens with 2-(4-carboxyphenyl)-5,6-dimethylbenzimidazole and fluorescence detection was adapted (Delvoux et al., 2007). Modern methods for measuring estrone have been discussed in previous sections of this chapter. Immunoassays are discussed in Section 8.4.1 and are listed in Table 8.4. Details of other methods are tabulated – GC-MS (Table 8.6), LC-MS (Table 8.7) – there are nearly 30 methods cited in these tables. Readers are invited to look at four of these as examples of the use of GC-MS/MS (Courant et al., 2007b – in pediatric plasma) and LC-MS/MS (Guo et al., 2008; Kushnir et al., 2008 – in serum; Xu et al., 2008 - in human peritoneal fluid). Methods for estrone using both LC- and GC-MS in the analysis of environmental samples are cited in Table 8.11. Kushnir et al. (2008) includes a comparison of LC-MS/MS results with those from three different immunoassay methods and gives data on concentrations of estrone and estradiol in men and women.

8.5.4 Estriol

This steroid is only a minor constituent of plasma and urine of non-pregnant women but rises to high levels during the course of pregnancy. Maternal urine estriol had long been monitored during late pregnancy to assess foeto-placental well-being though it has not been widely used since the 1990s (Beischer et al., 1995). Urinary steroid profiles (Shackleton, 1993) have been carried out during pregnancy as a means of diagnosing steroid sulfatase deficiency (Glass et al., 1998) and Smith-Lemli-Opitz syndrome (SLOS – Jezela-Stanek et al., 2006). Estriol arises in pregnancy due the metabolism of steroids in the three main steroidogenic compartments, fetal adrenal and liver, maternal adrenal and placenta. Maternal adrenal dehydroepiandrosterone (DHEA) sulfate is taken up from the maternal circulation and it is also made by the fetal adrenal. DHEA sulfate from both sources is 16α-hydroxylated in the fetal liver and then goes to the placenta where it is hydrolysed by the placental sulfatase enzyme

and converted into estriol, which then returns to the maternal circulation – an excellent description of this process is given by Rainey et al. (2004) and is succinctly summarized in Figure 5 of this publication. Steroid synthesis during pregnancy has also been reviewed by Kallen (2004) and Pasqualini (2005). As estriol arises during pregnancy by a steroidogenic co-operation between fetus and placenta, it would suggest that any malfunction in this smooth co-operation would likely lead to a fall in estriol production – indeed placental sulfatase deficiency is the most likely single cause of extremely low estriol excretion.

Monitoring of serum estriol levels during the second trimester as part of a multiple marker test for Downs Syndrome was recommended in 1988 (Wald et al., 1988) and, in combination with parallel measurements of alpha-fetoprotein, human chorionic gonadotrophin and inhibin A, is the best biochemical predictor (Canick and MacRae, 2005). Estriol can also be monitored in serum as a predictor of pre-term delivery, the largest contributor to perinatal morbidity and mortality throughout the world (Yeast and Lu, 2005, 2007). The rationale being that there is an increase in estriol levels immediately prior to the onset of labor and detection of an early surge of saliva to concentrations greater than or equal to 2,000 ng/L is an indication of impending delivery (McGregor et al., 1995). Mars et al. (2007) report salivary estriol values at 37 weeks gestation of 468 ± 219 (SD) ng/L with a maximum value of just under 1,000 ng/L – well below the levels suggested by McGregor et al. (1995). Venepuncture for acquisition of serum is also invasive and in the Third World, sterile syringes may be in short supply and thus saliva is recommended as a substitute for serum in this context (Voss, 1999), with the added benefit that saliva is said to reflect the concentration of unbound (or biologically available) steroid in serum/plasma. The question however remains as to how to respond to raised salivary estriol levels. While it may be predictive, Ramsey and Andrews (2003) suggest that intervention at this stage is not clinically justified on this basis and indeed Yeast and Lu (2007) suggest that although the salivary estriol assay has been approved by the FDA for use in singleton gestation, its use beyond this is unlikely to be effective. In addition the effect of estriol levels of betamethasone and uncertain effects of other drugs limits its predictive value.

The use of saliva as a matrix in which to measure estradiol has already been discussed above and it appears that the problems associated with salivary estradiol are identical to the difficulties in the assay of estriol in saliva. Reviewing the literature on salivary estriol over the last 10 years, a number of research papers have been identified (McGregor et al., 1995, 1999; Hendershott et al., 1999; Heine et al., 1999; Heine et al., 2000), all of whom use the same commercial assay for salivary estriol. There is limited information about this assay in McGregor et al. (1995) which gives no validation data or source for these data and quotes a 'sensitivity' of 330 ng/L. The only post-2,000 publications accessible to us, which describe in detail immunoassays for salivary estriol are those by Hedriana et al. (2001) and Suri et al. (2008). Although there was enthusiasm in the 1990s for the measurement of steroids in saliva, there has been little interest or developments in salivary estrogens over the last 10 years. While saliva appears an attractive non-invasive matrix and reflects the level of unbound steroid in the serum/plasma, it is only of use if strict collection methods are used and full details of assays used and validation and quality assurance information provided.

Immunoassays as well as LC and GC- and LC-MS and MS/MS methods for estriol are available, usually as part of an estrogen profile. From what has already been said, there is very little reason for the development of assays for estriol in serum or urine but if readers are interested, data are given in Section 8.4.1 for immunoassay and Tables 8.6 and 8.7 for GC- and LC-MS methods.

8.5.5 Catechol Estrogens

The phrase 'catechol estrogens' is used to refer to polar metabolites of estrone and estradiol further substituted by a hydroxyl group at C-2 or C-4. The compounds formed by such substitution bear hydroxyl groups on adjacent carbon atoms of the aromatic ring A and therefore resemble *o*-catechol. These compounds are extremely labile and are very readily oxidized. The catechol estrogens in the peripheral circulation are rapidly transformed to the corresponding 2-methyl ether derivatives by the COMT (catechol-*O*-methyl transferase) enzyme, located on the erythrocyte. Catechol estrogens and their formation have been discussed earlier in this chapter (see Sections 8.2.2 and 8.2.6). Figures 8.5 and 8.6 illustrate the formulae and metabolic inter-relationship of these compounds. There are quite a number of these estrogens but most of the early literature has dealt with urinary 2-hydroxy- and 16-hydroxy estrone as the 2/16 ratio has been considered as a useful predictor of breast cancer (*vide supra*). This section of this chapter is concerned with analytical methods and the value of this approach in recognition of increased risk of breast cancer is dealt with elsewhere.

Much effort has been devoted to the development of reliable and robust methods of analysis, which fall into two classes – measurement of the two urinary catechol estrogens by immunoassay and attempts to measure a wider spectrum of estrogen metabolites using GC and LC methodology. These estrogens are also susceptible to oxidation and need protection during processing, which is achieved by addition of ascorbic acid. Chattoraj et al. (1978) reported determination of 2-hydroxyestrone in acid-hydrolysed urine. An antiserum raised to 2-hydroxyestrone-17-(*O*-carboxymethyl)-oxime-BSA with equal cross-reactions to 2-hydroxyestrone and 2-hydroxyestradiol was used. Although lower values for 2-hydroxyestrone were obtained after purification by chromatography on Sephadex LH-20, the authors report results from assays which did not include this step. Values ranged from 50 to 175 nmol/24 h. Ball et al. (1979) examined the urinary excretion of conjugated forms of 2-hydroxy- and 2-methoxyestrone. Antisera provoked by immunization with these compounds, linked through C-17, were used with ^{3}H-labeled ligands. Mean values recorded were similar to those reported by Chattoraj et al. (1978). Others (Emons et al., 1979) reported a method for 2-methoxyestrone and its conjugates in plasma. The antiserum was raised to 2-methoxyestrone linked through C-17 to BSA; the ligand was 2-methoxy[^{3}H] estrone. The conjugated form was found to be some ten times more abundant than the unconjugated estrogen. The use of ^{125}I-labeled (*O*-carboxymethyl)-oxime-

ligand, 2-hydroxyestrone-17–[125]I]iodohistamine, was introduced by Berg et al. (1982) for analysis of 2-hydroxyestrogens in plasma. These workers used an antiserum with cross-reactions to the 2-hydroxy derivatives of estrone, estradiol and estriol and reported their results as '2-hydroxyestrogens'. Concentrations of 40–775 pmol/L were recorded. In each of these reports, the authors recognized the special conditions needed to handle catechol estrogens and went to some lengths to assess and validate their methodology. It must be pointed out, however, that these results are in disagreement with those reported by Kono et al. (1980). These authors, using an antiserum to 2-hydroxyestrone, reported plasma concentrations less than 50 pmol/L for both 2-hydroxyestrone and 2-hydroxyestradiol which had been purified before assay by chromatography on Sephadex LH-20. McGuinness et al. (1994) published a 'direct' RIA for urinary 2-OH-estrone after glucuronidase hydrolysis with a reported LOD of 345–828 pmol/L (100–240 ng/L).

There have been a large number of publications measuring 2-OH- and 16-hydroxyestrone, all of which use the Estramet 2/16 enzyme immunoassay (Immunacare, Inc.), based upon work done by Klug et al. (1994), which now uses a monoclonal antibody. Details of this kit are available from ImmunaCare's website (www.immunacare.com). Criticisms are made of the use of commercial immunoassay kits but in this case, full evaluations have been done by many laboratories using different validation approaches (Chen et al., 1996; Ziegler et al., 1997 [comparing results with a GC-MS method]; Bradlow et al., 1998 [comparing results to a GC-MS method]; Falk et al., 2000 [modified kit]; Spierto et al. 2001; Rinaldi et al., 2003 [reproducibility]). The EIA continues to be used by, *inter alia*, Wellejus et al. (2005) in a prospective study of urinary hydroxyl estrogens and breast cancer risk, Kabat et al. (2006) to assess breast cancer risk in a large cohort in the Long Island Breast Cancer Study, Salih et al. (2007) reporting lowered urinary 2-hydroxy estrogens in polycystic ovary syndrome, McCann et al. (2007) reporting a change in the 2/16 ratio after consumption of flaxseed, and Bradlow et al. (2006) comparing results from urine with those from plasma.

Immunoassays developed in the early 1990s provided valuable information but were primarily directed at 2- and 4-hydroxylated metabolites, whereas there are many more polar metabolites of estrogens which are of interest (see Fig. 8.5). The ELISA procedures for 2-OH-estrogens devized by Klug et al. (1994) and Bradlow et al. (1998) also give reliable results for the sum of 2-OHE1 and 2-OHE2. Comparisons between urinary and serum levels for both the free catechol estrogens and their methyl ethers are complicated by the fact that the methyl group is removed in the liver prior to conjugation and clearance. This increases the relative level of the free compounds in the urine and decreases the relative amount of the methyl ethers in the urine.

Mass spectrometry has been applied to this area of analysis by an increasing number of groups since 2000. Its value as a means of validating simpler immunoassay methodology has already been stressed – indeed it is mandatory. GC-MS methods have been used in a study of catechol estrogen production in rat liver microsomes (Sepkovic et al., 1994) and, using trimethylsilyl derivatization, in an *in vitro* study of

COMT isoforms (Dawling et al., 2001) or after pentafluoropropionyl derivatization to measure estradiol, catechol estrogens and methoxy compounds in human plasma using negative ion CI (Zacharia et al., 2004). GC-electron impact ionization-MS has been used for the measurement of catechol estrogens during *in vitro* studies on CYP1B1-catalysed hydroxylations of estradiol (Hanna et al., 2000; Hachey et al., 2003) using trimethylsilyl ether derivatives, monitoring two or three selected ions for each analyte and using deuteriated estradiol as an internal standard. GC and latterly GC-MS have provided valuable urinary steroid profiling for more than 20 years and this approach has been applied to the study of catechol estrogens.

LC-MS/MS also has the capability to provide similar profiles; however, the chromatographic resolution achieved by LC is often not as good as that using GC, although of course the latter requires derivatization, which has been perceived as a drawback to its use. Increasingly LC-MS/MS is also using derivatization to improve sensitivity by increasing ionization efficiency. Figures 8.12 and 8.13 illustrate this point, comparing a profile of catechol estrogens using GC-MS (Fig. 8.12) with a similar profile using LC-MS/MS (Fig. 8.13), where the relatively poor LC chromatographic resolution is compensated by the resolving power of the mass spectrometer. Eight catechol and guaiacol estrogens formed in rat brain have been determined using LC-MS/MS with negative ionization (Mitamura et al., 2000a, b). A semi-quantitative analysis of 2- and 4-hydroxyestradiol after formation of their ferrocene cyclic boronate esters using a quadrupole ion-trap MS^2 system and a novel ESI process has been described (Williams et al., 2001). LC with electrochemical detection has been used for the quantification of catechol estrogens and their conjugates in mammary tumor tissue from knock-out mice (Devanesan et al., 2001), in transgenic mice tissue (Yue et al., 2003), in the ovaries of catfish (Mishra and Joy, 2006) and in urine as a potential marker for prostate cancer using fluorescence detection as well as a new diamond-electrode (Katayama et al., 2007).

Xu's group have reported LC-MS or LC-MS^n methods after formation of *p*-toluenesulfonyl hydrazones (Xu et al., 2002, 2004). Alternatively dansyl derivatives (Roos and Medwick, 1980) can be made followed by LC-ESI(+)-MS/MS (Xu et al., 2005) Supercritical fluid LC-APCI(+)-MS using MRM (Xu et al., 2006) or LC-ESI(+)-MS/MS after extraction with methylene chloride for the free estrogens, followed by hydrolysis of the unextracted aqueous layer and hydrolysis to liberate the conjugates (Xu et al., 2007), can be carried out. There have also been a number of publications where a combination of LC-MS/MS and GC-MS (primarily to confirm identification have been used (Lee et al., 2002, 2003a; Lee and Zhu, 2004; Belous et al., 2007). LC-MS-ESI(+) after derivatization with *p*-toluenesulfonyl hydrazide has been used to measure 2- and 4-hydroxy estrones in human urine (Xu et al., 2002). Other LC-MS/MS methods have been published recently, using LC-APCI-MS/MS and switching between positive and negative ionization (Hsu et al., 2007), using negative/positive ionization to measure 40 estrogen-related compounds (estrogens, metabolites and adducts) (Gaikwad et al., 2008), and finally to apply this latter methodology to identify possible novel biomarkers of prostate cancer risk (Yang, L et al., 2008a).

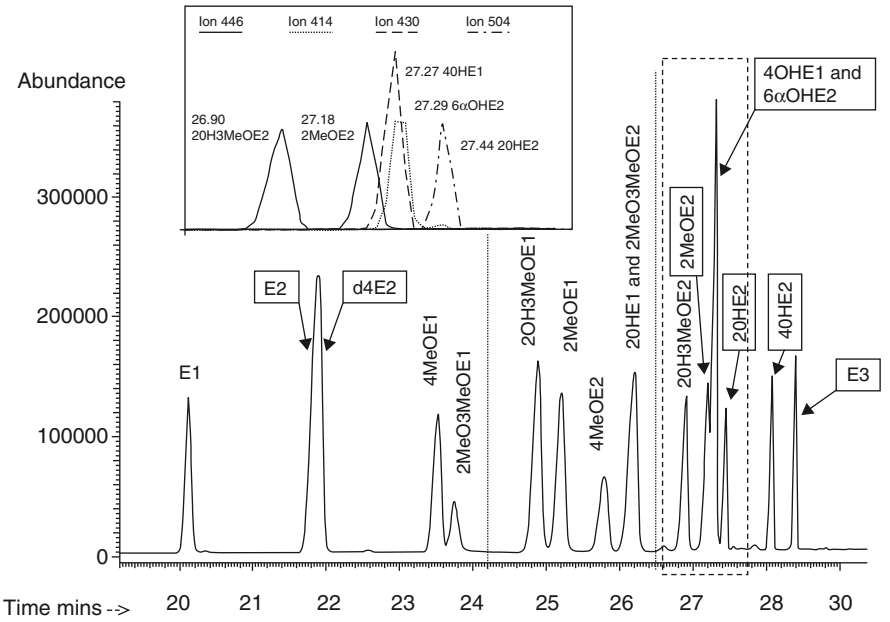


Fig. 8.12 GC-MS ion trap TIC illustrating the separation of an equimolar mixture of estrogens, their metabolites, and the deuterated internal standard (d4E2). The *vertical dotted lines* indicate the position of the three different ion collection groups: 19–24.2 min [*m/z* 229, 257, 285, 287, 314, 315, 342, 343, 372, 373, 416, 417, and 420]; 24.2–26.5 min [*m/z* 257, 315, 342, 372, 373, 388, 389, 430, 431, 432, 446, and 447]; and 26.2–31 min [*m/z* 283, 309, 311, 315, 345, 373, 414, 430, 431, 446, 447, 504, and 505]. The *inset* shows the single-ion chromatograms (*m/z* 446, 414, 430, and 504) for the area within the *dashed line* on the total ion chromatogram where the peaks overlap. All compounds except 2-MeO-3-MeOE1 are chromatographed as TMS derivatives (from Dawling et al., 2001, with permission; Copyright 2001 American Association for Cancer Research)

8.5.6 15α-Hydroxyestriol (Estetrol)

This estrogen, with hydroxyl groups at C-3, C-15α, C-16α and C-17β, is synthesized in significant quantities in the latter part of human pregnancy. Although in the late seventies there was considerable interest in this compound as an indicator of fetal well-being, as it was demonstrated >40 years ago that this steroid is produced during pregnancy (Schwers et al., 1965), interest has diminished over the succeeding years. A number of groups (e.g. Fishman and Guzik, 1972; Giebenhain et al., 1972; Korda et al., 1975; Tulchinsky et al., 1975; Kundu and Grant, 1976; Zucchelli et al., 1979) developed radioimmunoassaysassays for this compound in plasma with a fair degree of accuracy. Maternal plasma concentrations of 15α-hydroxyestriol between 20 weeks and term from lowest values of 0.13 ng/mL to around 2–5 ng/mL were reported (recently reviewed by Holinka et al., 2008). Antisera to estetrol have been developed (Nakagomi et al., 1999; Suzuki et al., 1999). Liquid chromatographic methods have

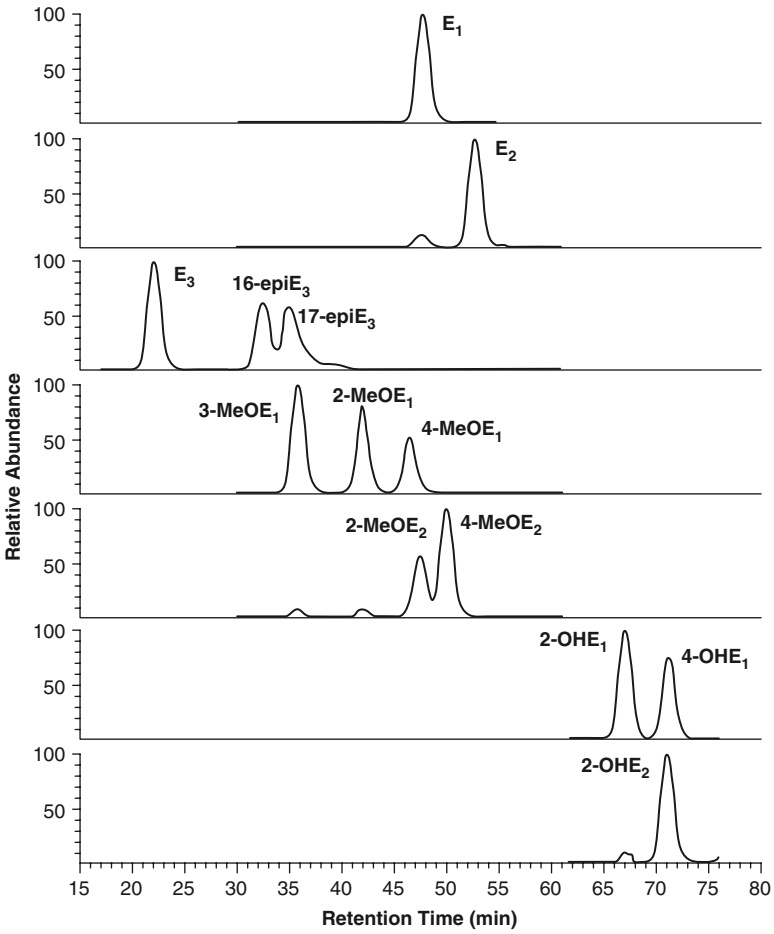


Fig. 8.13 Capillary liquid chromatography-electrospray ionization-tandem mass spectrometry selected reaction monitoring (SRM) chromatographic profiles of 13 estrogens and estrogen metabolites (EM) in a charcoal-stripped human serum sample that has been spiked with each EM to a final concentration of 40 pg/mL (from Xu et al., 2008, with permission; copyright 2008 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim)

also been published, using electrochemical detection (Hayashi et al., 1985), electrochemical and UV detection (Noma et al., 1991) and fluorimetric detection after pre-column derivatization with 2-(4-carboxyphenyl)-5,6-dimethylbenzimidazole (Katayama and Taniguchi, 1993). Estetrol has also been measured in human plasma using a temperature-dependent inclusion (β-cyclodextrin) LC-UV (DAD) method as part of a ten steroid metabolome study (Zarzycki et al., 2006). The same group has recently reported a study of the interaction of estetrol and estrone, estradiol and estriol with cyclodextrins and their hydroxypropyl derivatives during reverse-phase LC (Zarzycki et al., 2006) and Zarzycki (2008) has reported the use of a simple hori-

zontal chamber for thermostated micro-TLC, separating estrone, estradiol, estriol and estetrol in a 2D-TLC system.

A number of papers on estetrol have recently been published in a supplement to the journal *Climacteric* (2008), Supplement 1. There are nine articles and three appendices in this supplement and mostly by the same group of authors who declare an interest in that they are either financed by or involved in Pantarhei Bioscience, who are developing estetrol. One article is a review (Coelingh Bennink et al., 2008). We have no access to this journal and recommend readers in a similar situation to consult a review by the same three authors (Holinka et al., 2008).

8.5.7 Ethinyl Estradiol, Equilin, Equilenin

The A and B-ring aromatic equine estrogens equilin and equilenin and 17α-ethinyl estradiol have been given a separate section merely as representatives of estrogens which are used for treatment of post-menopausal symptoms (i.e. HRT) or used as contraceptive agents. There are many synthetic estrogens, the formulae of which and other data of interest are listed in standard reference works (e.g. Merck Index [Merck & Co, New Jersey] and Index Nominum – International Drug Directory [MedPharm Scientific Publishers, Stuttgart]) and in Hill et al. (1991). Measurement of these estrogens are of interest in three areas – in the pharmaceutical area where formulation and stability studies are necessary, secondly for biochemical and clinical studies of the pharmacokinetics of these estrogens in women and thirdly studies of environmental contamination by these estrogens excreted by women. The first area of interest is dealt with in Chapter 12 and the last area – environmental monitoring – is considered in some detail in Section 8.5.9 where methods of analysis are dealt with and in particular where data on GC- and LC-MS/MS methodology is tabulated (see Table 8.11). Estradiol is metabolized forming 2-hydroxy and 4-hydroxy catechol estrogens and these metabolites can form depurinating adducts which may cause damage to DNA leading to cancer. In a similar fashion both 4-hydroxy-equilin (Zhang and Henion, 1999) and 4-hydroxy-equilenin are formed *in vitro* in breast cancer cell lines (Spink et al., 2001) and equilenin DNA adducts have been identified in human breast tissue by nano-LC-nano-ES LC-MS/MS (Embrechts et al., 2001, 2003). DNA damage by 4-hydroxyequilenin has been reported in rat mammary tissue (Zhang et al., 2001) and breast cancer cell lines (Chen et al., 2000; Liu et al., 2002). Ethinyl estradiol is also metabolized in a similar way forming 2- and 4-hydroxylated products.

The A, B-ring aromatic estrogens are found almost exclusively in pregnant mare urine and are of analytical interest only because a purified extract of this urine is a major component of Premarin®. Several attempts have been made to assay these compounds using antisera against an equilin–albumin complex. Although it discriminates well against estrone, it significantly cross reacts with equilenin and 17-dihydroequilenin (Morgan et al., 1980). Other antisera have been developed but these too possess significant cross reactivity. Pre-assay chromatographic separation on silver nitrate-impregnated Celite columns was considered essential for successful and specific

immunoassay by Bhavnani et al. (1981). In the last 10 years no new immunoassays for equilin or equilenin have been published and GC- or LC-MS or MS/MS are now increasingly being used to assay these ring B aromatic steroids and their metabolites. GC-MS has been used to measure estrone and equilin in serum of females taking equine estrogens (Siekmann et al., 1983). Reverse-phase LC with electrochemical detection was used for an investigation into the metabolism of equilin sulfate in the dog (Chandrasekaran et al., 1995). Equilenin has been measured in human urine (Rule and Henion, 1999) using LC-APCI(+)-MS/MS, monitoring in MS3 a product ion of MH^+, *m/z* 209, probably arising from D ring cleavage. Equilin and equilenin have been assayed in buffer solution during *in vitro* studies of receptor binding using LC-MS/MS with ESI(−) (Sun et al., 2005). In the course of developing an LC-MS/MS method for serum estradiol, Xu and Spink (2008) investigated the MS/MS characteristics of pyridine-sulfonyl derivatives of equilin and equilenin.

Ethinyl estradiol is found in the blood of women taking oral contraceptives which incorporate this compound or its 3-methyl ether (most formulations contain around 20–35 mg/dose – see British National Formulary - on line as www.bnf.org). It appears to be metabolized to the 3-sulfate or 3-glucuronide esters and excreted as such into female urine (recently reviewed by Zhang, H et al., 2007) and methods have been described for analysis of both compounds. Women taking norethisterone (norethindrone – a synthetic progestin) may metabolize it *in vivo* to ethinyl estradiol (Chu et al., 2007). Antisera developed against the C3-compound cross react minimally with other estrogen metabolites (Warren and Fotherby, 1974; Akpoviroro and Fotherby, 1980). Antisera obtained using ethinyl estradiol linked to albumin at C-6 or C-7 have also been used (Rao et al., 1974). Despite the antiserum specificity, some form of further purification, usually on column chromatography, was necessary. LC has been used to separate ethinyl estradiol after plasma extraction before RIA (Tacey et al., 1994). A novel electrochemical method using a square wave adsorptive stripping voltammetric method has been applied (Ghoneim et al., 2006) to the measurement of ethinyl estradiol in pharmaceutical preparations and in human serum and plasma, reporting an LOQ of 2.9 nmol/L (858 ng/L). Assays have been developed using GC-MS (Kuhnz et al., 1993) and isotope dilution mass spectrometry (Siekmann et al., 1998). GC-MS has been used to validate a radioimmunoassay method (Kuhnz et al., 1993). A commercial inadequately described (PPD Development, Richmond, VA) GC-MS method using CI and SIM was used to measure ethinyl estradiol in plasma, after extraction and derivatization in a pharmacokinetic study (Boyd et al., 2003). GC-MS usually requires derivatization, most often as trimethylsilyl ethers and optimization of the formation of these derivatives has been studied by Arroyo et al. (2007) and applied to measurement of ethinyl estradiol, estrone and estradiol in bovine urine. As with other steroids, LC-MS/MS methods have also been recently developed and applied to ethinyl estradiol assay in rhesus monkey (Anari et al., 2002) and human plasma (Shou et al., 2004; Li et al., 2005; Licea-Perez et al., 2007), but all have required derivatization with dansyl chloride to improve ionization and Licea-Perez et al. (2007) used ultra performance (UP)-LC.

After cryogenic pulverization and simple extraction with acetonitrile, ethinyl estradiol was measured directly in cattle hair by LC-MS/MS after derivatization with

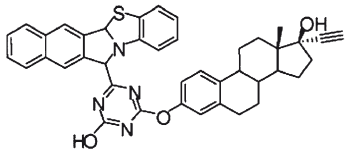
dansyl chloride to improve EI(+) ionization – two MRM transitions were used (Pedreira et al., 2007). A GC-MS method for analysis of cattle hair had previously been described (Durant et al., 2002b).

As ethinyl estradiol (and possibly norethisterone as well) taken by women is excreted in the urine and ends up unaltered in water treatment plant effluents where it can act as an estrogen disruptor in fish and other marine creatures, there have been intensive studies on ultrasensitive assays for the measurement of ethinyl estradiol in sewage and water treatment effluents. Such assays are discussed in Section 8.5.9, which primarily deals with modern sensitive LC- or GC-MS/MS methodology necessary for the detection of the very low levels of estrogenic steroids found in environmental samples. Limits of detection for ethinyl estradiol using LC-MS/MS and pre-column derivatization are in the region of 67 pg/L with an LOQ approximately 3 × the less sensitive (Matejicek and Kuban, 2008). Figure 8.14 shows the response in this system of 1 ng/L of the FBIBT derivative (structure shown in the figure) added to river water. Immunoassay methodology usually cannot match this sensitivity but when it does, specificity constraints restrict its use, although it may have value as an on-site method of detecting 'estrogenicity' and specific estrogens (e.g. Hinteman et al., 2006). Some methods of determining these compounds in plasma/serum are given in Table 8.10.

8.5.8 Estrogen Conjugates/Esters

8.5.8.1 Glucuronides and Sulfates

Estrogen glucuronides and sulfates are formed by estrogens in a similar fashion to other steroids. Estrone sulfate is quantitatively the most important circulating estrogen in serum/plasma and has been discussed in a previous section (Section 8.5.3). There are two approaches to the analysis of the conjugates – measurement of the intact compounds or removal of the conjugate by hydrolysis and analysis of the estrogen aglycone by previously discussed methods. Immunoassays of intact glucuronides have been developed but application of a more selective GC methodology has required separation and hydrolysis prior to assay. The advent of LC methods has allowed direct analysis of intact conjugates to be carried out. It is not entirely clear why this approach is necessary, although prior hydrolysis destroys information about the precise chemical nature of the estrogen conjugates under consideration. In quantitative terms, hydrolysis must be complete and non-destructive to the aglycone. Acid hydrolysis is frequently destructive and there seems little justification for this approach today. Solvolysis utilizes the ability of certain solvents, such as ethyl acetate, in the presence of H^+, to hydrolyse conjugated steroids, and is particularly applicable to steroid sulfates. After experimental investigation, it was concluded that solvolysis of plasma (2.5 mL), diluted 1:1 with water with 25 uL H_2SO_4 (66%, v/v) in the presence of $(NH_4)_2SO_4$ (1.1 g) provided optimal deconjugation of estrone sulfate with minimal contamination of the estrone produced by



17α-ethynylestradiol - FBIBT

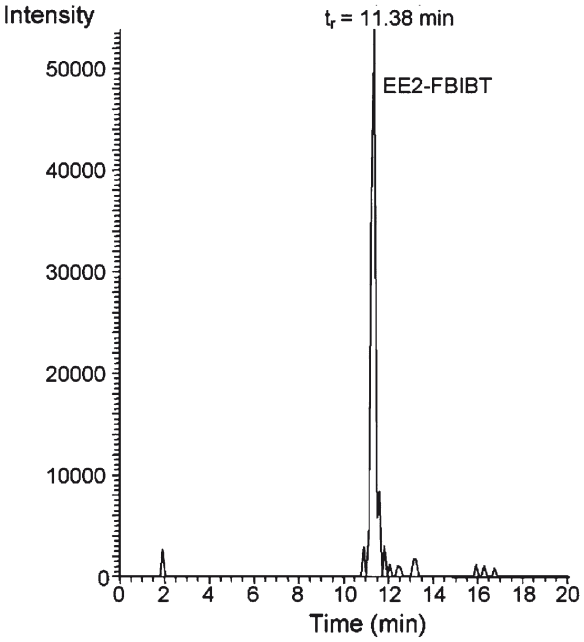


Fig. 8.14 Liquid chromatographic analysis of ethinyl estradiol (EE2) in river water. The chromatogram shown represents the product ion (*m/z* 620), monitored in MS3, formed from the MH+ ion (*m/z* 620) selected in MS1 and subjected to collision in the MS/MS system used. The peak represents the addition of 1 ng/L EE2 added to river water. The structure of the derivative formed with FBIBT [12-(Difluoro-1,3,5-triazinyl)-benz[f]isoindolo-[1,2b][1,3]benzothiazolidine] is given above the chromatogram (from Matejicek and Kuban, 2008, with permission; copyright 2008 Elsevier B.V.)

unwanted material. Towobola et al. (1980) preferred to extract estrone sulfate from plasma with ether-ethanol in the presence of $(NH_4)_2SO_4$ and carry out solvolysis in the ether-ethanol phase. Loriaux et al. (1971) isolated steroid sulfates by thin-layer chromatography from mixed conjugates extracted from serum. Estrone was then liberated by solvolysis with glacial acetic acid-ethyl acetate (1:9, v/v).

A variety of different enzyme preparations have been used to liberate estrogens from the respective sulfate and glucuronide conjugates. β-Glucuronidase from bacteria (Hobkirk et al., 1969; McGarrigle and Lachelin, 1983) is relatively specific for these conjugates, but other sources of the enzyme, for example, molluscs, also

Table 8.10 Some assays for synthetic steroids 1994–date

Analyte	Matrix	Method	Sensitivity	References
EE2	Plasma	Extraction-LC-RIA	2 ng/L	Tacey et al. (1994)
EE2	Plasma	CH_2Cl_2 extraction – RIA	43 ng/L	Agasan et al. (1994)
EQN	Urine	LC-MS/MS	Not given	Rule and Henion (1999)
EE2	Rat plasma	LC-ESI(−)-MS/MS	30 ng/L	Twaddle et al. (2003)
EE2	Water	ELISA	14 ng/L	Schneider et al. (2004)
EE2	Rhesus monkey plasma	LC-MS/MS	LOQ 5 ng/L	Anari et al. (2004)
EE2	Plasma	LC-MS/MS	2.5 ng/L	Shou et al. (2004)
Norethinderone and EE2	Plasma	LC-MS/MS	N – 50 ng /L; EE2 – 2.5 ng/L	Li et al. (2005)
EQ and EQN	Ultrafiltrate	LC-ESI(−)-MS/MS	Not given	Sun et al. (2005)
Norethinderone; Levonorgesterol; EE2	Plasma	UPLC-MS/MS	N&L 100 ng/L; EE2 10 ng/L	Licea-Perez et al. (2007)
Mestranol and EE2	Water	GC-MS	0.05–0.2 ng/L	Zuo et al. (2007)
EE2, EQ and EQN	Serum	LC-ESI-MS/MS	Not given	Xu and Spink (2008)

EE2, ethinyl estradiol; EQ, equilin; EQN, equilenin.

contain steroid sulfatases. Similarly, sulfatases from molluscs contain β-glucuronidase activity. Careful selection of the source of enzyme is essential if it is intended to assess in which conjugate form the liberated estrogen was present in the original material. The mixed enzyme preparations may be used for selective hydrolysis by inhibiting β-glucuronidase using saccharolactone and steroid sulfatase activity with phosphate. Today these enzyme preparations can be obtained from commercial suppliers (e.g. Sigma) and are pretty reliable.

For many years, estrogen glucuronides in urine or plasma were assayed as unconjugated estrogens following acid or enzyme hydrolysis. At first, applications of immunoassay techniques to estrogen conjugate assay followed a similar scheme (Goebelsmann et al., 1972; McGarrigle and Lachelin, 1983). If the glucuronic acid moiety of the conjugate was used to form the bridge to the immunogenic protein, antisera could be obtained which recognized not only the unconjugated estrogen but also the intact conjugate (Kellie et al., 1972; Samarajeewa and Kellie, 1975). In this way, antisera capable of recognizing, almost exclusively, estrone-3-, estradiol-3-, estradiol-17-, estriol-3- and estriol-16α-glucuronides were obtained, although significant cross reactivity with conjugates on other positions remained. In these new assays, the corresponding ^{3}H-labeled estrogen conjugate was used as the labeled ligand. Direct assay, in which extensive dilution of the urine sample is the only pre-treatment required, eliminated any destruction of estrogens that occurred during previous hydrolysis

procedures. Valuable information on the relative proportions of various estrogen conjugates in urine was also obtained by these procedures (Baker et al., 1977).

This approach of direct assay of estrogen conjugates received much attention for application to pregnancy urine in efforts to monitor feto-placental function. In late pregnancy urine, estriol 16α-glucuronide is the most abundant estrogen glucuronide, so most attention was directed to this analyte. Davis and Loriaux (1975) coupled estriol 16β-glucuronide to BSA and obtained an antiserum. As a label, these authors used [^{3}H]estriol, rather than the glucuronide, and found cross-reactions with unconjugated estrone, estradiol and estriol. This was unlikely to be detrimental because of the overwhelming abundance of estriol 16α-glucuronide in the samples.

Lehtinen and Adlercreutz (1977) used thyroglobulin as an antigenic protein, whilst Haning et al. (1977) used keyhole limpet hemocyanin, and Wright et al. (1978a) used BSA or bovine thyroglobulin. All techniques produced useful antisera. When [^{3}H]estriol 16-glucuronoside was used as a label, a more marked cross-reaction with unconjugated estriol was noted with the antiserum to the hemocyanin derivative. Use of C-2 or C-4 for attachment of hapten (estriol-16α-glucuronide) to antigenic protein (Agarose) or BSA was described by Di Pietro (1976) who recorded cross-reactions of <1% with other conjugated or unconjugated estrogens.

Stanczyk et al. (1980) applied these methods to the measurement of estrone-3-glucuronide, estradiol-3-glucuronide, estradiol-17-glucuronide, estriol-3-glucuronide and estriol-16α-glucuronide in urine from non-pregnant women. Estradiol-17-glucuronide was found to be the most abundant form of estrogen conjugate of those measured at mid-cycle. This is in contrast to the earlier data based on indirect assay (Brown, 1955) in which conjugated estrone is the more abundant form. Collins et al. (1979) also used these reagents but concentrated on the measurement of estrone 3-glucuronide as an index of follicular and luteal function. Direct assay of non-pregnancy urine was also reported by Wright et al. (1978b) using antisera described earlier. The estrogen conjugates can be labeled with [^{125}I]histamine which was linked covalently to the glucuronic acid residue (MacLean et al., 1981). A second antibody technique was used to separate free and bound ligand. These authors also applied the technique to monitor the response of infertile women to therapy with gonadotrophins.

The direct techniques, avoiding hydrolysis of estrogen conjugates have been applied to analysis of plasma, in particular to analysis of estriol-16α-glucuronide. Lehtinen and Adlercreutz (1977), who used an antiserum fixed to Sepharose particles in a urine assay, found the same procedure could be applied to pregnancy plasma, provided extra washes of the particles were included after separation of free and bound ligand. Kerr et al. (1977) used an antiserum with affinity for estriol-16α-glucuronoside and estriol. In use, binding sites for the latter antigen were obscured by exposure of the antiserum to estriol. An alternative approach was used by Wright et al. (1978a), who adopted their non-hydrolysis urinary technique to plasma. These authors extracted the unconjugated estrogens from the plasma and then subjected the extracted plasma to analysis for conjugates. A recent enzyme immunoassay (O'Connor et al., 2003) has been modified by replacing the polyclonal antibody used previously with a monoclonal, which has the advantage of providing a continuing supply of consistent antibody.

The availability of LC separation systems attached to MS/MS has allowed the analysis of intact glucuronides. However, although this capability exists and is increasingly used, hydrolysis of glucuronide and sulfate conjugates prior to assay is still used – the flow chart of one such method is illustrated in Fig. 8.15, taken from Xu et al. (2007). A similar approach was adopted in a recent RIA method (Geisler et al., 2008). Five estrogen sulfates (estrone-3-S, estriol-16-S, estradiol-3-S, estradiol-17-S, estradiol-3,17-S) were separated by LC-MS/MS without prior hydrolysis using negative ionization (Zhang and Henion, 1999) and the first three of these naturally occurring sulfates were measured in 100 μL of human urine with a LOQ of 200 ng/L. Although perhaps a retrograde step, but functional in the absence of the necessary MS equipment, LC-with fluorescence detection was used to determine estradiol-3- and 17-glucuronides in rat and human liver microsomes in a study on kinetics of glucuronide formation *in vitro* (Alkharfy and Frye, 2002). Estriol-3- and 16-glucuronides were determined in pregnancy urine by LC-MS/MS using column-switching and negative ionization MRM with an LOD of 10 μg/L (Yang et al., 2003). LC-MS/MS using negative ionization has been used in an *in vitro* study (Lepine et al., 2004) of formation of glucuronides of estradiol, estrone and catechol estrogen metabolites by isoforms of UDP-glucuronyl tranferase enzymes (UGTA1 and UGTA2), and the measurement of four glucuronide and three sulfate conjugates of estrone, estradiol and estriol in human urine using hydrophilic interaction chromatography (HILIC – Qin et al., 2008a). This HILIC methodology (Qin et al., 2008b) has been used for the measurement of the same seven estrogen conjugates in Canadian river water and, with the aid of column switching, simultaneous measurement of unconjugated estrogens derivatized with dansyl chloride. Figure 8.16 illustrates the complete separation of these estrogens which is achieved by a combination of chromatography and multiple reaction monitoring – the transitions used for MRM are given for each chromatogram. Similar measurements have been made in river sediments using LC-MS^n ion-trap technology with reported LODs of 1 ng/g (Matejicek et al., 2007). Capillary electrophoresis with ESI(−)-MS/MS has been used to quantitate estriol-3-glucuronide, 16-glucuronide and 3-sulfate in amniotic fluid (Cho et al., 2006). It is interesting to note the other advantage of tandem mass spectrometry, which has been applied by (Lampinen-Salomonsson et al., 2006) to use chemical derivatization and LC-MS/MS to determine the position of conjugation.

8.5.8.2 Depurinating Adducts of Catechol Estrogens and Glutathione Conjugates

Catechol estrogens are highly reactive and bind to such thiols as glutathione and 2-mercaptoethanol. Catechol estrogens stereospecifically react with thiols such as glutathione, 2-mercaptoethanol, tyrosinasel and aryl thiols. 2,3-catechols react with thiols almost exclusively at C-1 or C-4 with a 1/4 ratio (Jellinck et al., 1984a, b; Jellinck and Fishman, 1984). The corresponding 2,3-quinone reacts at C1/C4 with a 1:1 ratio. Aryl thiols also react with a 1/4 ratio. However the ratio for aryl thiols is altered by sub-

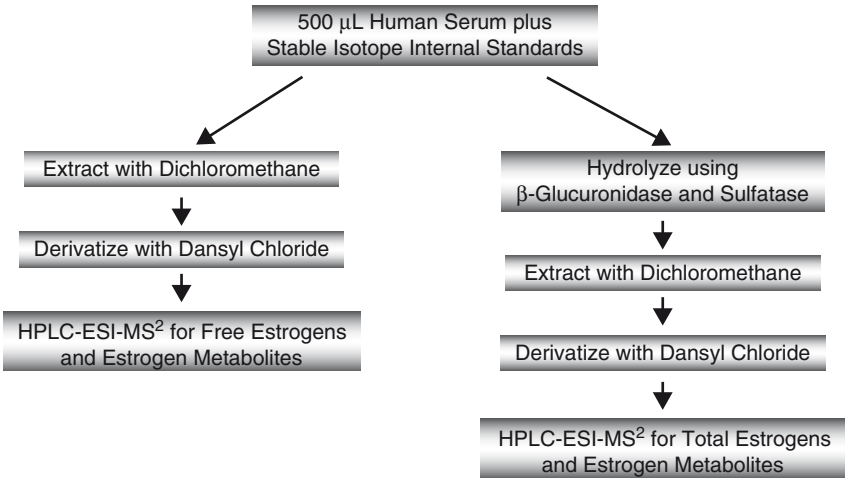


Fig. 8.15 Flow chart of method for the measurement of endogenous estrogens and their metabolites in human serum using LC-MS/MS (from Xu et al., 2007, with permission; copyright 2007 American Chemical Society)

stituents at the C-4 position of the arylthiol. A bulky substituent at C-4 results in a 0.25/1 ratio (Kalyanaraman et al., 1985). As mentioned earlier in this chapter, 3,4-catechol estrogens also form depurinating adducts with DNA which may form part of the *in vivo* carcinogenic process. The major depurinating adducts, 4-hydroxyestradiol-N7-guanine and 4-hydroxyestradiol-N3-adenine, can be detected in human breast tissue extract by luminescence and phosphorescence spectrometry (Markushin et al., 2003) and measured by LC-MS/MS formed during *in vitro* experiments using recombinant CYP1B1 by LC-MS/MS - reverse-phase LC is followed by ESI(+) and MRM, looking at the transitions of the pseudo-molecular ion of each analyte $[M+H]^+$ at two collision energies (Belous et al., 2007). A similar approach was adopted for the measurement of 40 estrogens, which included 23 depurinating adducts and *N*-acetylcysteine estrogen conjugates in the urine of 75 normal women and breast cancer patients – in this instance (Gaikwad et al., 2008), the estrogen conjugates were extracted using phenyl solid-phase cartridges and analysed in a UPLC-MS/MS system (Zhang, H et al., 2007) using both positive and negative ESI and MRM. A similar approach had been used by the same group to analyse human breast tissue from both normal and tumor sites using LC with electrochemical detection (Rogan et al., 2003; Lu et al., 2007) and nano-LC-ESI-MS/MS (Embrechts et al., 2003). An LC-ESI(+)-MS/MS-MRM method using deuteriated internal standard has been described for the measurement of three estrogen-glutathione conjugates formed during *in vitro* studies with recombinant CYP1B1 (Hachey et al., 2003). Use of MS (Ramanathan et al., 1998) and HPLC, MS and NMR (Cao et al., 1998) have been used in the structural elucidation of conjugates of estrogens with glutathione and estrogen quinone conjugates with cysteine, *N*-acetylcysteine and glutathione. Red wine drinkers will be pleased to learn that using the UPLC-MS/MS system (Zhang, H et al., 2007) resveratrol has been shown to prevent the formation of estrogen-DNA adduct formation in

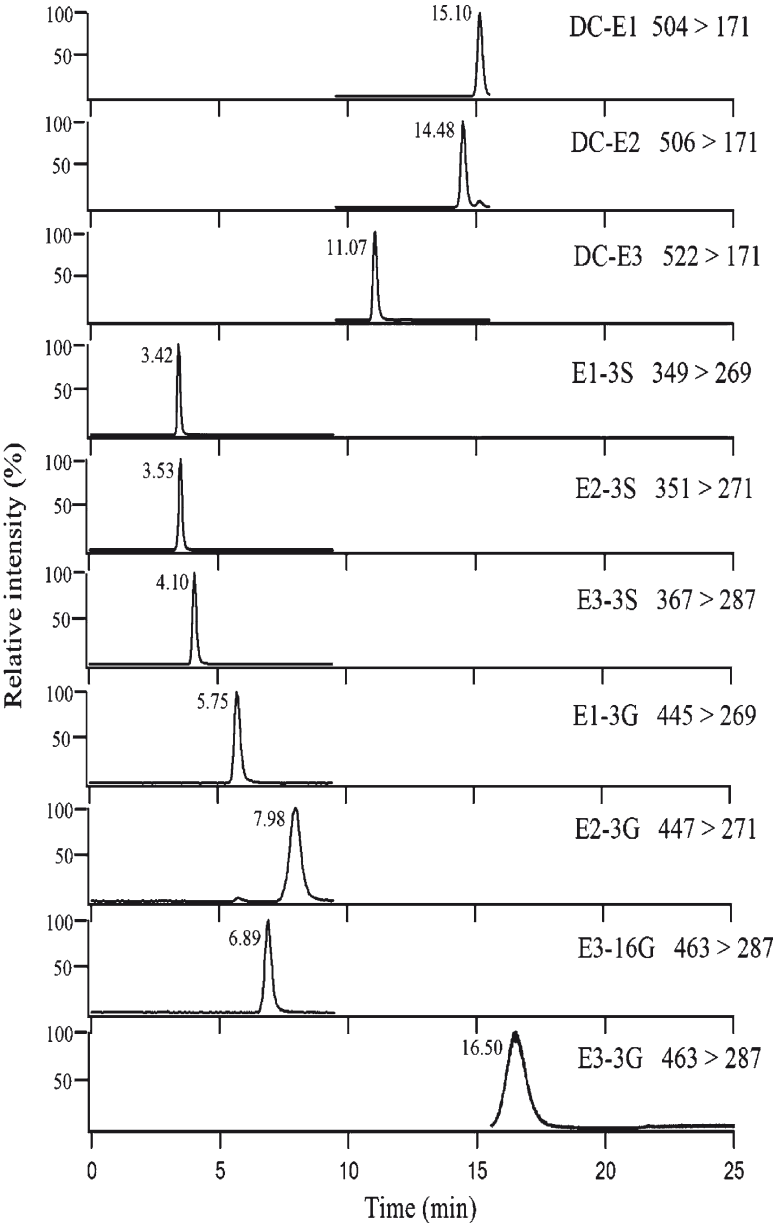


Fig. 8.16 Resolution of free and conjugated estrogens. Multiple reaction monitoring of dansyl chloride derivatives of free estrogens (E1, E2, E3) and underivatized estrogen conjugates after RP/HILIC-LC-MS/MS (from Qin et al., 2008, with permission; copyright 2008 Elsevier B.V.)

MCF-10F cells *in vitro* (Zahid et al., 2008). Immunoaffinity columns have been developed for extraction of 4-OH-E1(E2)-2*N*-acetylcysteine conjugates, which have then been quantified using capillary electrophoresis and laser-induced luminescence

(Markushin et al., 2005). Using LC-APCI(-)-MS, formation of 2- and 4-hydroxylated derivatives of 17α-estradiol have been demonstrated to be formed *in vitro* by incubation with a kidney adenocarcinoma cell line and that these catechol estrogens could form stable DNA adducts (Rizzati et al., 2005). Zhang and Gross (2008) have described the synthesis and purification of ^{15}N- and ^{13}C-labeled and unlabeled 4-hydroxy-estrone-1-N3Ade and have given suggested LC-MS fragmentations of the $[M + H]^+$ parent ion. The synthetic pathway is equally effective for use with estradiol and for formation of adduct with guanine. LC-MS/MS using these internal standards has been described for the identification of 4-OH-estrone-N3Ade in human breast tissue (Zhang Q et al., 2008).

8.5.8.3 Lipoidal Esters of Estrogens

Lipoidal esters of estradiol are a minor class of conjugates which have been found *in vivo* and are readily made synthetically (Kiuru and Wahala, 2006). Their biosynthesis was first described by Schatz and Hochberg (1981). Pahuja et al. (1995) have described the esters as 'the most potent of the naturally occurring estrogens' and described the origin of these esters in human ovarian follicular fluid, using [^{3}H] estradiol as a substrate for *in vitro* studies in follicular fluid or serum. After incubation, extraction was done with chloroform: methanol removed the estradiol products, which were purified on an alumina column, and eluting was done with benzene: ethyl actetate after washing with iso-octane: benzene. The estradiol fatty acid ester fraction was further purified by LC. Similar studies were carried out in granulosa cells and for the determination of endogenous concentrations of these esters in follicular fluid. Adlercreutz's group published a time resolved fluoroimmunoassay (Vihma et al., 2001) for the determination of estradiol fatty acids in serum and follicular fluid. After extraction with diethyl ether: ethyl acetate, the esters were isolated using Sephadex LH-20 chromatography, followed by saponification, Sep-Pak C18 extraction and further purification on Lipidex 5000 and LH-20 before quantitation using immunoassay. This method has subsequently been applied in a number of other investigations: estradiol fatty acid esters in lipoprotein particles (Tikkanen et al., 2002a, b), effects of estradiol by transdermal and oral routes on serum estradiol fatty acid ester concentrations (Vihma et al., 2003a) and determination of estradiol fatty acid esters in lipoprotein fractions of human blood (Vihma et al., 2003b) and adipose tissue and serum of pre- and post-menopausal women (Badeau et al., 2007). LC-MS/MS has recently been employed to demonstrate the formation of HDL estrone-fatty acid esters (Miilunpohja et al., 2006). Figure 8.17 shows the daughter ion spectrum of the oleate ester of estrone NH_4^+ adduct, which identifies the fatty acid. An interesting recent study has further highlighted the potential importance of this group of estrogen esters, suggesting that estradiol fatty acid esters are more tumorogenic than estradiol itself in human mammary gland (Mills et al., 2008). The existence of fatty acid esters of estradiol as a fraction of circulating estradiol was first reported by Janocko and Hochberg (1983) and their significance in human mammary tumors was discussed by Abul-Hajj and Nurieddin

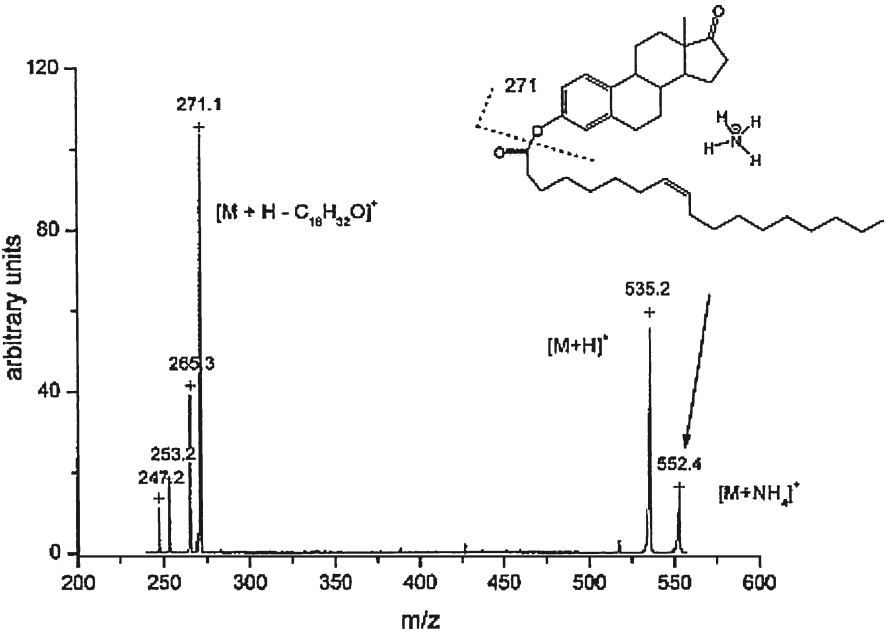


Fig. 8.17 Structural elucidation of estrone-3-oleate by LC-MS/MS. Daughter ion spectrum of estrone-3-oleate (as NH_4^+ adduct, 552.4 *m/z*). The ion produced from E1–3-oleate, the NH_4^+ adduct $[M + NH_4]^+$, was isolated and subjected to fragmentation. The resulting fragment ion spectrum is displayed here. The main fragmentations are loss of NH_3 (535.2 *m/z*) and loss of NH_3 and fatty acid as ketene (271.1 *m/z*). The loss of fatty acid yields the same product ion for all E1 esters therefore the ion at 271.1 *m/z* can be used as an indicator of this substance class. The small fragments at 265.3, 253.2 and 247.2 have not been identified (from Miilunpohja et al., 2006, with permission; copyright 2006 Elsevier)

(1983). Studies of the clearance rate of estradiol esters in comparison to that of estradiol itself (Hershcopf et al., 1985) suggested that these esters could be a source of free estradiol. The uterotropic effect of a saturated lipoidal ester of estradiol following oral administration to rats was described by Paris et al. (2001). Fatty acid esters of estradiol and estrone have been found using the LC-MS/MS in the mussel *Mytilus edulis* as a result of exposure to estrogens in sewage effluents (Labadie et al., 2007).

8.5.9 Environmental Estrogens

There is an increasing concern about the potentially adverse affects of polluting estrogenic substances on the environment and ultimately human and animal life – summarized comprehensively in the Introduction to Noppe et al. (2007). Estrogenic substances, of which there are many, are not always steroid estrogens and the analysis of such non-steroidal estrogens (e.g. bis-phenol, phytoestrogens) will not be dealt

with here. Polluting estrogens arise from two main sources – probably the majority from animal waste which is spread on fields as manure without any treatment to remove excreted estrogens or by direct excretion into water courses or via farm drains (e.g. Johnson et al., 2006) and from human waste, particularly from synthetic estrogens that are taken for contraceptive purposes and as hormone replacement therapy by post-menopausal women. The presence of estrone and estradiol has been demonstrated in streams running through farms by LC-MS/MS at concentrations ranging from 0.04–3.6 ng E2 equivalents/L (Matthiessen et al., 2006). The removal of these steroids in sewage treatment plants was, in the past, not regarded as necessary but over the last 20 years the demonstration of the presence of significant concentrations of steroid estrogens in the environment, has stimulated concern and multi-center studies (e.g. ENDIS-RISKS - Noppe et al., 2007) are underway. Estrogenic chemicals (including steroid estrogens) are only one component of the increasing complex cocktail to which human and animal life is exposed and the synergistic or additive effects of all the diverse components of the cocktail must not be neglected (Kortenkamp, 2007). Continued investigations into the concentration of estrogens in the environment and their long- and short-term effects remain important tasks. Table 8.11 shows examples of environmental estrogens that have been detected and their measurement by mass spectrometry. As concentrations are usually very low, sensitive methods are required for their measurement and these are usually GC or LC linked to mass spectrometry, although simpler non-MS methods are still used (e.g. Ribeiro et al., 2007, 2009 who describe development and use of an LC-diode array detection system for E1, E2 and EE2 in estuary water). LC with ultraviolet (UV) and electrochemical (ED) detection have been used to detect estrogens in river and waste water (Penalver et al., 2002, Wang et al., 2008) and LC with fluorescence detection in river water (Ying et al., 2002) – LODs reported by these workers ranged from 300–1,100 ng/L (UV), through 60–80 ng/L (ED) to 20–50 ng/L (fluorescence) and these are orders of the magnitude higher than LODs achieved with GC and LC-MS (*vide infra*). There have also been some useful reviews of methodology in this area (Croley et al., 2000 – application of mass spectrometry, Lopez de Alda and Barcelo, 2001b – analytical methods for estrogens in waste waters, Petrovic et al., 2002 – mass spectrometry, Lopez de Alda et al., 2003 – use of LC-MS/MS, Petrovic et al., 2004 – TIE [toxicity identification and evaluation], Stuart, 2007 – use of GC-MS, Rodriguez-Mozaz et al., 2007 – SPE-LC-MS/MS compared to biosensors, Noppe et al., 2008 – steroid hormones [including estrogens] in edible matrices). The impact of mass spectrometry in this area is regularly reviewed (Richardson, 2006) and for the years 2006–2007 has been reviewed by Richardson (2008).

The majority of methods used for estrogen analysis in environmental samples are based upon a mass spectrometric end-point and variations in the methodology are primarily concerned with extraction/purification processes prior to GC- or LC-MS. The degree of care in pre-purification required is dependant upon the matrix under examination – sewage sludge, soil or animal feeds may need more pre-purification than wastewater, though in the latter case, the concentrations of estrogens may be very low, so the problem is concentration rather than clean up.

Table 8.11 Mass spectrometry in the measurement of estrogens in environmental matrices (2000–date)

Analyte(s)	Matrix	Extraction	Mass spectrometry	References
Gas chromatography-mass spectrometry				
17β-E2 and EE2	Surface water from ditches/canals	Filtration with glass fiber/ SPE (C18 or PS-DVB polymer) or EtAc liquid extraction	Underivatized or *t*-butyldimethylsilyl ether derivatives. Voyager GC-EI(+)-MS	Mol et al. (2000)
E1, 17β-E2, EE2	Wastewater from Suffolk/ Essex treatment works	SPE – C18 disks	*t*-butyldimethylsilyl ether derivatives. Thermoquest GCQ Ion-trap GC-EI(+)-MS and MS/MS	Kelly (2000)
E1, 17ß-E2, E3, EE2	River water from Tama River, Japan	SPE-ENV/124 cartridges after filtration	3-PFB-17-TMS derivatives. HP-5973 -NCI using methane as reagent gas, monitoring $[M\text{-}57]^-$ ions	Nakamura et al. (2001)
	River water, creeks feeding Lake Constance and Danube	Ethinyllbenzene-divinylbenzene co-polymer for extraction	3-PFB derivatives. Same GC-MSD	Kuch and Ballschmiter (2000, 2001)
	River Thames Water	C18-SPE cartridges or polymer impregnated disks (SDB-XC)	Per-PFB derivatives HP5895 MS. NCI	Xiao et al. (2001)
	Ground water and swine lagoon samples	Oasis-HLB cartridges	3-PFB-17-TMS derivatives. Finnigan TSQ-7000 NCI & MS/MS	Fine et al. (2003)
E1, 17β-E2, EE2 and mestranol	Activated and digested sewage sludge	Extraction with MeOH and acetone; gel and silica chromatography HPLC	di-TMS derivatives; Varian Saturn 4D GC-MS/MS-EI(+)	Ternes et al. (2002)
E1, 17α and β-E2, E3, EE2	Water from WWTPs on River Seine up- and down-stream of Paris	Filtered. SPE-C18 and final solvent EtAc extraction (x4) + *Helix pomatia* hydrolysis	Pentafluoropropionyl derivatives –HP5973 GC-MS-SIM	Mouatassim-Souali et al. (2003); Cargouet et al. (2004)
E1, 17β-E2, EE2, 16α-OH-E1	Sediments from River Ouse	Microwave assisted MeOH extraction, centrifuged; silica gel mini-columns	TMS derivatives; Thermoquest Polaris Q ion-trap MS. EI(+) –SIM	Liu et al. (2004b)
		OASIS-HLB		Liu et al. (2004a)

(continued)

Table 8.11 (continued)

Analyte(s)	Matrix	Extraction	Mass spectrometry	References
E1, E2	Surface water from sites on River Elbe	Study of SPE sorbents – OASIS-HLB chosen; water filtered	TMS derivatives; GC-Finnigan MAT ion-trap MS. GC-EI(+)-MS	Weigel et al. (2004)
DES, E1, E2, mestranol, EE2, E3	River water and samples of in- and e-ffluent STP	SPE using OASIS-HLB catridge after filtration followed by 500 mg silica cartridge	TMS ether derivatives formed – several reagents studied. Varian Saturn-2000 ion-trap GC-MS and MS/MS	Quintana et al. (2004)
		Polyacrylate fibres and derivatization on fibre		Carpinteiro et al. (2004)
E1, E2, EE2	Water samples from River Tama, Japan	Polydimethylsiloxand coated-stir bar absorptive extraction	Underivatized Es and acetates. Thermal desorption from stir bar and HP-5973 GC-MS EI(+) SIM	Kawaguchi et al. (2004); Kawaguchi et al. (2006)
E1, E2, EE2	WWTPs in Sussex and Almeira	Filtration, C18 SPE	TMS ethers. Thermoquest GCQ ion-trap GC-MS-EI(+)-SIM or GC-MS/MS	Hernando et al. (2004)
E1, E2, E3, EE2, MeEE2	Surface water and WTP effluent samples in areas in Paris and other cities	Evaluated several SPE cartridges. Oasis HLB used after filtration	TMS ethers on HP 5973-EI(+)-SIM	Labadie and Budzinski (2005)
E1, E2, EE2	Surface water at various points in the Scheldt estuary	Bakerbond Speedisk – C18. Purification with Si cartridges in series with NH2 cartridge in series	Thermofinnigan Trace GC-2000 ion-trap MS/MS after TMS formation	Noppe et al. (2005)
E1, E2, EE2	Water samples from STP	Automated C18-SPE. STP inffluent water needed extra Silica gel clean up	LC-MS/MS using Micromass Quattro tandem MS with ESI(–). MRM	Zuehlke et al. (2005)
E1, E2	Water sample from Sajiao district in China. Analysis of fish serum	Supelco polyacrylate fibre	Derivatization using BSTFA. HP5973 GC-MS	Yang et al. (2006b)
		Automated system		Yang et al. (2006a)

E1,17α- and 17β-E2, E3, mestranol, E3	Pearl River estuary sediments	Solvent extraction using Soxhlet and ultra-sound, followed by Silica gel column	PFP-derivatives by HP5972 MSD	Peng et al. (2006)
E1, 17α- and 17β-E2, E3	Flushed dairy manure wastewater	Carbograph SPE (Andreolini et al., 1987), followed by C18SPE (×2)	TMS ethers; HP5973 GC-MSD EI(+)	Hanselman et al. (2006)
E1, E2, E3, EE2	Septic, soil and groundwater matrices	Filter through glass fibre paper, SPE on Strata-SAX in line with Strata-X. + SIL cartridges	TMS ethers. Saturn 2000 GC-MS^n ion-trap EI(+)- Fullscan MS or MS/MS	Stanford and Weinberg (2007)
E1, E2, EE2, E3	River sediments from Moldau River up- and down-stream from Prague	Homogenize. Solvent (Hx-acetone) extraction, OASAIS-HLB	Underivatized estrogens. GC × GC-HS-ToF-MS, monitoring M^+ ions	Hajkova et al. (2007)
E1, E2, 17α- and 17β-E2	Environmental soil samples	Pressurized liquid extraction using a Dionex Extractor 2000. Acetone gave best recovery. C18-SPE	TMS ethers; HP5973 GC-MS-EI(+)	Beck et al. (2008)
E1, E2, EE2	Environmental water samples	Use of PTV (programmable-temperature vaporization) enabling large volumes to be injected onto the GC	Underivatized estrogens. HP5973 GC-MSD-EI(+)	Hu et al. (2008)

(continued)

Table 8.11 (continued)

Analyte(s)	Matrix	Extraction	Mass spectrometry	References
E1, E2–17β and 17α, E3, EE2 and mestranol	River and wastewater and sediments in Thessaloniki, Greece	SPE on Oasis-HLB, clean-up with Florisil. Solid sample used ultrasonic solvent extraction	TMS ether derivatives. GC-MS using a Polaris Q ion-trap MS. EI(+)	Arditsoglou and Voutsa (2008)
E1, E2–17β and 17α, EE2, E3, mestranol	Water from various sites on Pearl River, China	Filtration through glass-fibre paper, SPE using ENVI-18 cartridges	TMS derivatives; GC-MS using Micromass Platform I-MS. SIM	Peng et al. (2008)
E1, E2, E3, EE2	Influent and effluent from Wuhan STP	Filtration through glass-fibre filter 0.7 μm. SPE with Oasis HLB cartridges	TMS derivatives; Agilent 5973 MSD. EI(+). SIM	Jin et al. (2008)
E1, 17α- and 17β-E2	Milk, meat and eggs	Milk extracted with solvent first. Enzyme hydrolysis with *Helix pomatia*. ChromP SPE. Liquid:liquid partition with *n*-pentane	TMS ethers; Quattro GCQ-GC-MS/MS using EI(+)	Courant et al. (2008)
Liquid chromatography-mass spectrometry				
E1, E2, E3, EE2	In- and e-ffluent water from sewage treatment plants in Rome	Filtration. Graphitised carbon black SPE (Carbograph 4)	LC-MS/MS using a Sciex API 2000 triple quad with negative turbo-ion spray. Methanolic NH_3 added post-column to promote ionization of estrogens. MRM	Baronti et al. (2000)
Plus sulfate and glucuronide conjugates				Gentili et al. (2002) and D'Ascenzo et al. (2003)
E1, E2, E3, EE2, mestranol	Water from sewage treatment plants and rivers in Spain	Filter. C18SPE	LC-diode array-MS. HP1100 API-ES MSD. ESI (-) and APCI(-) for estrogens. $[M-H]^-$	Lopez de Alda and Barcelo (2000)
			Automated LC-DAD system	Lopez de Alda and Barcelo (2001a)

E1, E2, EE2	Effluent from STP in Long Island, New York	Immunosorbent – monoclonal Abs to E1 and E2 bound to control-pore glass	LC-MS using Micromass Platform LCZ MS with Z-spray. ESI(−) monitoring $[M-H]^-$ ions	Ferguson et al. (2001)
E1, E2, E3, EE2	River sediments in Catalonia, Spain	Ultrasonic solvent extraction of lyophilized sediment. Purification on C18 cartridges	LC-diode array-MS- ESI(−) for estrogens	Lopez de Alda et al. (2002)
E1, E2, EE2	Waste water from STPs	Filtration through glass fibre paper. SPE with C18 cartridges. Eluate dried and taken up in EtAc and washed with saline. Purified in Florisil columns	ThermoFinnigan LCQ using negative ion ESI and APCI. ESI more sensitive than APCI	Ingrand et al. (2003)
E1, E2, E3, EE2, E2-3S, E2-3G + other estrogen and conjugates	Water samples from Tamagawa River in Tokyo and Lake Kasumiguara	Filter through glass fibre paper. SPE with EDS-1. Oasis-HLB also examined. Free Es purified on Florisil column	LC-MS/MS using Micromass Quattro Ultima using ESI(−). Addition of triethylamine to mobile phase increased ionization	Isobe et al. (2003)
E1, E2, E3, E2-17G, E1-3S, EE2	Water samples from each stage of treatment in St Joan Despi waterworks	SPE using PLPR (cross linked styrene-divinylbenzene polymer) cartridges	LC-DAD-MS/MS usingMicromass Quattro LC. ESI(−)-MRM	Rodriguez-Mozaz et al. (2004)
E1, E2, E3, EE2, mestranol	STP effluent in Hamburg, Germany and river water from River Bilina, Czech Republic	Yeast recombinant screen. LC-MS/MS to determine Es. Liquid extraction with CH_2Cl_2. Phenogel size exclusion chromatography	LC-MS/MS using a Sciex API 3000. E2 and mestranol detected using ESI(+) - other Es using ESI(−) mode. MRM	Heisterkamp et al. (2004)

(continued)

Table 8.11 (continued)

Analyte(s)	Matrix	Extraction	Mass spectrometry	References
E1, E2, E3, EE2	Coastal waters of Baltic Sea, outer and inner; one site in vicinity of outflow from STP	Filtration with glass-fibre paper. SPE with Oasis-HLB cartridges. Purification on silica gel columns	LC-MS/MS using Sciex API 4000 with Turbo ion-spray using MRM. $[M-H]^-$ in M1	Beck et al. (2005); Beck et al. (2006)
E1, E2, E3, EE2	River water from Toghui River in Beijing	SPE on C18 cartridges followed by NH_2-SPE	Micromass ZMD MS with Z-spray ion source with ESI(−) probe	Hu et al. (2005)
E1, E2, E3, EE2	River water samples at six sites in Okayama City, Japan	In-tube absorption using a Supel-Q PLOT column in series with analytical column. Operated by valve switching	LC-MS/MS using Sciex API 4000. using turboIon spray in negative ion mode. MRM using $[M-H]^-$ in MS1	Mitani et al. (2005)
SO_4 and gluc conjugates of E1 and E2	Influent and effluent water from STP	Filter through glass-fibre paper. SPE on Oasis-HLB cartridge. Cleaned up using DEAE anion-exchange column	LC-MS/MS using a Micromass Quattro LC fitted with a Z-spray ESI source. Negative ions used. MRM. Also used LC-ToF MS (Micromass LCT)	Reddy et al. (2005)
E2	Water from River Hirose, Sendai, Japan	MIPs, using 6-oxo-E2 as template, on-line	Shimadzu LC-MS 2010A system – ESI(−) -SIM	Watabe et al. (2006)
E1, E2, E3, EE2, E1-3S	Settled sewage and final effluent water samples in UK	Filter through glass-fibre paper. C18 SPE. PLgel size-exclusion chromatography and anion exchange (Varian-NH_2)	LC-MS/MS using a Quattro Premier XE-MS using Z-spray ESI(−). MRM	Koh et al. (2007)
E1, 17α- and 17β-E2, E3, E1-3G, E2-17G, E1-3S, E2-3S, E3-3S, EE2	River sediments from River Svrata in Brno and River Loucka, Czech Republic	Soxhlet or microwave-assisted extractions with MeOH:water. Oasis-WAX catridge	LC-IT-MS/MS using Finningan LCQ Advantage ion-trap MS-ESI(−). MRM	Matejicek et al. (2007)
E1, E2, EE2	River sediments from 4 locations in river Ouse	Microwave assisted solvent extraction. SPE on Strata-X-AW and clean-up on silica gel catridges	LC-ToF- and LC-MS/MS using Micromass ToF-MS or Quattro Premier triple quad MS. ESI(−). MID and MRM	Labadie and Hill (2007)

E1, E2, EE2	River water and effluent from STP in Taipei, Taiwan	50 mm Bakerbond C18 Speedisks and filtered thru PTFE filters Derivatization as Dansyl-, perfluorbenzyl- and (2-fluoro-1-methylpyridinium-*p*-toluenesulphonyl- derivates compared with no derivatization	LC-MS/MS using ThermoFinnigan TSQ 7000 MS. ESI(−). MRM	Chen et al. (2007) Lin et al. (2007)
E1, E2–17α and β, E3, EE2 + 13-16 E conjugates.	Concentrated animal feeding operations lagoons from swine, poultry and cattle operations in Southern USA	Centrifuged and filtered thru glass-fibre paper; free Es analysed by GC-MS Fine et al. (2003); direct analysis of conjugates Gentili et al. (2002)	LC-MS/MS using Finnigan TSQ; ESI(−). MRM	Hutchins et al. (2007)
E1, E2, EE2	Influent and effluent from STP near Lyon, France	Automated pre-column SPE on Oasis-HLB, elution and derivatization with dansyl chloride	LC-MS/MS using Sciex API 3000 MS using Turbospray (+) ionization. MRM using $[M+H]^+$ ions in MS1	Salvador et al. (2007)
17α- and 17β-E2, E1, E3, norethindrone, levonorgestrel and EE2	Effluent water from two STPs near Lyon	Filter through glass-fibre filters and μM nitrocellulose filters. SPE investigated using C18, Strata-E and StrataX, ENV+ cartridges, followed by Oasis HLB and – MCX. Bakerbond silica gel cartridges used for clean up	LC-DAD-MS/MS with ESI and API ionization (? Agilent 100MSD). Use if ESI(+) using SIM of $[M+H]^+$	Vulliet et al. (2007)

(continued)

Table 8.11 (continued)

Analyte(s)	Matrix	Extraction	Mass spectrometry	References
E1, 17α- and 17β-E2, E3, EE2	Water sample from Svratka river, Brno	SPE on Oasis HLB followed by separation on ENVI-Florisil column. Derivatization with FBIBT in needle of automated injector	LC-MS/MS using a Finnigan LCQ ion-trap Advantage Max MS system. ESI(+). MRM using $[M+H]^+$ in MS1	Matejicek and Kuban (2008)
E1, 17α- and 17β-E2, E3, EE2	Surface and ground water from Rhone-Alpes, France	Filter through glass fibre filter and 0.45 μm Nitrocellulose filters. Automated SPE extractio using Strata- C18E	3200 QTrap triple quadrupole MS. Using ESI(−). MRM selecting $[M-H]^-$ in MS1	Vulliet et al. (2008)
E1, E2, E3 and sulfatee and glucuronide conjugates	N Saskatchewan River	SPE extraction with Oasis HLB cartridges. Separation of free estrogens and derivatization with dansyl chloride. Column switching allowing free E derivatives to be separated by RP-LC and E-conjugates by HILIC	Hybrid quadrupole/linear ion trap – API4000Q. Free estrogens and conjugates in negative ion mode and derivatized free estrogens in positive ion mode. MRM	Qin et al. (2008b)

E1, estrone; E2, estradiol (17α- or 17β-); E3, estriol; EE2, ethynylestradiol; SPE, solid-phase extraction; TMS, trimethylsilyl; PFB, pentafluorobenzoate; EI, electron impact; ESI, electrospray ionization; MS, mass spectrometry; MS/MS, tandem MS (MS1-collision cell-MS2); MeOH, methanol; SIM, single ion monitoring; EtAc, ethyl acetate; BSTFA, bis-trimethylsilyltrifluoroacetamide; PFP, pentafluoropropionyl; Hx, hexane; HS-ToF, high speed time of flight; MSD (Agilent name – mass selective detector); NH_3, ammonia; MRM, multiple reaction monitoring (similar to SRM: selective reaction monitoring); API, atmospheric pressure ionization; APCI, atmospheric pressure chemical ionization; DAD, diode array detection; Abs, antibodies; CH_2Cl_2, methylene dichloride; FBIBT, 12-(difluor-1,3,5-triazolinyl)-benz[f]isoindolo-[1,2-b][1,3]benzothiazolidine; MIP, molecularly imprinted polymer; HILIC, hydrophilic interaction liquid chromatography.

Many papers have described solid-phase extraction (SPE) where estrogens are absorbed onto SPE cartridges (Lopez de Alda and Barcelo, 2001c); Rodriguez-Mozaz et al. (2007), on stir bars or SPME and derivatized on the adsorbent followed by thermal desorption and GC-MS analysis. The sensitivity is as low as 0.2 mg/L (Moeder et al., 2000; Fine et al., 2003; Kawaguchi et al., 2005). A summary of GC- and LC-MS methods used since 2000 is given in Table 8.11. All but two of LC-MS/MS methods in this table have used underivatized estrogens for MS/MS, preferring to generate negative ions ($[M\text{-}H]^-$) but Salvador et al. (2007) derivatized with dansyl chloride and Matejicek and Kuban (2008) with 12-(difluor-1,3,5-triazolinyl)-benz[f]isoindolo-[1,2-b][1,3]benzothiazolidine (Fujino and Goya, 1992) and both these latter two methods used positive ionization. Part of the treatment in sewage plants may involve ozonation to remove unwanted components from the influent. GC-MS has been used in one of the few studies of the effect of such ozonation on the degradation of the estrogens E1, E2 and E3 in three STPs in Japan (Zhang H et al., 2008).

A survey of the LODs reported for these methods suggests that for both GC- and LC-MS methodologies LODs have improved between 2000 and 2008. LODs for GC-MS reported in 2000 were 50–300 ng/L whereas by 2008, LODs had fallen to 0.03–0.05 ng/L. Sensitivities of the LC-MS methods are, at their lowest in 2008, reported as in the 0.02–0.231 ng/L (Stavrakakis et al., 2008) and 0.07–0.300 ng/L (Matejicek and Kuban, 2008). There is for both GC- and LC methodologies a surprising variation in the reported LODs with little obvious explanation of why this should be. In this context, we have accepted only quoted LODs where it is clear that LOD is defined as the value achieved by multiplying the noise level at zero concentration by a factor of 3 (Armbruster et al., 1994) and it may be that the observed variation is due to different interpretations of LOD by different authors.

Other methods not using LC, GC or mass spectrometry have been applied to the determination of estrogens in environmental samples: one useful approach has employed a measure of total estrogenicity using a yeast recombinant assay (e.g. Routledge and Sumpter, 1996 and, more recently, Bovee et al., 2004), measuring total estrogenicity in E2 equivalents (e.g. Fernandez et al., 2007; Fernandez et al., 2008 – estrogenicity in Canadian wastewater and at STPs). Other approaches to the measurement of estrogenicity have been used (E-SCREEN - Soto et al., 1995) or a luminescence enzyme-linked receptor assay (Seifert, 2004). These estrogenicity assays are useful in that they quantify the total estrogen activity, the steroid estrogen components of which can be assessed by parallel use of LC-MS (Bovee et al., 2006) in animal feed or GC-MS (Nielen et al., 2006) in calf urine or both GC- and LC-MS (Muller et al., 2008). In order to exert an 'estrogenic' effect it is usually accepted that binding to one or the other of the estrogen receptors is required and this has also been used as a means of assessing oestrogenicity in water using surface plasmon resonance (SPR – reviewed by Habauzit et al., 2007 and applied by Habauzit et al., 2008), which relies on the ability of the ligand to institute dimerization of the ER and binding to the ERE, which can be detected and is quantitative. SPR has been used in the development of a miniaturized on-site detector for the presence of estrone-3-glucuronide in environmental aqueous samples (Sesay and Cullen, 2001).

Other methods have usually involved some kind of immunoassay: an ELISA used in municipal waste waters and validated by GC-MS – MS/MS (Huang and Sedlak, 2001), a 'highly specific' RIA for estrone used in Pacific coastal waters (Atkinson et al., 2003), a bench top fluorimetric IA (Glass et al., 2004), E2 assay in river water using a fluorimetric IA (Matsumoto et al., 2005), rapid- and high-throughput microplate magnetic chemiluminescence IA for E2 in water samples (Zhao and Lin, 2005), assay of E2 and EE2 in STPs using two IAs (Hinteman et al., 2006), evaluation of four commercial ELISA assays by LC-MS/MS (Farre et al., 2006) and a comparative study of an ELISA kit with LC-MS/MS and a new UPLC-QToF-MS method (Farre et al., 2007). It is not clear exactly how valuable these assays are, apart from their convenience, allowing assays to be done on site rather than in the laboratory (e.g. Glass et al., 2004). Hanselman et al. (2004) concluded at the end of a comparative study of three IAs for the measurement of E2 in flushed dairy manure wastewater that there was too much interference for these assays to be useful and that effort should be directed to the development of GC-MS.

While not strictly within the remit of this section, it is worth noting that anxiety was being expressed nearly 10 years ago about the possible effect of estrogens in food, whether endogenous or exogenous, on human development and health (Andersson and Shakkebaek, 1999), particularly in children (Aksglaede et al., 2006). The measurement of steroid estrogens as well as other non-steroidal estrogenic compounds in various foods is therefore of legitimate interest and various methods, already discussed above, have been developed to address and evaluate this problem with regard to steroid estrogens (e.g. Courant et al., 2007a, b, 2008; Noppe et al., 2008).

8.6 Quality Control

All assays must be validated fully before reliance can be placed on results. Commercial assays must be used with care and data from the supplier should be scrutinized. Laboratories should not accept everything they are told by manufacturers and must have in place some scheme for checking that claims are accurate. Co-operation with other laboratories using the assay is always valuable. Assays using reagents from other trusted laboratories may not always work in their new home/environment as well as expected and the reason for this is not always obvious. One obvious avenue is the quality of the standards used for quantitation – internal standards may have an effect on precision but primary standards must be pure and they must be stored properly to prevent deterioration and checked at regular intervals. Standards bought from commercial suppliers or provided by colleagues must always be checked. Even when assays, however simple or complex they are, become operational, they must be subject to stringent regular quality control and good laboratory practice. Suitable internal quality control samples covering the concentration range used must be used in every assay batch to ensure that the method continues to perform in an accurate and precise manner. In most cases, quality control is based on the evaluation of precision. QC data can be plotted

using Levy-Jennings charts (Westgard, 2004) to determine trends, shifts, and random error and control of outliers. The application of Westgard rules (Westgard, 2004) allows for the detection of random analytical errors versus systematic analytical errors. Accuracy or bias is a difficult concept and it is not easy to establish a universal method for one laboratory to establish in isolation whether their results are accurate. Performance should be monitored externally and all laboratories should belong to a recognized external quality assurance scheme, if one exists. If not, it is always valuable to collaborate with other laboratories doing the same assays by exchanging samples. In the case of steroids, the 'gold' standard for assessment of accuracy is a comparison with results by GC-MS or LC-MS but not a surrogate, and if such methodology is unavailable, the All Laboratory Trimmed Mean (ALTM), often is very close to the 'real' answer if the number of independent laboratories contributing to the ALTM is sufficiently large.

In the United Kingdom there is a recognized External Quality Assurance Scheme for Steroids (UK NEQAS) and Table 8.12 shows the relative performance for serum estradiol assay as of February 2006 (Middle and Kane, 2009). It should be noted that this is a snapshot and changes in method can alter performance over a period of time. Laboratory results are compared against the ALTM to provide an assessment of bias. It can be seen that there is a wide variety of bias for the different methods despite all being standardized against a substance readily available as a pure solid. Similar differences between methods are shown by other quality assessment schemes such as the Endocrinology Proficiency Testing programme run from the New York Department of Health Wadsworth Center (Table 8.13).

As can be seen from the differences in bias the results from different laboratories using the same assays show considerable variation in values for the same samples and this is even greater when comparing different methods. This should be borne in mind when comparing results from different laboratories.

Performance evaluations for many of these automated systems when applied to estrogen assay have been published (Rodriguez-Espinosa et al., 1998; Draisci et al., 2000; Tello and Hernandez, 2000; Koenn and Ndah, 2003; Taieb et al., 2003a, b; Yang et al., 2004; Massart et al., 2006; Taieb et al., 2007) (see Table 8.5).

Table 8.12 UK NEQAS performance for estradiol by automated immunoassay analysers in February 2006 (Data used with permission from Dr. J. Middle, UK NEQAS Organiser; http://www.ukneqas.org.uk)

Instrument	N	Median bias	Interquartile range
All methods	300	+3.4	–11.2 to +10.4
Beckman Access	20	+0.1	–3.9 to +3.4
Bayer Advia Centaur	72	+13.4	+9.2 to +17.6
Abbott Architect	28	–1.4	–3.9 to +0.9
Wallac Delfia	10	–7.1	–9.1 to –5.3
Roche Elecsys	82	+7.9	+5.1 to +11.0
DPC Immulite	6	–28.2	–29.6 to –28.0
DPC Immulite 2000	52	–24.6	–26.5 to –22.5

Table 8.13 Adapted from Clinical Chemistry and Hematology Laboratory, Wadsworth Center, NY State Department of Health (www.wadsworth.org/chemheme/chem/endo)

Summary of participant performance (mean and standard deviation)

10 September 2007

(Estradiol pg/mL)

Instrument	N	Specimen E06	Specimen E07	Specimen E08
All methods	162	61.4 ± 27.48	144.2 ± 51.7	145.7 ± 37.67
AxSym	6	46.2 ± 10.73	129.6 ± 16.38	106.0 ± 13.78
Access	9	121.5 ± 17.7	129.8 ± 8.97	263.7 ± 17.7
Advia Centaur	41	76.2 ± 14.12	234.7 ± 40.06	137.9 ± 17.52
Elecsys	12	46.9 ± 6.77	130.4 ± 7.82	111.1 ± 8.42
Immulite	16	47.8 ± 10.73	129.9 ± 15.35	163.4 ± 20.56
Immulite 2000	24	53.9 ± 8.19	126.8 ± 11.96	161.6 ± 10.24
Vitros Eci	8	33.0 ± 2.61	92.1 ± 6.58	152.5 ± 9.76

8.7 Concluding Remarks

Remarkable advances in estrogen analysis were made in the period 1970–1980 as RIA replaced earlier methods. During that time, the techniques for raising antisera capable of recognizing selected estrogens with a high degree of specificity were developed and tested; different labels were evaluated; and a variety of systems for the separation of bound ligand from the free were assessed. These RIAs had improved levels of sensitivity, specificity, precision and simplicity compared to the previous methods. Most of the RIAs were directed to analysis of estrogens after isolation from their original matrix. Advances in immunoassay such as non-extraction (or 'direct') or non-isotopic techniques quickly found their way into commercial kits and then into automated immuno-analysers. This gave the user both benefits and pitfalls. The ease of use overshadowed the loss of freedom to adjust the assay system to match the demands of a particular problem. Given the capital outlay involved, commitment to the use of the kits may place too much reliance on the products of just one manufacturer. Additionally, widespread use of commercial methods may impair the maintenance of the ability and experience necessary to develop methods tailor-made for a particular analyte (or estrogen).

In future, analytical methods for estrogens in reproductive or general endocrinology which can be applied to initial diagnosis or screening procedures are likely to become much less complicated. Improvements in automation mean that large numbers of samples can be analysed easily. Nevertheless, since the first edition of this book, the predicted change to positive or negative end-points at physiologically relevant concentrations has not occurred. The early enthusiasm for salivary estrogen measurement has not been widely accepted and readers of this chapter are left to consider for themselves possible reasons for this lack of enthusiasm. It is possible that the "analyser on a chip" may be the future, especially if measurement of substances in the environment becomes more important. Such devices would be easily transportable

or even implantable to allow continuous monitoring over time but removal of devices from the laboratory is fraught with problems. Regrettably, despite many advances in automation the measurement of estradiol remains an analytical challenge especially at low concentrations (27 ng/L – 100 pmol/L) that may be relevant in post-menopausal women at risk of developing breast cancer – an analytical problem multiplied in women being treated with aromatase inhibitors or SERMs.

In the research laboratory or in non-clinical environments the problem is different. There will be the need to show that the compound of interest is estrogenic. At this fundamental level some form of bioassay is likely. Once a compound or mixture is shown to be estrogenic, then isolation of groups of these compounds will be necessary, followed by separation, detection and measurement of the individual components. Structural information as well as quantitation can be obtained by GC-MS and large scale analyses of identified compounds will likely be done by automated LC-MS/MS Automation of the pre-prepared clinical specimens is making large-scale analyses of individual estrogens by the latter technique feasible but more needs to be done before a throughput equivalent to the current immunoassay analyzers is possible.

There is however a general expectation that the steroid immunoassays are likely to assume less importance in the future and therefore less effort will be devoted by manufacturers to the production of new methodology and/or automated systems for immunoassay. Mass spectrometers have come to be widely used in laboratories as they have become more compact and computerized systems have provided simple control and data handling. A bench-top GC-MS is no longer a rare sight in clinical laboratories and we predict that future developments will be in the area of automated and simplified LC-MS/MS systems. This view is supported by a recent review of developments in LC-MS/MS in the clinical laboratory over the last 10 years and suggested goals for future developments (Vogeser and Seger, 2008).

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Chapter 9
Anabolic Steroids: Metabolism, Doping and Detection in Human and Equestrian Sports

A.T. Kicman, E. Houghton and D.B. Gower

9.1 General Introduction

This chapter highlights the important aspects of detection of doping with synthetic anabolic steroids and discusses some of the problems with, and solutions to, the detection of misuse of the naturally occurring ones.

9.1.1 Anabolic Activity

Androgens, such as testosterone (T), possess both androgenic and anabolic activities. With structural modifications, the anabolic effects of androgens can be enhanced but, even so, these cannot be divorced entirely from their androgenic effects. Hence, a more accurate term for anabolic steroids is anabolic-androgenic steroids, but for simplicity, the shorter term is used within this chapter. Figure 9.1 displays the mainstream anabolic steroids and their diagnostic metabolites. For a comprehensive list of trade names, together with the name and country of corresponding manufacturers, Index Nominum: International Drug Directory. Stuttgart: Medpharm Scientific Publishers, 2008. Structures of other anabolic steroids are displayed in Figs. 9.2 and 9.3.

The clinical usefulness of anabolic steroids in reversing the catabolic state of patients, such as those with severe burns or wasting diseases, has not been realized on the basis of the conclusions of previous reports. As a result, many anabolic steroids developed in the last century have been withdrawn as licensed products in numerous countries world-wide and their uses are limited to the treatment of specific diseases. For example, various anaemias and also hereditary angioedema can be treated with anabolic

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steroids. Oxymetholone is still considered to have beneficial effects on damaged myocardium (Tomoda, 1999) is, Further, T preparations (and also mesterolone but seldom used, if at all)) are prescribed for hormone therapy in male hypogonadism. Nandrolone decanoate is still indicated for osteoporosis in postmenopausal women (but it is not an advocated treatment). However, consideration of their therapeutic efficacy for anabolic purposes may need to be revisited, especially for the treatment of sarcopenia (loss of

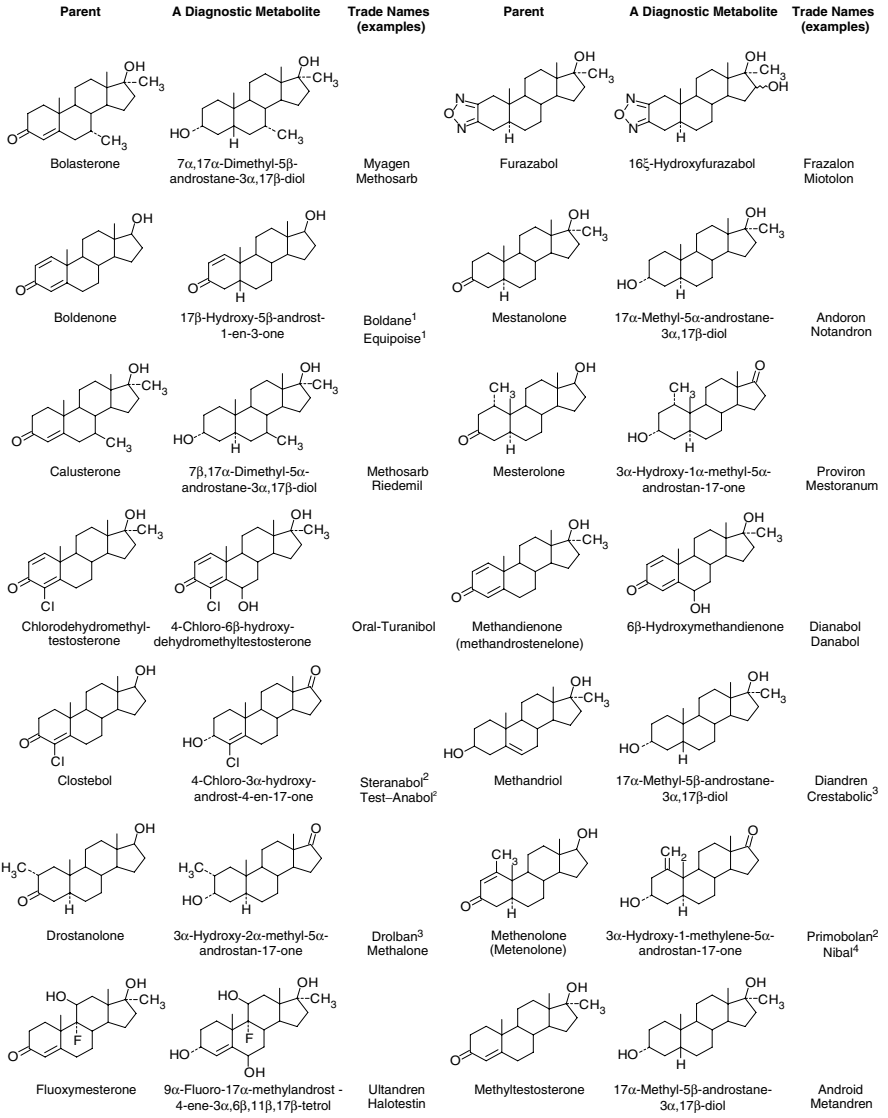


Fig. 9.1 Structures of anabolic–androgenic steroids with corresponding diagnostic metabolites and examples of registered trade names (figure continued overleaf). Superscripted numbers against trade names refer to 17β-hydroxyl-esterified preparations: [1]undecylenoate; [2]acetate; [3]propionate; [4]heptanoate; [5]decanoate; [6]hexahydrobenzylcarbonate

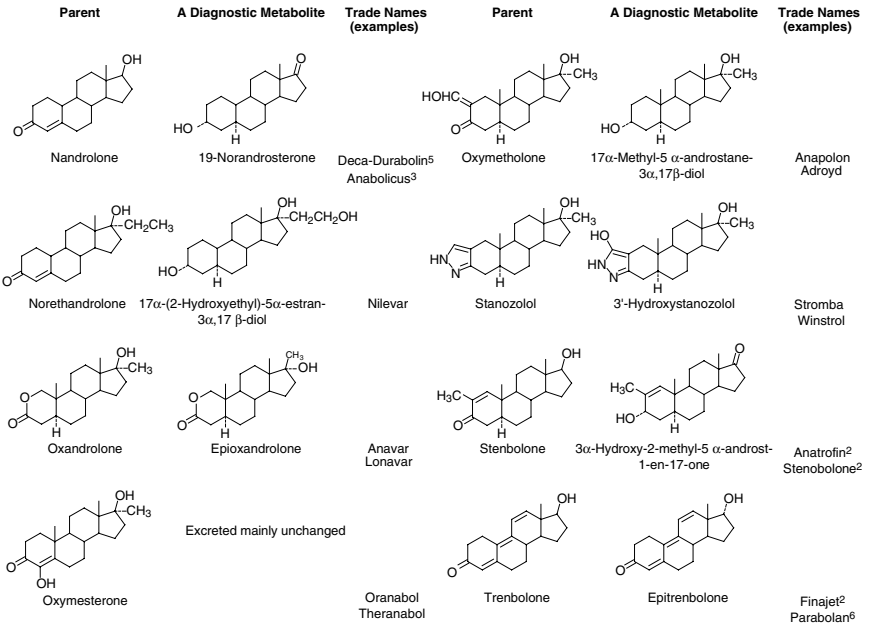


Fig. 9.1 (continued)

muscle mass and strength). Anabolic steroids, such as T and oxymethelone, appear to be extremely useful in the treatment of HIV-related muscle wasting (Hengge et al., 1996; Bhasin and Javanbakht, 1999; Bogin and Shaw-Stiffel, 1999; Bhasin et al., 2000), and nandrolone decanoate has been demonstrated to be effective in countering sarcopenia in patients receiving dialysis (Johansen et al., 1999, 2006). Trestolone (7α-methyl-19-norT; MENT) may be a promising new androgen therapy, for example in age related sarcopenia (Bhasin et al., 2006; Solomon and Bouloux, 2006).

With regard to sport, it has been conclusively demonstrated that administration of supraphysiological doses of T to men can significantly increase fat-free mass, muscle size, strength and power (Bhasin et al., 1996, 2001), and these effects are likely to be enhanced for synthetic steroids, which have favorable anabolic properties compared to T (based on the rat mytrophic:androgenic index and nitrogen balance studies). The mechanisms of action on skeletal muscle may include a direct anabolic effect on androgen receptors, an anti-catabolic effect by countering the actions of cortisol, and also an indirect anabolic effect via an androgenic effect in increasing aggression and competitiveness, as discussed by Kicman and Gower (2003). In the equine, reports on the efficacy of anabolic steroids are contradictory, but increases in body weight and improved performance have been demonstrated, both in training and on the race-track, and also in horses with orthopaedic conditions (e.g., Snow, 1993).

9.1.2 Control of Anabolic Steroids

In the UK, anabolic steroids are controlled under Schedule IV Part 2 of the Misuse of Drugs Act; the act includes most of the anabolic steroids, together with clenbuterol

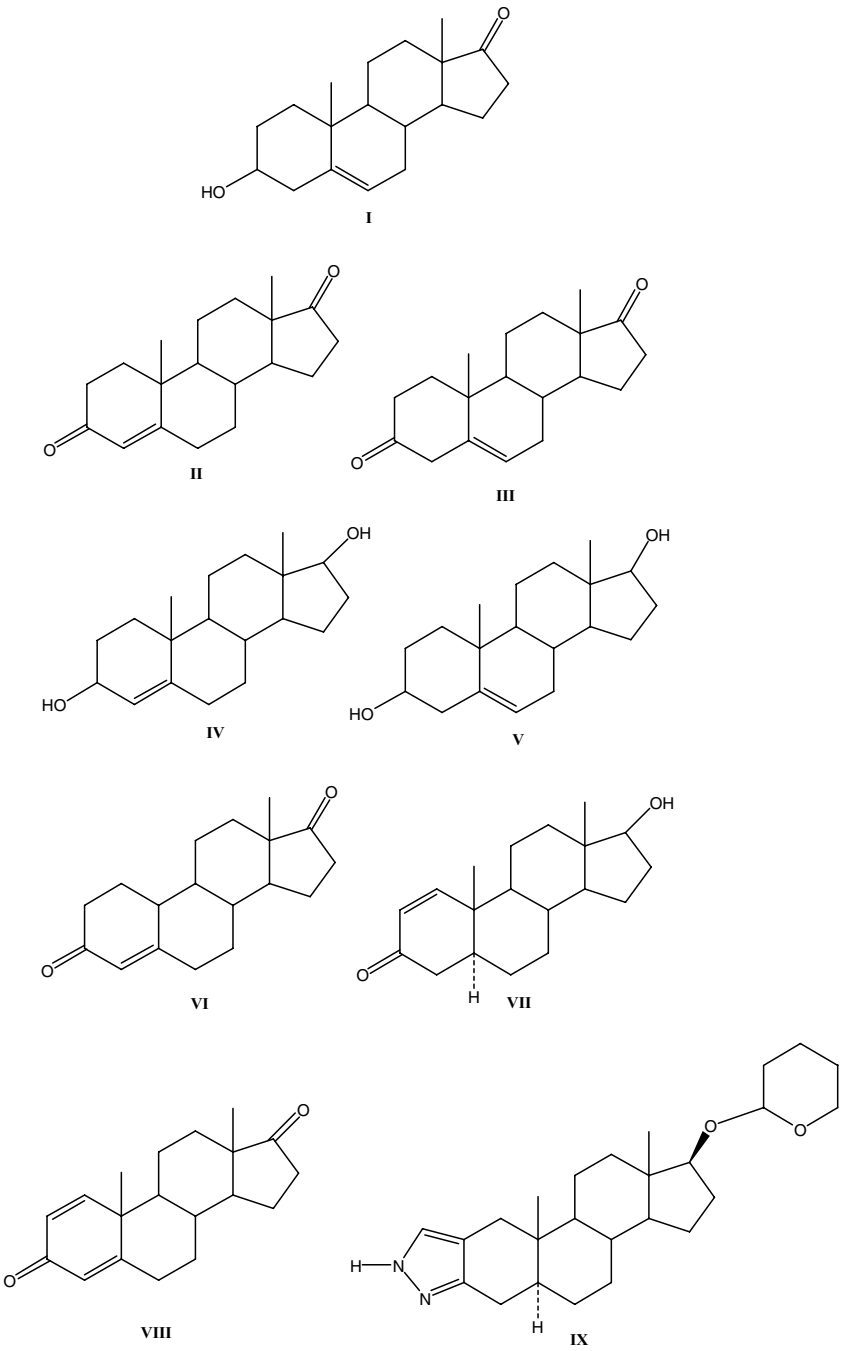
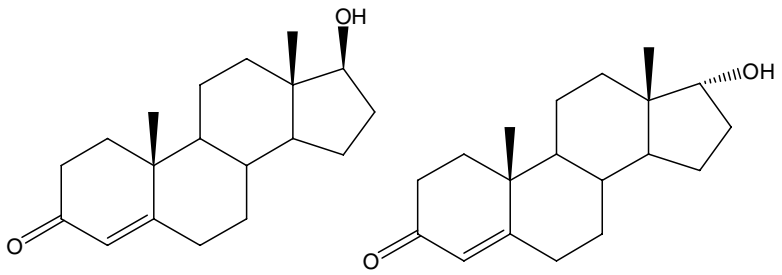
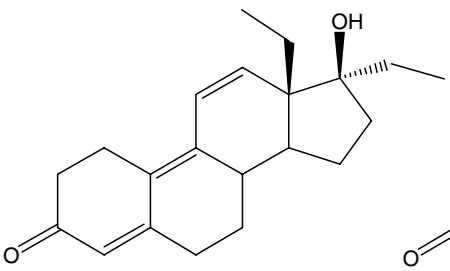


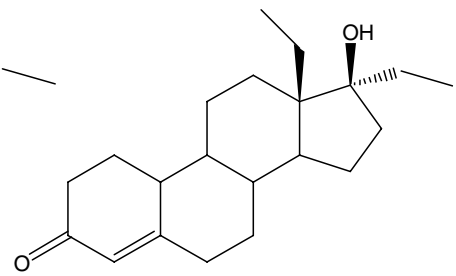
Fig. 9.2 The 'supplements' (I) DHEA, (II) and (III) androstenedione with (delta 4 and 5 versions, respectively), (IV) and (V) androstenediol (delta 4 and 5 versions, respectively), (VI) 19-norandrostenedione (only delta 4 version displayed), (VII) androsten-1-one-17b-ol-3-one (the D^1-analog of testosterone), (VIII) boldione and (IX) prostanozolol



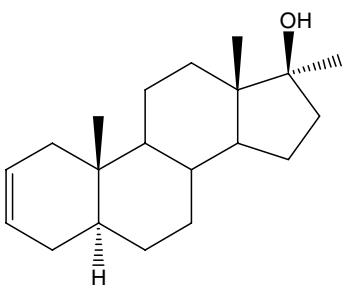
Testosterone with Epitestosterone



Tetrahydrogestrinone



Norbolethone



Madol

Fig. 9.3 Designer drugs. Testosterone administered with epitestosterone to normalise the T/E ratio; Tetrahydrogestrinone, Norbolethone, Madol

(adrenoreceptor stimulant) and growth hormones. There is no restriction on the possession of these substances when they are part of a medicinal product and are for self-administration. However, prosecutions of intent to supply have been made of individuals found in possession of large quantities of these substances without a prescription for them. A Home Office license is required for importation and expor-

tation of anabolic steroids, except in cases of small quantities for legitimate purposes. Other countries have similar types of control.

In human sports, the International Olympic Committee (IOC) Medical Commission introduced anabolic steroids as a banned class in April 1974 following the development of a screen for the 17α-alkylated orally active drugs; for history of introduction of anabolic steroid testing (Kicman and Gower, 2003); Van Eenoo and Delbeke, 2006). The name of this banned class was amended to anabolic agents in the 1990s to incorporate out of competition testing for clenbuterol and other β_2-agonists, which are also considered to have weak anabolic activity. In 1999, the World Anti-doping Agency (WADA) was set up as a foundation under the initiative of the IOC with the support and participation of intergovernmental organizations, governments, public authorities, and other public and private bodies fighting against doping in human sport. Under WADA, the rules and technical documents concerning anabolic steroids (and other drugs) are constantly evolving, and for up to date information, the reader is strongly advised to access the WADA web site (www.wada-ama.org/en/).

In equine sports, a drug control program for anabolic steroids was introduced into the UK in 1976. Initial efforts to control anabolic steroids concentrated on the control of the sustained-release veterinary preparations of nandrolone, T, boldenone, and trenbolone. These studies have been extended to the control of stanozolol and other 17α-alkyl orally-active steroids. In countries where effective drug control programs have not been implemented there appears to be fairly widespread misuse of these agents.

9.1.3 Misuse of Anabolic Steroids in Sport and Society

The abuse of drugs, especially hormones, by both professional and amateur sportsmen and women for enhancement of their performance is of concern. The extensive media coverage that sometimes arises following the failure of a drug test by an athlete tends to fuel speculation that the abuse of performance-enhancing drugs in sport is widespread, despite relatively low incidence of positive dope tests. Although the extent of doping is debatable, few would dispute that the urge to succeed, and the rewards of success, both financial and otherwise, have provided powerful incentives to competitors to look for every possible means of improving their performance, despite the risks of denunciation and penalties. Less widely publicized is the use of such drugs amongst both adults and adolescents in society, in particular the use of anabolic steroids for cosmetic benefits. Such use of anabolic steroids may not be so surprising in an age where dietary supplementation is a huge industry, which promises a healthy image, youthful looks, and longevity. Anabolic steroid administration may be regarded (wrongly) as a relatively harmless pharmacological manipulation that can aid the development of bulging muscles and 'six-pack' stomachs that appear to be so eagerly displayed by some popular icons on television and within magazines aimed at young people. Surveys estimate that up to 5% of

gym users in the UK are users of anabolic steroids, the proportion being much higher (25–50%) amongst competitive bodybuilders, the majority being men. Although anabolic steroids are prescription-only medicines, they are relatively easy to obtain via the 'underground market' or the Internet. In drug control in sport, of the 223,898 samples analyzed in 2007 by 33 WADA[1] accredited laboratories, 4,402 (2%) failed the drug test (A-sample), of which 2269 (646 exogenous and 1,623 'endogenous') were positive for anabolic steroids.

The Olympic Games held in Athens in 2004, of the 23 specimens confirmed for the presence of prohibited substances, 16 were related to anabolic steroids (Tsivou et al., 2006). Comparison of the adverse findings for wordwide testing in 2004 with those of 10 years earlier shows that there has been little change in the ranking at the top of the table (Table 9.1), and this trend continues in 2007 with testosterone being the most common finding followed by nandrolone, stanozolol, and methandienone. It is important to note that the named compounds listed under adverse findings reflect interpretation by WADA of the steroid that had been probably administered rather than what the laboratory declares, which is often a diagnostic metabolite present in the urine sample, for example, 19-norandrosterone is the chosen diagnostic metabolite of nandrolone (19- norT). T, nandrolone, and stanozolol are all licensed for medicinal use and, therefore, perhaps it is not entirely unexpected that these compounds find their way on to the underground market and commonly are the causes of adverse findings. The current availability of some of the other anabolic steroids may appear to be limited because many of them being withdrawn as licensed products in numerous countries, including the UK and USA. Examples of such steroids are methandienone and clostebol, but these continue to be produced and supplied as legitimate pharmaceutical preparations in many other countries.

9.1.4 Non-pharmaceutical Anabolic Steroids

A current cause for concern is the recent manufacture of analogs of established anabolic steroids. Under the WADA rules, these steroids are considered to belong within the class of anabolic agents as they are related in structure to anabolic steroids. For an in-depth description of the structure, metabolism, and detection of doping with these steroids, the reader is referred to the review by Van Eenoo and Delbeke (2006).

It appears that many of the novel anabolic steroids are supplied not so much to circumvent the sports drug tests as to avoid statutory controls of countries regarding the manufacture and supply of drugs, that is, they are often widely marketed as nutritional/dietary supplements to tap the bodybuilding market. Examples

[1] WADA was formed in November 1999, under the initiative of the International Olympic Committee to bring together all parties involved in the fight against doping in human sport. Prior to that, laboratories were accredited by the International Olympic Committee. The 2006 statistics were the latest available at the time of completion of this chapter.

Table 9.1 Comparison of the anabolic steroids identified in the class of anabolic agents in 2004 and 1994

2004		1994	
'A-samples' analyzed = 169,187 Total adverse findings = 3,305 (1.7%) Anabolic steroid adverse findings = 1,191 (0.7%)		'A-samples' analyzed = 93,680 Total adverse findings = 1,278 (1.4%) Anabolic steroid adverse findings = 793 (0.8%)	
Ranking	Anabolic steroid findings (%)	Ranking	Anabolic steroid findings (%)
Testosterone	32.9	Testosterone	35.3
Nandrolone	28.5	Nandrolone	26.1
Stanozolol	19.0	Methandienone	12.1
Methandienone	5.3	Stanozolol	11.7
Methyltestosterone	2.7	Methenolone	8.6
Methenolone	1.8	Dihydrotestosterone	4.4
DHEA	1.7	Boldenone	3.8
Boldenone	1.6	Methyltestosterone	2.5
Mesterolone	1.5	Drostanolone	1.3
Androsterone[a]	1.4	Mesterelone	1.1
Clostebol	0.8	Oxandrolone	0.9
Delta-1-androst-3,17-dione	0.8	Dehydrochloromethyl testosterone	0.6
Drostanolone	0.4	Fluoxymesterone	0.4
Delta-1-testosterone	0.3	Oxymethelone	0.4
Oxymethelone	0.3	Clostebol	0.4
Androstenedione	0.3	Norethandrolone	0.1
Boldione	0.3	Formebolone	0.1
Oxandrolone	0.3		
Methandriol	0.1		
Trenbolone	0.1		

These findings are often based on the detection of a diagnostic metabolite. The statistics include all analyses conducted by accredited laboratories for in- and out-of-competition testing in the years 2004 and 1994.

[a]Androsterone is not an anabolic steroid but an adverse finding can be based on abnormal carbon isotope ratio.

of 'dietary supplements' are dehydroepiandrosterone (DHEA), androstenedione, androstenediol, and their 19-nor equivalents (these steroids are prohormones), and analogs of T and stanozolol called 1-testosterone and prostanozolol, respectively (Fig. 9.2). It is the consequence of their widespread availability that a minority of athletes will also use these steroids in an attempt to improve sporting performance, and therefore, antidoping laboratories have to incorporate such compounds into their drug screens. Only time will tell whether these compounds become a major problem for sport.

The origin of steroids as dietary supplements began in the USA. Prior to 1994, within the USA, dietary supplements were regulated in the same manner as foods, and therefore, their manufacturing, quality, and labeling were monitored by the Food and Drug Administration. However, many considered that such regulation

was too restrictive and as a result, Congress passed the Dietary Supplements Health and Education Act in 1994, which placed dietary supplements in a special category of "foods". US law defined the 'dietary supplement' as a product taken by mouth that contained 'dietary ingredient' intended to supplement the diet. Dietary ingredients may include vitamins, minerals, herbs, or other botanicals, amino acids, and other substances, for example, enzymes and steroids. In the same year that the Dietary Supplements Health and Education Act was passed, Morales et al. (1994) published the effects of replacement therapy of dehydroepiandrosterone (DHEA) in men and women of advancing age, and the media likened DHEA to the 'fountain of youth'. Before that, DHEA was not considered as a steroid of abuse probably due to its limited availability, even though the physician, Di Pasquale, mentioned as early as 1990 that DHEA 'is used by some athletes as an anabolic agent' (Di Pasquale, 1990). With such media attention, DHEA became widely available in the USA as a dietary supplement and it was reputedly used by some athletes before and during the Olympic Games in Atlanta in 1996. Although DHEA would have been considered a prohibited substance under the Anti-Doping Code, in December 1996, the IOC Medical Commission addressed concern as to whether it came under the banned class of 'Anabolic Agents' by explicitly adding it as a named example. Around that time, other 'dietary supplements' became increasingly available, these are being androstenedione, androstenediol and their corresponding 19-nor analogs, as the '4-ene' or '5-ene' geometric isomers (or sometimes containing both isomers). Advertisements claimed a number of benefits from administration of these products, but notably that improvements could be gained in muscle mass because these steroids are prohormones that can be converted within the body to T and nandrolone, respectively. The same approach is used to market boldione as the prohormone of anabolic steroid boldenone. Such products were not considered as falling under the legislation for pharmaceuticals, even though Phase I metabolism results in conversion of the prohormone into a biologically active androgen.

Established legislation in some countries, for example, Australia, UK, restricts the supply of steroid prohormones, and in the USA, recent legislation has recently restricted the sale of androstenedione (FDA White Paper March 11, 2004). This is a promising start in an attempt to reduce the availability of such compounds. Even so, currently there are some newer 'dietary supplements' that are now available and can be ordered via the World Wide Web.

In the field of drug control in sport, designer drugs are considered (by the authors) as ones that are manufactured specifically to circumvent the doping tests, that is, they are supplied in clandestine fashion and are not compounds that are advertized for the bodybuilding market. With respect to anabolic steroids, there are few examples to draw on. Classified documents (Franke and Berendonk, 1997) saved after the collapse of the German Democratic Republic revealed that since 1983 a pharmaceutical company had produced preparations of epitestosterone propionate exclusively for the governmental doping program. Epitestosterone is a steroid with no anabolic activity, but its administration with T simultaneously or sequentially enables an athlete to manipulate the test for T administration if the test is based solely on determination of the T/E ratio (see Section 9.10.2). Much more recently, the 'BALCO

Affair' attracted much media attention due to high profile of athletes involved, not least because of a transdermal preparation (The Cream) was supplied that contained T and epitestosterone, as well as a sublingual preparation of a new anabolic steroid tetrahydrogestrinone (THG) (Catlin et al., 2004), coded as 'The Clear' (information on the structure-activity and metabolism of tetrahydrogestrinone are in Sections 9.3 and 9.5.2.5, respectively). Underground chemists recently appeared also to be accessing information concerning other steroids that were synthesized several decades ago by pharmaceutical companies but were never marketed. Such steroids that have been detected to date are norbolethone (Catlin et al., 2002) and madol (Sekera et al., 2005) (madol is also refered to as desoxymethyltestosterone by the WADA accredited laboratory in Montreal, who detected the administration of this steroid around the same time as the laboratory at UCLA).

9.1.5 Anabolic Steroids in Food

In slaughter animals, the misuse of anabolic steroids for promoting growth and feeding efficiency can have potentially serious implications in human sport. In contrast to the USA, where androgen administration for such purposes is legally restricted to esters of trenbolone and T (the site of injection in the ear is discarded after slaughter to prevent accidental ingestion), within the European Economic Community such administration is totally prohibited (EC directive 88/146). Total prohibition is more liable to result in contaminated meat because the site of injection is likely to be in areas of an animal where the depot is difficult to detect, such as tail base or neck muscle that is turned into low-grade minced tissues. Veterinary residue analysis programs for hormonal growth promoters may combat this illicit activity to some extent. Subsequent ingestion of a depot in meat can result in unequivocal identification of banned substances in urine (Debruyckere et al., 1992, 1993; Kicman et al., 1994).

Currently, there is no accepted analytical method capable of distinguishing between a sample from an abuser and one from an athlete who has eaten contaminated meat. The probability of an athlete testing positive from eating contaminated meat is not known, but it is likely to be extremely low. Even so, it has been recommended that athletes avoid eating poor quality processed meats. Likewise, that they should shun offal from the boar and horse as these species produce significant amounts of nandrolone endogenously (Le Bizec et al., 2000).

9.2 Adverse Effects on Health

In human sports, there are many reviews that deal with the health hazards induced by anabolic steroid administration (e.g. Kicman and Gower, 2003; James et al., 2004). The adverse effects may be manifested with pharmacologically recommended doses but can be of much greater consequence for those who administer excessive amounts over long periods of time.

Sustained disturbances of the hypothalamic–pituitary–gonadal axis can cause prolonged infertility, testicular atrophy, and secondary amenorrhea. In women, some of the effects of virilization may be irreversible, for example, deepening of the voice and clitoral hypertrophy. Several studies have concluded that users have altered serum lipoprotein profiles, as found in groups with a larger risk of developing coronary artery disease. Liver dysfunction and hepatotoxicity are associated with the use of the 17α-alkylated steroids and are considered as a contributing factor to the rare formation of hepatocellular carcinoma in competitors. Although some users desire the androgenic effects of anabolic steroids for increased aggression in enhancing training and competitiveness, in others, major mood disturbances can arise including psychosis and violent activity. This is possibly the most dangerous feature of anabolic steroid abuse.

Apart from trestolone, many of the newer steroids available are not manufactured by the pharmaceutical industry (which adheres to stringent regulatory controls). This fact, and because their administration is not subjected to medical supervision, may increase the risk of damage to health. It is logical to assume that the hazards would be similar to those already documented regarding chronic administration of anabolic steroids. Even so, little is known about the pharmacological activity of most of these newer steroids and hence the extent of the risk is largely unknown. Their lack of regulatory control increases the risk that a steroid analog with acute toxicity may be introduced onto the market.

Of the side-effects observed in the human (adverse effects on liver, cardiovascular and reproductive systems and premature epiphyseal cell closure), it is the effect on the reproductive system in the horse that has received the greatest attention due to the potential value of these animals at stud. Long-term administration of anabolic steroids leads to various side-effects. When geldings were treated with nandrolone phenylpropionate for several weeks they developed behavioral changes, including increased aggressiveness. Fillies and mares, treated with anabolic steroids for 1 year, developed virilization and exhibited a change to a male behavior pattern.

9.3 Structural Modifications

The clinical advantages of pure anabolic agent were recognized many years ago and work was undertaken by a number of groups and drug companies to modify the T molecule with a view to maximizing the anabolic effect and minimizing the androgenic activity. Some of the structural modifications that have been introduced into T are shown in Fig. 9.4.

Oral activity can be conferred by substitution of the 17α-H on the steroid nucleus with a methyl or ethyl group to make the 17α-alkylated steroids. This substitution prevents deactivation of the steroid by first-pass metabolism, the alkyl function hindering oxidation of the 17β-hydroxyl group. Oral activity can also be conferred by attachment of a methyl group at C-1, as in methenolone or mesterolone, but these two anabolic steroids are relatively weak in activity. Parenteral preparations do not require a 17α-alkyl group but the 17β-hydroxyl group is esterified with an acid

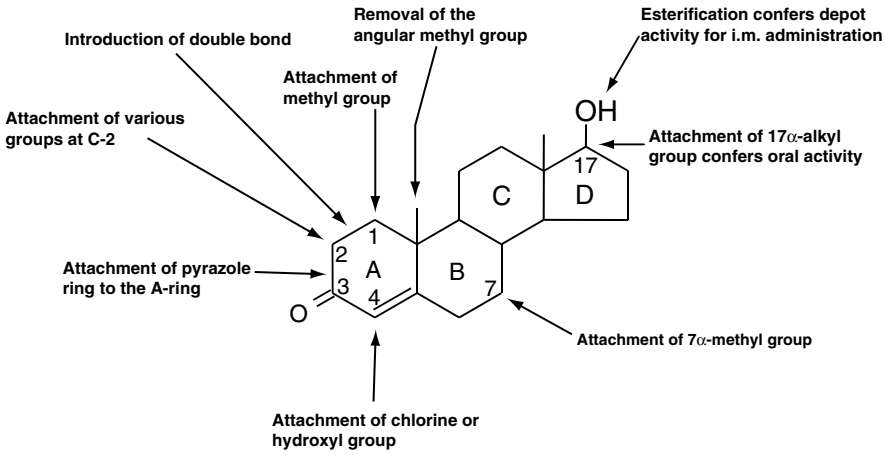


Fig. 9.4 Structural modifications to the A- and B-rings of testosterone which increase anabolic activity; substitution at C-17 confers oral or depot activity (i.m. = intramuscular)

moiety (Van der Vies, 1993) to prevent rapid absorption from the oily vehicle, usually arachis oil plus a small amount of benzyl alcohol.

For the so-called dietary supplements and designer steroids, the evaluation by scientific experimentation of the structural-activity relationships, pharmacological and adverse effects would be very time consuming. Fortunately, such data is not a necessary prerequisite before their use can be banned in sport. Under WADA rules, these steroids are considered to belong within the class of anabolic agents as they are related in structure to anabolic steroids. Of these, perhaps the most interesting is the designer steroid, tetrahydrogestrinone (THG), which was identified from the contents of a spent syringe by the accredited laboratory in California in 2003 (Catlin et al., 2004). This drug was supplied by an American nutritional supplement company, the Bay Area Laboratory Co-operative (BALCO), under a clandestine product name called 'The Clear'. Its manufacture can be easily achieved by the catalytic hydrogenation of the ethynyl group of gestrinone (Fig. 9.5), the latter being a legitimate progestogen used for the treatment of endometriosis. This relatively simple synthetic step hides the cunning thinking that probably lay behind the design of THG.

Pioneering work in the development of synthetic progestogens in the 1950s, which resulted in the birth of the oral contraceptive 'pill', sheds light on part of the rationale of hydrogenating gestrinone. The progestational activity associated with progesterone was found to be greatly increased by removal of the angular C-19 methyl group to make 19-norprogesterone. Likewise, substitution of the 17α-H with an ethynyl group on T produced a substance with more progestational than androgenic activity (Djerassi, 1992, 2006), and being 17α-alkylated conferred high oral bioavailability. Logic dictates therefore that substitution of the 17α-H with an ethynyl group on nandrolone, a 19-nor anabolic steroid with some progestational activity, will result in a potent orally active progestogen, this being called norethisterone

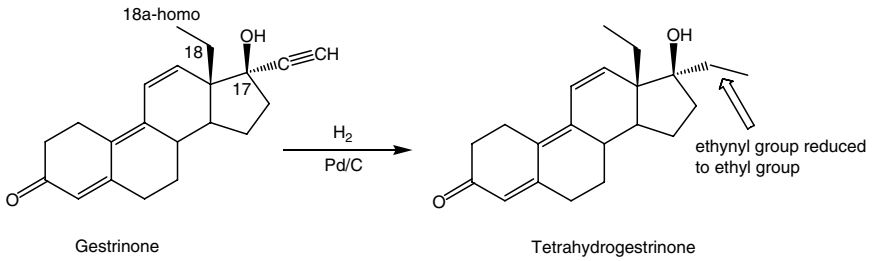


Fig. 9.5 Catalytic hydrogenation of gestrinone to form tetrahydrogestrinone (THG). An example of a catalyst is palladium on carbon (Pd/C), as described in a procedure employed by Catlin et al. (2004)

(norethindrone), a steroid that is still used in some contraceptives today (for the interested reader, the synthetic route is described by Djerassi et al. in a seminal paper in 1954). However, substitution with an ethyl group on nandrolone rather than ethynyl group results in another anabolic steroid known as norethandrolone, which also has oral activity. It is perhaps not surprising that there is an overlap between progestational and androgenic activity of these 19-norsteroids, especially given the close homology of androgen and progestogen receptors, but which activity predominates depends on whether the alkyl substituent is ethynyl or ethyl. Gestrinone, is another compound that lacks the C-19 angular methyl group but has a 17α-ethynyl group, and in light of the above, it follows that reduction of the ethynyl group to the tetrahydro product should make tetrahydrogestrinone a 'potent' androgen. This is indeed the case, as subsequently THG was found to be a highly potent androgen (and progestogen) in an *in vitro* bioassay system expressing human steroid receptors (Death et al., 2004) and it promotes muscle accretion in orchidectomized male rats (Jasuja et al., 2005). Of interest is that despite the presence of the 17α-alkyl function, that should make the steroid resistant to first pass metabolism, instructions from BALCO laboratories were to place a few drops of the liquid preparation under the tongue, that is, a sublingual route of administration. Finally, of particular note, is that THG is invisible in the GC-MS screen that employs pert-rimethylsilylation and selected ion monitoring for anabolic steroids, and therefore screening for this steroid is performed using LC-MS/MS (see Section 9.8.2).

9.4 Routes of Administration

The major routes of administration of anabolic steroids are oral, parenteral, and transdermal. Data concerning the pharmacokinetic parameters of many xenobiotic anabolic steroids is not described in the published literature, thus making it difficult to predict what length of time will pass before an individual may test negative following cessation of administration. Interpretation is also confounded by the variability in the

size and frequency of the doses used for doping. As a general guide, because oral and transdermal formulations are short acting, detection of doping is particularly challenging usually within a week of cessation of their administration, whereas many parenteral preparations with sustained release can be detected for much longer.

Orally active formulations, apart from T undecanoate, include steroids with a 17α-alkylated group, a 1-methyl substituent (methenolone) or 1α-methyl substituent (mesterolone). With the exception of stanozolol and oxymetholone, the 17α-alkylated steroids have been withdrawn as pharmaceutically licensed products for human therapy in the UK, but nevertheless they are abundantly available as tablets worldwide.

Parenteral preparations for human and veterinary use on the UK market are mainly esters of T and nandrolone. In addition, esters of boldenone and trenbolone (Fig. 9.1) have been licensed for veterinary purposes only; notwithstanding, athletes have also been known to administer veterinary anabolic steroids. For parenteral administration, esters include cyclohexylpropionate, decanoate, laurate, and phenylpropionate for nandrolone; acetate, cypionate, decanoate, enanthate, isocaproate, phenylpropionate, propionate and undecanoate for T, undecylenate for boldenone and acetate for trenbolone. The ester formulations in vegetable oils are sustained-release preparations. The mechanism of action of the nandrolone esters and other anabolic steroids and the effect of drug delivery systems on their biological activity have been studied by Van der Vies (e.g. Van der Vies, 1993). The duration of action of the esters depends upon the rate of absorption from the site of administration. This is dependent on the chain length of the acid moiety and also the formulation. In fact, it has been shown to be related to the partition coefficient of the derivatives between the oil used in the formulation and plasma. In general, the longer the chain length of the acid moiety, the more slowly the preparation is released into circulation, thus prolonging the duration of action, but also increasing the window of detection with respect to doping, for example adverse findings for nandrolone metabolites can occur many months following a single administration of the decanoate ester. Following release from the intramuscular (i.m.) depot, the esters are hydrolyzed either in plasma or in specific tissues to yield the parent steroid, the active anabolic agent.

Non-pharmaceutical water-based T suspensions for injection are advertized on bodybuilding web sites and cheats in sport may find these attractive as, in theory, these should be relatively short acting. Nonpharmaceutical-based preparations, whether oil or water based, may be a particular hazard to health as the contents may not have been prepared under sterile conditions.

Transdermal formulations are invariably T based, legitimately designed for replacement therapy, and include the patch and hydroalcoholic gels, to be applied on a daily basis. Other short-acting T preparations include those designed to be administered by the sublingual or buccal route. Such short-acting formulations are of particular concern in sport, as the exogenous source of T is rapidly eliminated following cessation of treatment. Increased out-of-competition testing helps to combat the cheat who is using short-acting preparations and ceasing administration prior to competition in anticipation of testing.

9.5 Metabolism of Anabolic Steroids

9.5.1 General Aspects

Since many, but by no means all, the anabolic steroids are extensively metabolized, with little or no unchanged steroid being excreted in the urine, it is important that the metabolic pathways should be elucidated for drug-monitoring purposes. In a very comprehensive paper, Schänzer and Donike (1993) describe the metabolism of anabolic steroids in man, chemical synthesis of major metabolites, and their GC retention times (determined on methylsilicone OV-1 and methyl 5% phenyl silicone SE-54 columns) with the electron impact mass spectra of trimethylsilyl (TMS) ether derivatives. Some veterinary steroids, mibolerone (17β-hydroxy-7α, 17-dimethylestr-4-en-3-one) and trenbolone are also abused by some sports competitors, and urinary metabolites of these in man have been characterized (Bowers, 1996; Uralets et al., 1996). In the horse, numerous detailed studies have been performed by Houghton and his colleagues and other workers since the late 1970s to understand how the parent steroids of the proprietary preparations are metabolized.

An overview of the Phase I and Phase II metabolic pathways of anabolic steroids in the human and equine has been published earlier (Gower et al., 1995; Schanzer, 1996; Kicman and Gower, 2003). For many of these steroids, there can be several diagnostic metabolites, Fig. 9.1 showing examples of one principal diagnostic metabolite for each. In the analytical context, it should be noted that not all anabolic steroids give rise to unique metabolites, that is, common glucuronidated metabolites can be produced due to shared pathways of metabolism. 17α-methyl-5α-androstane-3α,17β-diol is a major metabolite of mestanolone, methylT and oxymetholone, probably because the latter two steroids are converted in the body to the mestanolone intermediate. 17α-methyl-5β-androstane-3α,17β-diol is a major metabolite of 17α-methylT, methandienone and methandriol, the latter two steroids being endogenously converted to the 17α-methylT intermediate.

9.5.2 Phase I Reactions

The pathways elucidated involve the oxidation, reduction, hydroxylation, and epimerization and metabolic pathways of T, nandrolone, boldenone, trenbolone, 17β-hydroxyl-17α-methyl steroids (e.g. 17α-methyl-T, methandienone and stanozolol), are described below in more detail to illustrate the extent of metabolism that can occur.

The properties and importance of cytochrome P450 enzymes (CYPs) in C_{19} Phase I reactions have been described in Chapter 6, and, in general, the enzymatic processes also apply to anabolic steroids. The CYP enzymes, of which there are more than 500 have been classified into a super-family, 74 families being present in humans. During the past 15 years, numerous research groups have investigated

CYPs as a means of catabolism of anabolic steroids. As noted earlier (Chapter 6) in the context of metabolic processes, 6β-hydroxylation of T is well known. Rendic et al. (1999) have extended studies of this hydroxylation to a number of important anabolic steroids, all containing the 3-oxo-4-ene grouping, including 17α-methylT, methandienione, boldenone and 4-chloro-1-dehydro-17α-methylT. In their *in-vitro* studies, the authors used three CYP enzymes because of their known activity in androgen metabolism. Two formats of human recombinant CYP3A4 and CYP2C9 and one format of human 2B6 were used, and GC-MS analysis was employed for characterization of the metabolites formed. Both the CYP3A4 enzyme formats catalyzed 6β-hydroxylation of the steroids selected. However, in contrast, neither format of CYP2C9 catalyzed 6β-hydroxylation and CYP2B6 catalyzed 6β-hydroxylation of only traces of T and 17α-MeT. The authors suggest that it is the electronic effect of the 3-oxo-4-ene grouping that contributes to selective binding the steroid within the active site of CYP3A4 and, hence, selectivity of 6β-hydroxylation.

9.5.2.1 Testosterone and Nandrolone

Following the administration of radiolabeled testosterone, nandrolone, boldenone, and trenbolone (parent drugs not esters) to the equine (Houghton, 1977; Houghton and Dumasia, 1979, 1980; Dumasia and Houghton, 1981, 1984, 1988; Dumasia et al., 1983), the rates of urinary excretion of radioactivity vary markedly (Table 9.2). Despite this difference, following the administration of [4-^{14}C]-nandrolone and [4-^{14}C]T, the metabolic pathways of these two steroids were very similar. Major metabolites arise by reduction in the A-ring and at C-3, oxidation at C-17 and subsequent reduction of the 17-oxo group with epimerization to yield a series of estrane-3,17-diols and androstane-3,17-diols from nandrolone and T, respectively. Reduction of the A-ring produces both 5α- and 5β-isomers, whereas reduction at C-3 yields predominantly the 3β-hydroxy isomers (Fig. 9.6). Additional oxidation at C-16 then produces a series of $C_{18}O_3$ and $C_{19}O_3$ metabolites from nandrolone and T, respectively (see Gower et al., 1995 for references).

In contrast with the horse, in the human 4-ene-3-oxo reduction yields predominantly 3α-hydoxylated steroids, and there is no further metabolism following oxidation at C-17 to produce 17α-hydroxy epimers. The major metabolites of

Table 9.2 Administration of radiolabelled steroids to horses: urinary excretion of radioactivity

Steroid	Radioactivity excreted in urine (%)			
	0–24h	Total (time, h) $	0–24h urine	
			% in glucuronide fraction	% in sulfate fraction
Nandrolone	60–65	70–90 (72–90)	50–60	23–29
Boldenone	32–36	50–55 (90–110)	60–62	25–30
Testosterone	23–26	48–54 (200)	18–20	62–65
Trenbolone	48–55	55–74 (72–96)	60–65	22–25

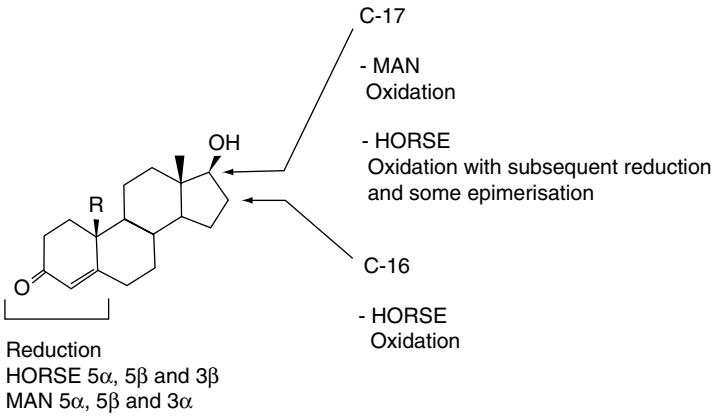


Fig. 9.6 Metabolism of testosterone (R=CH_3) and nandrolone (R=H)

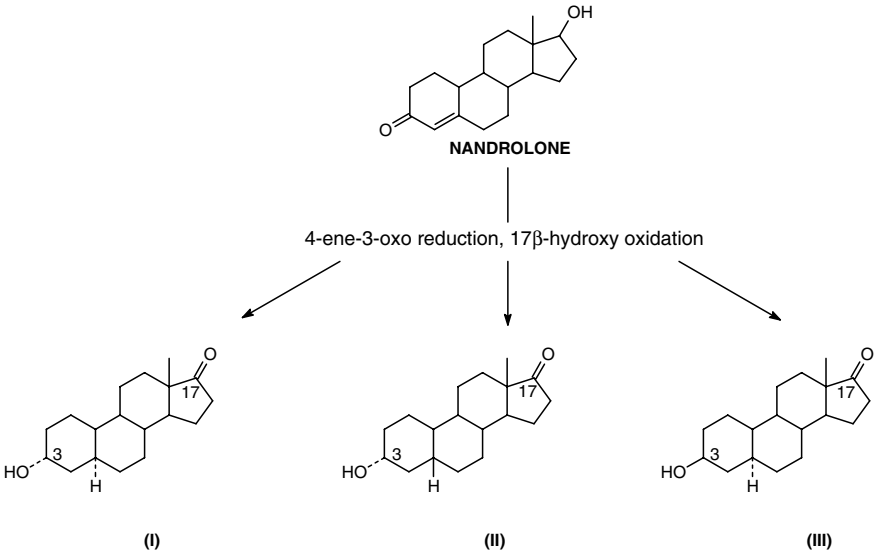


Fig. 9.7 Structures of the metabolites of nandrolone: (I) 19-norandrosterone (3α-hydroxy-5α-estran-17-one), (II) 3α-hydroxy-5β-estran-17-one and (III) 19-norepiandrosterone (3β-hydroxy-5α estran-17-one)

nandrolone and T are consequently the tetrahydro-17-oxo steroid metabolites, these being 19-norandrosterone (3α-hydoxy-5α-estran-17-one) and 19-noretiocholanolone (3α-hydoxy-5β-estran-17-one), and androsterone (3α-hydoxy-5α-androstan-17-one) and etiocholanolone 3α-hydoxy-5β-androstan-17-one, respectively. Following i.m. injection of an aqueous suspension of nandrolone, only about 0.4% of nandrolone is excreted into urine conjugated with glucuronic acid (a Phase II reaction; see below), but about 30% is excreted as the tetrahydro-17-oxo steroid metabolites (Masse et al., 1985); the structures are displayed in Fig. 9.7. After

glucuronide hydrolysis, the abundance of 19-norandrosterone is usually found to be greater than that of 19-noretiocholanolone, and hence it is this metabolite that is targeted for confirmatory analysis. Only a relatively small amount of the aglycone of 19-norepiandrosterone is present, not least because whatever is excreted is likely to be predominantly as a sulpho-conjugate, consistent with excretion of other 3β-hydroxylated steroids (such as epiandrosterone). The presence of the 3β,5β isomer, 19-norepietiocholanolone (3β-hydroxy-5β-estran-17-one) has not been observed, unlike the metabolism of T where the 3β,5β isomer, epietiocholanolone, is a minor metabolite.

9.5.2.2 Boldenone

The rate of urinary excretion of boldenone (1-dehydroT) in the horse (E. Houghton, unpublished data) is intermediate between those of nandrolone and T, following theraputic doses of these steroids as esters (Table 9.2). The 1,4-diene-3-one structure of the A-ring stabilizes the steroid to reductive metabolism and the major Phase I metabolic pathway results in formation of the 17α-epimer. However, partial and complete reduction of the 1,4-diene-3-one functionality coupled with metabolism at C-17 produces a complex series of minor metabolites (Dumasia and Houghton, 1988). In addition to hydroxylation at C-16, hydroxylation at C-6 also occurs, and three isomers of a 6,16-dihydroxy metabolite have been identified in equine urine from the nonconjugated fraction.

Metabolism of boldenone in humans (Schanzer and Donike, 1992, 1993; Schanzer, 1996; and references therein) occurs by partial or complete reduction of the 4-ene-3-oxo group, oxidation at C-17 and some hydroxylation at C-6, the 1-ene group being stable to reductive metabolism. This, and the partial reduction of the 4-ene-3-oxo group, produces a major metabolite, 17β-hydroxy-5β-androst-1-en-3-one. Two other major metabolites characterized in human urine are 3α-hydroxy-5β-androst-1-en-17-one and 5β-androst-1-ene-3α,17-diol (Fig. 9.8).

9.5.2.3 Trenbolone

After administration to horses of trenbolone as the acetate (E. Houghton, unpublished data), urinary excretion was complete within 72–96 h (Table 9.3). As in the case of boldenone, the extension of conjugation of the 4-ene-3-oxo group through introduction of unsaturation at the 9- and 11-positions stabilizes the A-ring to reductive metabolism. The major metabolic pathway both in the horse (E. Houghton et al., unpublished results) and in humans (de Boer et al., 1991) is epimerization at C-17. In the horse hydroxylation at C-16 also occurs, producing three 16-hydroxy isomers (E. Houghton et al., unpublished observations). A similar metabolic route has been observed earlier in the bovine.

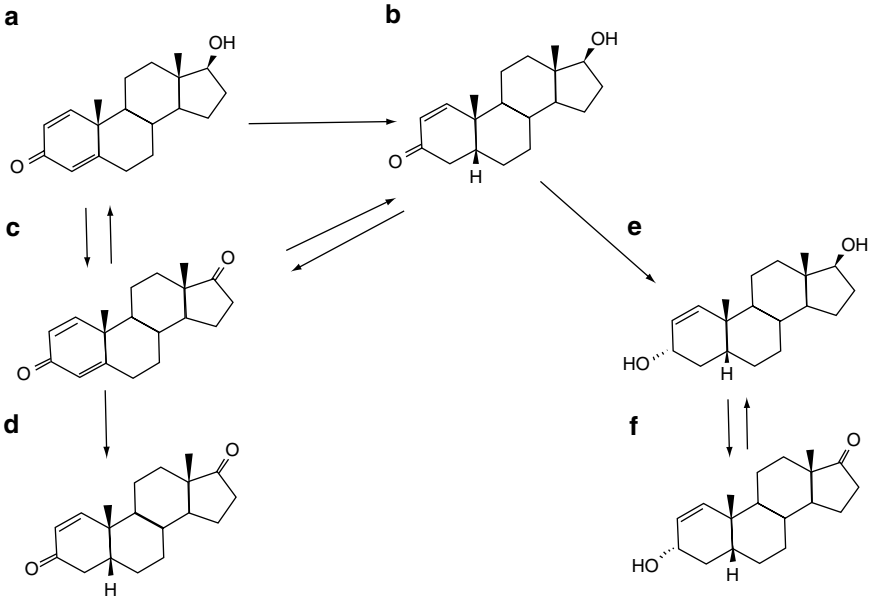


Fig. 9.8 Metabolism of boldenone (**a**) in man; (**b**) 17β-hydroxy-5β -androst-1-en-3-one; (**c**) androsta-1,4-diene-3,17-dione; (**d**) 5β-androst-1-ene-3,17-dione; (**e**) 3β -androst-1-ene-3α,17β-diol; (**f**) 3α-hydroxy-5β-androst-1-en,-17-one (from Schanzer and Donike, 1993, with permission of authors and Elsevier Science B.V)

Table 9.3 *In vivo* metabolism of some orally active anabolic steroids in the horse

Steroid	Major metabolites/metabolic routes	References
Ethylestrenol[a]	Norethandrolone	Gourdie et al. (1995)
	19-Nor-17α-pregnane 3,17β-diol	Kim et al. (1996)
17α-Methyl testosterone	Reduction of the 4-en-3-one group	Stanley et al. (1997)
	Hydroxylation at C-6/C-16/C-15	
	Epimerisation at C-17	
Fluoxymesterone[b]	Hydroxylation at C-16	Stanley et al. (1997a)
Oxymetholone[c]	Mestanolone	Tang et al. (2000)
	5α-Androstane-17α-methyl-3β,17β-diol	
	5α-Androstane-17α-methyl-3α,17β-diol	
Mestanolone[d]	5α-Androstane-17α-methyl-3β,17β-diol	Tang et al. (2000)
	5α-Androstane-17α-methyl-3α,17β-diol	
Danazol[e]	2-(Hydroxymethyl)ethisterone	Tang et al. (2001)
	6-Hydroxyethisterone	
	Ethisterone	
Normethandrolone[f]	Reduction of the 4-en-3-one group	Fox et al. (2001)
	Epimerisation at C-17	
	Hydroxylation at C-6/C-16	

(continued)

Table 9.3 (continued)

Steroid	Major metabolites/metabolic routes	References
Methandienone[g]	Reduction of the 4-ene	McKinney et al. (2001a)
	Hydroxylation at C6/C16	
	Reduction of the 1-en-3-one group (slow)	
Norethandrolone[h]	19-Nor-17α-pregnane-3,17β-diols	McKinney et al. (2001b)
	19-Nor-17α-pregnane-3,16,17β-triols	
	19-Nor-17α-pregnane-3,17β,20-triols	
	19-Nor-17α-pregn-4-en-3-one-17β,20-diols	
	19-Nor-17α-pregnane-3,17β-diol-21-oic acids	
	19-Nor-17α-pregn-4-en-3-one-17β-ol-21-oic acid	
Methenolone[i]		
Acetate	Deacetylation	Ho et al. (2005)
	Epimerisation at C-17	
	Hydroxylation at C-16	
	Oxidation at C-17	
17α-Methyl testosterone	Reduction of the 4-en-3-one group	Dumasia (2003)
	Hydroxylation at C-6/C-16	
	Epimerisation at C-17	
	Hydroxylation at C-20	
Clostebol[j]		
Acetate	4-Chlorotestosterone	Leung et al. (2005a)
	4-Chloroandrost-4-ene-3α,17β-diol	
	4-Chloroandrostane-3α,17β-diol	
Mesterolone[k]	1α-Methyl-5α-androstan-3α-ol-17-one	Leung et al. (2005b)
	1α-Methyl-5α-androstan-3β-ol-17-one	
	1α-Methyl-5α-androstane-3β,17β-diol	
	1α-Methyl-5α-androstane-3,17α-diol	
	1α-Methyl-5α-androstane-3,16-diol-17-one	

[a] Ethylestrenol – 17β-hydroxy-17α-ethylestr-4-ene
[b] Fluoxymesterone – 9α-fluoro-11β,17β-dihydroxy-17α-methylandrost-4en-3-one
[c] Oxymethanolone – 17β-hydroxy-2-(hydroxymethylene)-17α-methylandrostan-3-one
[d] Mestanolone – 17β-hydroxy-17α-methylandrostan-3-one
[e] Danazol – 17α-pregna-2,4-dien-20-ynol[2,3-d]isoxazol-17β-ol
[f] Normethandrone – 17β-hydroxy-17α-methylestr-4-en-3-one
[g] Methandienone – 17β-hydroxy-17α-methylandrosta-1,4-dien-3-one
[h] Norethandrolone – 17β-hydroxy-17α-ethylestr-4-en-3-one
[i] Methenolone – 17β-hydroxy-1α-methyl-5α-androst 1-en-3-one
[j] Clostebol – 4-chlorotestosterone10
[k] Mesterolone – 17β-hydroxy-1α-methyl-5α-androstan-3-one

9.5.2.4 Orally Active Alkylated Steroids

The metabolism in humans of stanozolol (Masse et al., 1989a, b; Schanzer et al., 1990, 1992, 1996; Bi and Masse, 1992; Bi et al., 1992a) and other anabolic steroids of this group, for example, methandienone (also known as methandrostenolone dianabol) (Schanzer et al., 1991; Bi and Masse, 1992; Bi et al., 1992a), has received considerable

attention during the past few years. Hydroxylation of stanozolol occurs at positions 3′, 4, and 16 (Fig. 9.9) and produces a series of mono-and di-hydroxy metabolites, 16α- and 16β-hydroxylation being the major metabolic pathways. Epimerization at C-17 has also been observed. Preliminary studies of the metabolism of stanozolol in the equine, using high-performance liquid chromatography-tandem mass spectrometry for the analysis of urinary extracts (Muck and Henion, 1990), demonstrated the presence of a hydroxylated metabolite. Metabolism studies following both oral and intramuscular administration of stanozolol to the horse, have shown hydroxylation to occur at 3′

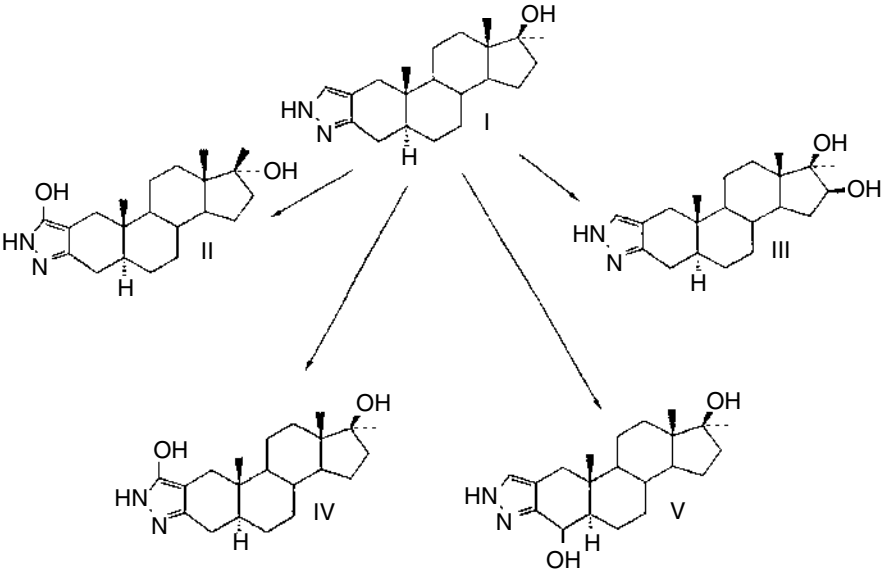


Fig. 9.9 Metabolism of stanozolol (I) in man; (II) 3′-hydroxy-17-epistanozolol; (III) 16-hydroxy-stanozolol; (IV) 3′-hydroxystanozolol; (V) 4 -hydroxystanozolol (from Schanzer and Donike, 1993, with permission of authors and Elsevier Science B.V)

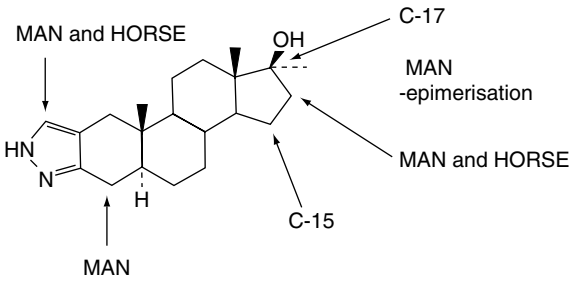


Fig. 9.10 Summary of the metabolism of stanozolol in man and horse. The *arrows* indicate hydroxylation positions (C-3′, -4 and -16)

and 16 positions as in man. In addition, evidence has been obtained for the presence of a 15-hydroxy metabolite (Teale and Houghton, 1991) (Fig. 9.10).

17-Epimerization mentioned above for stanozolol has been documented by several groups as being an important route for biotransformation of 17β-hydroxy-17α-methyl anabolic steroids. For example, it has been shown that 17-epi-4-chloromethandienone is a major metabolite of 4-chloromethandienone in humans (Durbeck et al., 1983) (Fig. 9.11). In a similar way, methandienone, oxandrolone, and stanozolol (as noted above) are all converted to the 17-epimers after administration to human volunteers (Masse et al., 1989a-c). It should be noted, however, that 17-epimerization is not a reaction specific to parent anabolic steroids, since some urinary metabolites of steroids such as methandienone, methylT and stanozolol are also affected (Bi and Masse, 1992 and references therein).

Schoene et al. (1994) have made a detailed study of the equine metabolism of 17α-methylT. After oral administration of a 1:1 mixture of the steroid and its $^{2}H_{3}$-analog to two thoroughbred horses, urines were collected for 72 h. Analysis of metabolites revealed that the main Phase I metabolic processes (Fig. 9.12) were complete, or partial reduction of ring-A and the 3-oxo group, 15- and 16-hydroxylation, 17-epimerization and hydroxylation at at least two other sites, postulated as C-6 and C-11. Both glucuronidation and sulphation of metabolites were found to be common features.

Subsequent to the study of Schoene et al. (1994), considerable effort has been devoted to the study of the metabolism of orally active anabolic agents in the horse. The major metabolites or major metabolic processes of the steroids studied are

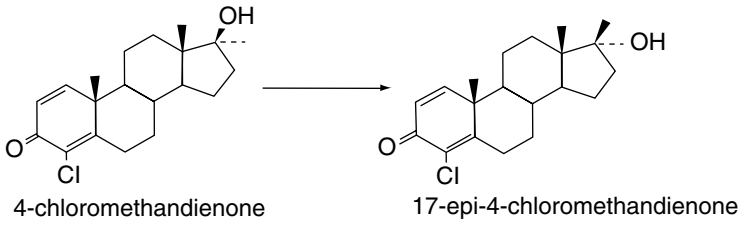


Fig. 9.11 Epimerization at C-17 of 4-chloromethandienone (4-chlorodehydromethyltestosterone)

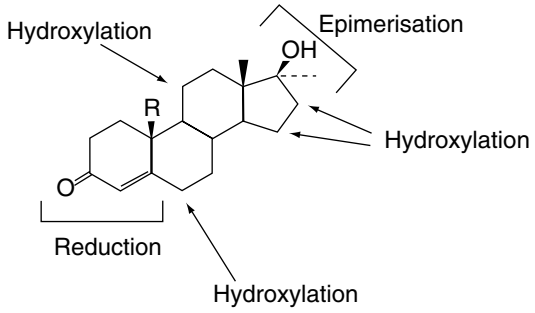


Fig. 9.12 Phase I metabolic reactions for 17α-methyltestosterone based on results of Schoene et al. (1994), with permission of authors and the publishers of Endokrinologie

Table 9.4 Distribution of urinary analytes of anabolic steroids between the glucuronide- and sulfate-conjugated groups and their detection periods following administration of proprietary preparations

Anabolic preparation	Analyte and conjugation mode[a]	Detection period[b] (days)
Nandrolin (nandrolone phenylpropionate)	Estrane-3β,17α-diol (G)	21
	Nandrolone (S)	
	Estrane-3β,17β-diol (S)	18
	Estrane-3β,17α-diol (S)	
Adroject (testosterone phenylpropionate)	Androstane-3β,17α-diol (G)	7
	Testosterone (S)	19
	Androstane-3β,17β-diol (G)	
Vebonol (boldenone undecylenate)	Boldenone (S)	40
Finajet (trenbolone acetate)	17α-Trenbolone (G)	3

[a]G = glucuronic acid conjugate; S = sulfate conjugate.
[b]Based on obtaining a satisfactory full-scan electron-impact mass spectrum.

summarized in Table 9.4 (Gourdie and Beresford, 1995; Kim et al., 1996; Stanley et al., 1997; Tang et al., 2000, 2001; Fox et al., 2001; McKinney et al., 2001a, b; Dumasia, 2003; Ho et al., 2005; Leung et al., 2005a, b). In general, the *in vivo* Phase I metabolic processes are similar to those identified for the C_{18}/C_{19} steroids; reduction of the 4-en-3-one group, epimerization at C-17 and hydroxylation at various sites including C-6 and C-16. In addition, Gourdie and Beresford (1995) observed the 3-hydroxylation of ethylestrenol to yield norethandrolone prior to its metabolism to 17α-ethyl estrane-3,17-diol isomers.

Also of particular interest is the observation by McKinney et al. (2001b) of the hydroxylation in the 17α-ethyl side-chain of norethandrolone to yield a number of 20-hydroxylated metabolites and 21-oic acids. This oxidation of the 17α-alkyl side chain has also been observed by Dumasia (2003) following oral administration of 17α-methylT to horses. Urine samples were hydrolyzed using the enzyme preparation *Eschericia coli* (glucuronidase), the steroid metabolites extracted by solid phase extraction and the extracts derivatized (methyloxime/TMS ether) prior to GC-MS analysis. Some isobaric $C_{20}O_3$ and $C_{20}O_4$ metabolites were detected showing an initial fragment ion by GC-MS analysis corresponding to loss of the radical of mass 103 amu (CH_2OTMS). This loss is characteristic of a metabolite arising by hydroxylation of the 17α-methyl group.

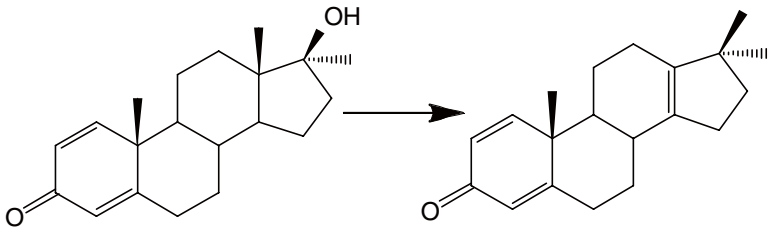
The group from the Hong Kong Jockey Club Racing laboratory (Tang et al., 2000, 2001; Ho et al., 2005; Leung et al., 2005a, b) have performed a series of studies for a number of steroids comparing the *in vitro* metabolism with horse liver microsomes with the *in vivo* metabolism following oral administration to horses. With danazol (Tang et al., 2001), both the *in vitro* and *in vivo* metabolism were complex but comparable. Of the 12 metabolites of danazol identified in the *in vitro* incubation study, nine were identified in the *in vivo* study. Other steroids studied by this approach are, oxymethelone and mestanolone (Tang et al., 2000), methenolone

acetate (Ho et al., 2005), clostebol acetate (Leung et al., 2005a, b) and mesterolone (Leung et al., 2005b).

6β-Hydroxylation is a major metabolic pathway in humans for methandienone, 4-chlorodehydromethylT and fluoxymesterone. The metabolites are excreted as unconjugated steroids. Indeed, 6β-hydroxy-methandienone was one of the earliest metabolites identified by GC-MS from extracts of urine samples collected from athletes (Ward et al., 1975).

Another major catabolic pathway for 17β-hydroxy-17α-methyl steroids in the human is that of C-13 demethylation. By a retropinacol rearrangement in acidic conditions, such steroids are converted into the corresponding 18-nor-17,17-dimethyl-13(14)-ene steroids (Bi and Masse, 1992; Bi et al., 1992a and references therein) (Fig. 9.13). Although the 18-nor derivative of methandienone was thought earlier to be an artefact arising from decomposition of the parent steroid under acidic conditions, it was shown subsequently using *in vivo* techniques that the18-nor compounds are, in fact, dehydration products of the 17β-sulfate of the parent compounds. Earlier results of Edlund et al. (1989b) had shown that the half-lives of tertiary sulfates are of the order of minutes in acidic conditions (equine urine or aqueous buffers). As a result of their observations, these workers proposed that the elimination of the 17β-sulfate group of methandienone results in the formation of the 18-nor-13(14)-ene derivative through rearrangement of the resultant carbonium ion. Subsequent nucleophilic attack by a water molecule would then yield the corresponding 17-epimer. The presence of the vicinal C-13 methyl group leads to rearrangement, yielding the 18-nor-13(14)-ene derivative. Bi et al. (1992a) have synthesized the 17β -sulfates of various 17β-hydroxy-17α-methyl steroids, including methandienone, methylT, mestanolone, oxandrolone and stanozolol, and have shown, using ^{13}C-NMR techniques, that when subjected to buffered hydrolysis the sulfates were converted to the corresponding 17-epimers plus the 18-nor-17, 17-dimethyl-13(14)-ene steroids (Fig. 9.14).

In studies of methandienone metabolism in humans, prolonged administration at high doses led to the unmetabolized drug being excreted in the urine and also contributed to the formation of a previously unreported metabolite: 3β,6ε,17β-trihydroxy-17α-methyl-5ε-1-androstene. Much more recently, and by contrast, Schanzer et al. (2006) have identified and characterized a long-term urinary



Methandienone

18-nor-17,17-dimethyl-1,4,13(14)-androstatrien-3-one

Fig. 9.13 C-13 demethylation of methandienone

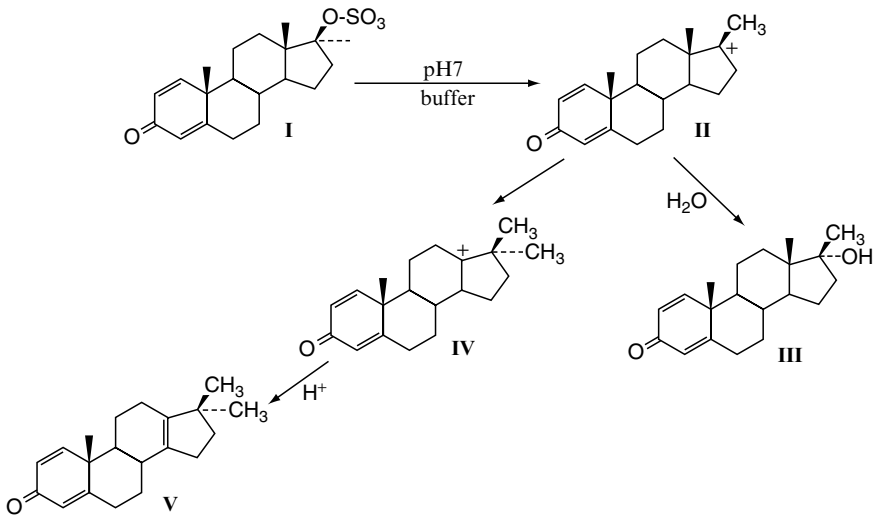


Fig. 9.14 Mechanism for epimerization of methandienone 17α-sulfate (I); tertiary carbocation (II); 17 β-methyl methandienone (III); carbocation (IV); 17,17-dimethyl-18-nor-13(14)-ene steroid (V) (from Bi et al, 1992a, with permission of authors and Elsevier Science, B.V.)

metabolite of methandienone that can be detected following a low dose of the drug. The C-13 demethylated metabolite concerned, characterized by GC-MS (including MS/MS) and also LC-ESI-MS-MS, was shown to be 18-nor-17β-hydroxymethyl,17α-methyl-androst-1,4,13-trien-3-one; the mechanism has not yet been fully elucidated but presumably involves a retropinacol rearrangement of a sulpho-conjugate as described above. Remarkably, following a single administration of methandienone (5 mg) to healthy male volunteers, this metabolite could be detected in the urine for as long as 19 days. Schanzer and his co-workers stress that the detection period is greater than that normally expected compared with commonly employed strategies.

Numerous studies have been concerned with the metabolism in humans of several other orally active synthetic anabolic steroids. For example, most of the metabolites of methenolone are excreted in the urinary glucuronide and sulfate fractions. Epimerization and oxidation at C-17 with concomitant reduction of A-ring functional groups occurred. Hydroxylation at several positions was confirmed, including C-2, C-6 and C-16 (Goudreault and Masse, 1990). Studies of the metabolism of stenbolone acetate (17β-acetoxy-2-methyl-5α-androst-1-en-3-one) in humans have confirmed 17-oxidation and A-ring reduction, with or without concomitant hydroxylation at C-16 (Goudreault and Masse, 1991). Also in humans, 18-hydroxylation was proven as a metabolic route for mesterolone, methenolone and stenbolone (Masse and Goudreault, 1992). Two more novel metabolites were isolated from the urine of a man who had ingested oxymetholone (Bi et al., 1992b). In these studies, steroids were isolated by solid-phase (Sep-Pak C_{18} cartridge)

extraction. Acidic metabolites were extracted with ether at acidic pH (1–2). GC-MS of the methyl ester-TMS ether derivatives afforded identification of 17β-hydroxy-17α-methyl-2,3-seco-5α-androstane-2,3-dioic acid and 3β, 17β–dihydroxy-17α-methyl-5α-androstane-2β-carboxylic acid.

9.5.2.5 Tetrahydrogestrinone – an Unusual 17-Alkylated Steroid

The detection of THG administration (a discussion of the structure–activity relationship of this steroid is in Section 9.3) relies currently on targeting the parent steroid in urine extracts following hydrolysis with β-glucuronidase. Catlin et al. (2004) commented in their seminal paper of 2004 that 'Given how straightforward it is to detect and identify THG in urine, and the media coverage it has received, it is unlikely that THG will be found again in a sport drug test.' Even so, it is of academic interest to briefly review its metabolism because it is an unusual anabolic steroid in terms of structure, and also because fresh human hepatocytes isolated from the liver tissue of patients undergoing partial hepatectomies were employed (Levesque et al., 2005). *In vitro* metabolism studies have been, to date, rarely applied for identification of metabolites of anabolic steroids and not with hepatocytes which would contain the gamut of metabolic enzymes (in the past, some elegant work has been done with liver microsomes – see Phase II metabolism, Section 9.3.3). This *in vitro* approach holds much promise in that it avoids administration studies in humans with unapproved xenobiotics, particularly the plethora of so-called steroid supplements/prohormones that are being manufactured that may be potentially harmful.

Incubation of THG in the presence of human hepatocytes and characterization of resultant metabolites by NMR, and HPLC coupled to photodiode array, fluorescence, MS or MS/MS detectors, showed introduction of hydroxyl groups at C-18 and another most likely at C-16ε. Further, conjugation with glucuronic acid at these hydroxyl groups, as well as that attached to C-17, were observed. A sum-

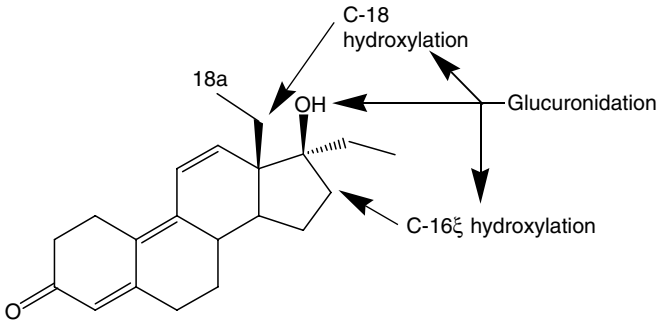


Fig. 9.15 Metabolism of THG in human hepatocytes, based on the results of Levesque et al. (2005)

mary of the metabolic biotransformations is displayed in Fig. 9.15. The investigators contrast their findings with what is known regarding metabolism of trenbolone in the human, which has a similarly conjugated double bond system but lacks the ethyl and ethynyl side chains at C-13 and C-17. Further, the authors comment that secondary carbon (C-18) of the ethyl group attached to C-13 of gestrinone and THG are more prone to P450-mediated oxidation than primary carbon (C-18a) of this group.

9.5.2.6 Dietary Supplements

These steroids are supplied for oral administration, and are therefore subject to first pass metabolism, a very important factor as to the extent the steroid is deactivated or converted to a more active form. Some of the putative metabolites of dietary supplements have been identified by MS; the interested reader is referred to the review by Van Eenoo and Delbeke (2006).

With respect to T prohormone supplements, as recently reviewed by Brown et al. (2006), these are modeled on steroids that are endogenously produced, that is, androstenedione, androstenediol and DHEA. However, supplements of DHEA or androstenedione may be of little or no benefit to healthy young men who wish to improve their strength and sporting performance if, as would be expected, any anabolic effect is primarily mitigated through peripheral conversion to T. Ingestion of DHEA can result in an increase in circulating DHEA and androstenedione but it is not resolved as to whether there is an increase in plasma T, for example, Brown et al. (1999). This is not surprising because in the adult male the overall peripheral contribution of these precursor steroids to circulating T is small, approximately 95% originating directly from testicular secretion. Any contribution from exogenous DHEA or androstenedione will be largely moderated by the large amount of T contributed by the testis. Following very large doses of DHEA (1,600 mg/day for 28 days) to five young eugonadal men, free and total serum T did not increase (Nestler et al., 1988). With regard to androstenedione, chronic administration of 300 mg/day to male volunteers between 19 and 40 years resulted in considerable interindividual variability to changes in serum T. King et al. (1999) showed no significant increase in serum T over an 8-week administration period (n = 10) whereas Leder et al. (2000) reported a significant increase after 7 days of administration (n = 14), but only a few of those subjects (n = 4) had serum T concentrations that exceeded the reference range. For androstenediol, Uralets and Gillette (1999, 2000) administered the Δ^4 and the Δ^5 form to male volunteers and have measured urinary concentrations of T in the former study. Following administration of the Δ^4 form (100 mg, p.o.) to three male volunteers, two showed large increases in urinary T concentration up to 20 h post-administration. However, plasma T was not assayed and it is probable that the increase in urinary T concentration (and urinary T/E ratio – see Section 9.10.4.3) in the adult male may be predominantly due to increased hepatic

metabolism of androstenediol to T glucuronide rather than any large increase in circulating T.

In the young adult female, an increase in performance may be possible following ingestion of these supplements, as circulating T would be expected to increase. The plasma concentration of T (0.7–2.6 nmol/L) is very approximately 1/10th that found in men and the proportion arising from peripheral conversion is much greater. Even though only 12–14% of androstenedione is converted peripherally to T (Horton and Tait, 1966; Bardin and Lipsett, 1967), this amount accounts for about half the circulating T in the female. As the peripheral contribution to blood T is far greater in the young adult female than the male, ingestion of modest amounts of DHEA, androstenediol or androstenedione is likely to raise circulating T. There are a few studies describing modest to large increase in circulating T following administration to women (Leder et al., 2002; Kicman et al., 2003; Bassindale et al., 2004; Brown et al., 2004). An investigation where the androstenedione was mixed and ground with lactose to aid dispersion in the gut showed that the plasma T concentrations increased from ~1 nmol/L to a maximum mean of 25.1 nmol/L at 75 min and remained significantly different from control values between 30 min and 8 h post-administration (Bassindale et al., 2004). The mean exposure (as determined by area under curve) to T was greater than an order of magnitude compared to the control period and the plasma concentrations observed were similar to those encountered in abuse of T for anabolic purposes. A similar profile would be expected with chronic administration but the risk of virilization precludes such a study.

As a corollary, Thieme et al. (2003) have made a detailed study of the metabolism of the delta-4 forms of 19-norandrostenediol (estr-4-ene-3,17-diol) and 19-norandrostenedione (estr-4-ene-3,17-dione) in human male subjects by transdermal administration. There is no suggestion that these steroids are available commercially at this time as transdermal preparations but the study is of academic interest. Nandrolone, 19-norandrostenedione, 19-norandrostenediol were quantified in peripheral blood serum, urine, beard hair, and sweat (eccrine and apocrine) taken from various body sites. Significantly, apocrine sweat from the axillae were devoid of the administered diol but contained two metabolites, nandrolone and 19-norandrostenedione, possibly formed by microbial action in the apocrine glands. Such findings are in agreement with earlier results in which Gower et al. (1997) showed the presence in human male apocrine sweat of bacteria containing steroid transforming enzymes.

9.5.3 Phase II Catabolic Reactions

9.5.3.1 Introduction

Phase II reactions occur in a similar way to the metabolism generally described for androgens (Chapter 6). The mechanism of Phase II reactions, that of conjugation with UDP glucuronic acid (UDPGA) to yield glucuronides and with

3′-phosphoadenosine-5′-phosphosulfate (PAPS) to yield sulfates, has been discussed by numerous authors (e.g., Gower, 1984).

9.5.3.2 Properties and Mode of Action of Uridine Diphospho Glucuronosyl Tranferases (UGTs) in Glucuronidation

UGTs are membrane-bound enzymes of the endoplasmic reticulum. A detailed account of this family of enzymes is given in Chapter 6. During the past 15 years, it has been shown that the human genome encodes at least 16 different UGTs, which are divided into families 1 and 2 as well as subfamilies 2A and 2B, depending on the degree of sequence identities and genomic organization. The UGTs are expressed mainly in the liver, although some of their enzymes occur also in other tissues.

Kuuranne et al. (2003b) have made in-depth studies of glucuronidation catalyzed by recombinant human hepatic UGTs of anabolic steroids or their metabolites (Table 9.5). In these experiments, liver microsomes were incubated separately in the presence of each of the steroids together with each of the recombinant UGTs, reactions being initiated by the addition of UDPGA (UDP glucuronic acid). The expression of human UGTs 1A1, 1A3, 1A4, 1A5, 1A6, 1A9, 2B4, 2B7 and 2B15 in bacillo-virus infected insect cells has been described by Kurkela et al. (2003), while cloning and expression of UGTs 1A7, 1A8 and 1A10 is detailed by Kuuranne et al. (2003b). After incubation, reaction mixtures were centrifuged and supernatants cleaned up by liquid chromatography. Characterization and quantification of products was achieved by electrospray MS-MS. Table 9.6 summarizes the results obtained. Of the large number of conclusions drawn by the authors, the major ones were (1) a high degree of regio-selectivity was found in that UGTs 1A8, 1A9 and 2B15 preferentially catalyzed the glucuronidation of the 17β-hydroxyl group, while most of the other UGTs catalyzed the same reaction but at C-3α as well as C-17β; (2) stereoselectivity was also observed with regard to 19-norandrosterone and the corresponding 5β-diastereometric isomer, 19-noretiocholanolone (both metabolites of 19-norT (nandrolone)) in that the results demonstrated that glucuronidation of 19-noretiocholanolone was mostly performed at higher rates, and by more UGTs, than glucuronidation of 19-norandrosterone. The orientation of the proton attached to C-5 has a dramatic effect on the steroid ring structure, as the A/B-cis junction of 5β-steroids changes the spatial ring geometry into a sharply bent form. Furthermore, the 3α-bond is equatorial around ring A in 5β-steroids, but axial in 5α-steroids. In contrast, however, no such stereoselectivity was observed if the analogous A-ring reduced metabolites of methylT were used as potential substrates; (3) UGTs readily catalyze glucuronidation at the 17 position of 5α-androstane-3α,17β-diol whereas methylT did not serve as a substrate due to steric hindrance presumably caused by the adjacent 17α-methyl group.

Of the seven members comprising the UGT2B family, UGT2B17 has been shown to be particularly active in catalyzing the glucuronidation of androgens, including T, 5α-DHT and perhaps surprisingly etiocholanolone (as this has no androgenic activity) (Turgeon et al., 2001). Further studies by Murata et al. (2003)

Table 9.5 Structures and nomenclature of steroid glucuronides. From Kuuranne et al, 2003, with permission

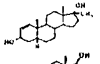
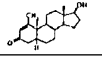
Abbreviation	Compound	Structure	Sites of glucuronidation	Precursor
5α-N	5α-Estran-3α-ol-17-one		3α-OH[a]	Nandrolone
5β-N	5β-Estran-3α-ol-17-one		3α-OH[a]	Nandrolone
5α-ME	1-Methylen-5α-androstane-3α-ol-17-one		3α-OH	Metenolone
MT	17α-Methylandrost-4-en-17β-ol-3-one		17β-OH	Methyltestosterone
5α-MT	17α-Methyl-5α-androstane-3α,17β-diol		3α-OH[a]	Mestanolone, methyltestosterone, oxymetholone
5β-MT	17α-Methyl-5β-androstane-3α,17β-diol		3α-OH[a]	Metandienone, methandriol, methyltestosterone,
5β-EPIM	17β-Methyl-5β-androst-4-ene-3α,17α-diol		3α-OH, 17α-OH	Metandienone
TES	4-Androsten-17β-ol-3-one		17β-OH	Testosterone
NAN	Estr-4-en-17β-ol-3-one		17β-OH	Nandrolone
5α-1-ME	1-Methyl-5α-androst-1-en-17β-ol-3-one		17β-OH	Metenolone

Table 9.6 Glucuronidation of anabolic androgenic steroids by recombinant human UGTs

UGT	5α-N	5β-N	5α-ME	MT	5α-MT	5β-MT	5β-EPIM	TES	NAN	5α-1-ME	5α-A
1A1	+	++	—	—	—	—	—	++	++	—	+++
1A3	++	++	+++	—	++	++	+	++	++	+	+++
1A4	+++	+++	++++	—	+++	++++	—	+++	+++	++++	+++++
1A6	—	—	—	—	—	—	—	—	—	—	—
1A7	—	—	—	—	—	—	—	—	—	—	—
1A8	—	—	—	—	—	—	—	++	+++	++	+++
1A9	—	—	—	—	—	—	—	+	++	++	+++
1A10	—	++	—	—	—	—	++	+++	++++	++++	++++
2B4	++	+++	++++	—	++	++	++++	+	+	+	+++
2B7	+++	+++++	++++	—	+++	+++	++++	+	++	+++	++++
2B15	—	—	—	—	—	—	—	+++	++	+++	+++++

The formation of a given glucuronide was estimated as the ratio of peak areas of analyte and internal standard. A minus sign means that glucuronide formation, if to any extent, was below the detection limit, whereas each additional "+" indicates an approximate ten-fold increase in activity. Experimental conditions: 50 μM substrate, 5 mM UDPGA, 5 mM saccharic acid lactone, 0.5 mg/ml protein, and 2 h of incubation time (from Kuuranne et al. 2003, with permission).

have revealed the existence of a deletion in the UGT2B17 gene, the deletion being characterized by Wilson et al. (2004).

Jakobsson and co-workers (2006) have explored further this gene deletion and have stated that the object of their work was to evaluate the contribution of the UGT2B17 deletion polymorphism made to individual and inter-ethnic variations in metabolism, and also to the urinary excretion of C_{19} steroids. For the latter part of the work, urine samples were provided by healthy men, 122 Caucasian (Swedish) and 74 Asian (Korean). The glucuronides and unconjugated fractions of 12 C_{19} steroids, including T, 5α-DHT, epi-T, were measured. Concentrations of several steroid glucuronides, including T were very much less in Korean subjects than in Caucasian individuals (highly significant differences). However, there were no significant differences in the epi-T glucuronide between groups. Consequently, the T/E ratios were 0.15 and 1.8 in the Korean group and the Swedish group respectively. As a result of these studies showing lowered C_{19} steroid glucuronide excretion in the Korean subjects compared with the Swedish group, it was suggested that this could well be due to slower formation of glucuronides. Hence the glucuronization process was investigated in detail, as below. It was also found that the natural loss of glucuronide excretions showed bi-modal patterns in both groups of subjects, suggesting a monogenic inheritance.

In attempts to explain the differences in T glucuronide excretion mentioned above, the deletion polymorphism of UGT2B17 noted by Murata et al. (2003) and characterized by Wilson et al. (2004), was further explored. Jakobsson et al. (2006) found that all the individuals with UGT2B17 del/del genotype had negligible or no T glucuronide in their urine. In fact, this genotype was seven times more common in the Korean group (66.7%) than in the Swedish group (9.3%). Further support for these findings was obtained from *in vitro* work with human liver microsomes, which showed that the del/del gene type was associated with significantly lower glucuronidation rates. Large differences in T excretion between Asian and Caucasian men associated with an UGT2B17 polymorphism has obvious implications for the T doping test based on the T/E ratio, determined following hydrolysis with β-glucuronidase, which currently works to an absolute reporting threshold (T/E = 4), rather than taking into account inter-ethnic variations in metabolism.

Urinary steroid sulfate concentrations were not measured in these studies, but it is well worth noting that Borts and Bowers (2000) found that concentrations of both glucuronide and sulfate conjugates of T and E were lower in a Chinese population compared with a Caucasian mixed group.

As Bi and Masse (Bi et al., 1992a) emphasize, at least two points should be noted with regard to the conjugation of steroids and their metabolites: (a) in contrast to man, generally sulphation is equally as important as glucuronidation in the horse; and (b) conjugation of the 17β-hydroxyl group of 17β-hydroxy-17α-methyl C_{19} steroids is far more likely to be by PAPS than via UDPGA. The reason for this is that the steric hindrance between the 17α-methyl group and the fairly bulky UDPGA molecule prevents glucuronidation. The much smaller PAPS molecule, being less sterically hindered, is relatively vulnerable to nucleophilic attack by the 17β-hydroxyl group. In keeping with this idea are the results of Masse et al. (1989a-c)

on the urinary conjugates of methandienone, oxandrolone, and stanozolol in human subjects. However, glucuronidation can occur with other, unhindered, functional groups, for example, with the enol function in 4-en-3-oxo steroids like methandienone and 17α-methylT and the pyrazole moiety of stanozolol.

In both the human and the horse, the anabolic steroids and their metabolites are extensively conjugated. In humans, the major metabolites of T, androsterone, and etiocholanolone, and the major metabolites of nandrolone, 19-norandrosterone and 19-noretiocholanolone are conjugated with glucuronic acid, as reviewed previously (Gower et al., 1995). In the horse a major metabolic pathway is sulphation of the parent steroids, T and nandrolone. Also, in contrast to the human, the major metabolites are the 3,17-diols, which are conjugated with either glucuronic acid or sulfate. The mode of conjugation is dependent to some extent upon stereochemistry. T and nandrolone (17β-OH) and the 17β-diol metabolites tend to be conjugated with sulfate, whereas the 17α-diol metabolites are conjugated to a large extent with glucuronic acid (Table 9.4). As might be anticipated with an anabolic steroid such as boldenone, where there is a free 17β-hydroxyl group, conjugation with UDPGA is a major metabolic pathway in man (Schanzer and Donike, 1992), and the major metabolites, 17β-hydroxy-5β-androst-1-en-3-one, 5β-androst-1-ene-3α,17β-diol, 3α-hydroxy-5β-androst-1-en-17-one and 6β-hydroxy-5β-androst-1-en-3,17-dione, are also conjugated as glucuronides. The major metabolic pathway in the horse (7–10% of the dose) is conjugation of boldenone with sulfate, as noted in a previous review (Gower et al., 1995). The 17α-epimer and the A-ring reduced metabolites are conjugated with glucuronic acid. Similarly with trenbolone and stanozolol, as noted above, in humans the primary mode of conjugation is with glucuronic acid, whereas in the horse, metabolites are conjugated with both glucuronic acid and sulfate.

Another Phase II reaction of importance is the proposed formation of an unstable tertiary sulfate resulting in epimerization of steroids with a 17β-hydroxy-17α-methyl configuration, the 17α-hydroxy-17β-methyl epimer being detected in human urine following drug administration (e.g., Durbeck et al., 1978, 1983; Durbeck and Buker, 1980; Masse et al., 1989a, c, 1991; Schanzer et al., 1991; Bi and Masse, 1992). The mechanism proposed by Edlund et al. (1989b) is based on elimination of the unstable sulfate group, which can also cause the migration of the angular methyl group attached at C-13, resulting in the formation of 18-nor-17, 17-dimethyl-13(14)-ene steroids (see also Section 9.5.2.4).

9.6 Collection, Handling and Storage of Biological Samples

9.6.1 Urine

In human sports, urine is the chosen biological fluid for analysis, the collection procedure being comprehensively described under the WADA ‘Guidelines for Urine Sample Collection’. Appropriate doping control officers witness a urine sample being delivered into a collection vessel, not least to ensure that no manipulation of

the sample occurs. Much time and effort has been devoted to ensuring that the sample kits and chain-of-custody documentation can withstand legal challenges.

The athlete is requested to select two coded glass bottles, each assigned a unique code. The urine is then divided between the two bottles, ideally into about 70 mL for screening purposes (usually designated the 'A-sample') and 30 mL for confirmatory analysis (the 'B-sample'). In some cases, the volume of urine available for analysis is considerably smaller. The bottles are sealed using tamper-proof lids and then sent to the laboratory within a sealed shipping container. The sampling officer is usually requested to measure and record the specific gravity and pH of urine remaining in collection vessels, prior to disposal of that urine. On arrival at the laboratory, the 'A-sample' seal is broken and the urine is subjected to screening analysis to detect any prohibited substances and, when found, a fresh aliquot of the A-sample is taken for confirmatory analysis. If the 'A-sample' fails a drug test, the 'B-sample seal' is broken at a later date, and the analysis repeated in the same laboratory, competitors having the option to witness this process with representatives of their choice, for example, an independent scientific expert and a legally qualified individual.

As specimens are collected at many locations within a country, delays during transit can be as much as several days. These samples are usually at ambient temperature and, as no preservative is added, general degradation of the sample may occur, for example, increase in pH due to ammonia formation. Markers of general degradation include a pH above that physiologically possible (pH > 8.3) and/or a large presence of 5α-androstanedione and free steroids which were originally glucuronidated, for example, androsterone, etiocholanolone (Ayotte et al., 1997). However, the absence of signs of general degradation is not conclusive proof that sample integrity has been maintained. Once received, storage of samples in WADA accredited laboratories is usually at −20°C. In clinical chemistry, maintaining sample integrity prior to analysis is extremely important and as a corollary, the apparent lack of precaution in preservation of sports samples in transit is completely alien to that philosophy. However, if preservative is added to sports samples, it has been argued that an adverse finding may be challenged on the basis that such samples failed the test because of adulteration with a foreign material. This argument appears to be weak, given the WADA protocol for blood collection, where blood can be drawn into a tube containing a serum separator gel and a clotting activation factor.

Although anabolic steroids are not thermally labile there has been some concern about the possibility of urinary microbial production of T causing an adverse finding (Bilton, 1995; Honour, 1996; Birchard, 1998) using the approved test for detecting doping with T. Steroid transforming micro-organisms are common in nature and urine can contain bacteria and yeast due to normal commensals and natural exfoliation. Also, in the adult female, symptomatic and asymptomatic urinary tract infections can be present, and urine can be directly contaminated during collection by normal gastro-intestinal flora present on the perineum. However, De la Torre et al. (2001a; Bilton, 1995; Honour, 1996; Birchard, 1998) did not find T production following inoculation of urine with selected organisms but they consider that microbial contamination may hamper interpretation of results. Kicman et al. (2002) have shown that an increase in urinary T can occur, following inoculation of urine with a strain

of *Candida albicans*, but the increase is minor and hence of little evidential value for any individual sport case. It is difficult to comment further as to the possibility of how much T can be formed by different strains of *C. albicans*. Interpretation will be helped by isotope ratio MS (see Section 9.10.5) of urinary steroids to determine whether T is of exogenous origin as well as comparing the T liberated by glucuronidase hydrolysis and/or that in the free steroid fraction to total T. Notwithstanding, overcoming the 'quasi-legal' difficulty of adding a suitable chemical preservative to samples to reduce the possibility of microbial action appears to be a straightforward way of addressing this specific issue.

Within horseracing, urine is also the preferred biological fluid for drug testing although a greater emphasis is now being placed upon blood (usually plasma) samples and in some racing jurisdictions both are collected. Within the European environment, urine must be collected whenever possible and blood is the preferred alternative if urine cannot be collected within a reasonable period of time.

Selection of horses for sampling is also at national discretion but generally countries will provide comprehensive powers for raceday stewards to select horses for sampling with flexibility in selection.

The majority of racing jurisdiction adopt a split sample policy providing the A- and B-samples, as in human sports drug testing, the B-sample being maintained with all seals and identification in tact and stored under suitable conditions for the purpose of referee analysis. B-analysis can be performed in the primary laboratory in the presence of an expert witness or, unlike human sports testing, by an independent referee laboratory; in some racing jurisdiction the latter procedure is mandatory.

In Great Britain, the racing authorities have taken steps to minimize sample degradation through the installation of refrigeration facilities in the sampling units of all 59 racecourses. Thus all samples can be cooled immediately after voiding and retained in the refrigerator until collected by the courier for transport to the laboratory. In addition the sample packaging system incorporates an icepack to maintain the samples in a chilled state during transport to the laboratory and the refrigeration units have deep freeze capacity for the racecourses to maintain a supply of frozen icepacks.

In contrast to human athletics, there is no universal sampling kit within the horseracing industry but within all racing jurisdictions, steps are taken to ensure the chain of custody and integrity of the samples are maintained at all times from the point of selection of the horse for sampling to the delivery of the samples to the laboratory. Within Great Britain, a combined blood and urine sample collection kit has been designed by HFL, Ltd., Newmarket in collaboration with veterinary officers associated with the racing industry (Haywood and Dunnett, 1994). The kit contains two urine bottles and 4 × 25 mL heparinized blood collection syringes, hypodermic needles and antiseptic wipes and thus allows for split sample collection for either urine or blood for referee analysis purposes. The kits also contain printed self-adhesive bar coded labels for use on the sample bottles and associated paper work.

The kit was designed to be leak proof and able to withstand the rigorous legal challenges associated with sample security. This is a tamper evident kit designed

with a multi-layer approach such that if one layer fails, the overall integrity of the collection procedure is not jeopardized. The first level of security is an aluminium diaphragm with a high density polyethylene backing which is hermetically sealed to the bottle. The second level of security is a tamper -evident tear-band cap screwed firmly onto the bottle. Each labeled bottle is then placed inside a uniquely numbered, bar-coded plastic security bag with a strong self-adhesive seal that reveals a void warning if any attempt is made to open the bag. Sample bottles sealed in the security bags are then packed in a high density expanded polystyrene box which incorporates the ice-pack. The polystyrene boxes are then sealed in outer cartons for shipment to the laboratory in a sealed nylon freight bag. At the time of writing, modifications to the kit are being considered, including reducing the size of the urine bottles.

9.6.2 Additional Biological Matrices for Analysis

As well as urine, biological matrices for analysis of anabolic steroids could include blood (already collected in horse racing as the preferred alternative if a urine sample cannot be provided), saliva, sweat and hair. Of these, blood, and hair analysis appear to be the most promising as useful adjuncts to urinalysis but much work needs to be done to establish whether these techniques as suitable ancillary methods for evidential analysis for anabolic steroids, as part of drug control in sport. The analyses of anabolic steroids in blood and hair also have relevance to forensic analysis, particularly when a post-mortem urine sample is not available.

9.6.2.1 Blood

Blood sampling has been performed and is increasingly being established as an additional biological matrix for analysis in doping control. Currently, the collection of blood in human sports is performed to detect blood doping (autologous and non-autologous), detection of erythropoietin administration by monitoring its effects (Robinson et al., 2001b; Sharpe et al., 2002), and sometime in the future, for the detection of human growth hormone administration (McHugh et al., 2005). Generally anabolic steroids and their metabolites are far more concentrated in urine than in blood, making it much easier to detect their presence in urine. Whether blood collection will facilitate detection of administration of anabolic steroids remains to be seen but it could be helpful in assessing whether natural androgen administration has occurred, such as by the detection of esterified forms of T (Peng et al., 2000).

9.6.2.2 Scalp Hair

Scalp hair analysis for anabolic steroids has gained interest as a possible approach for drug control in sport over the past 15 years (and appears to be of increasing importance

in forensic analysis, particularly post-mortem). Not only is scalp hair easily obtained but the samples are stable with no need for temperature and pH control or addition of preservative during transportation to the laboratory. At least 60 pharmaceuticals and drugs of abuse, together with some anabolic steroids such as nandrolone and stanozolol, can be measured (Rivier, 2000). In addition, there are reports of the detection of T and its esters, nandrolone and its esters and methandienone in scalp hair after parenteral administration. Especially useful is the fact that a number of samples can be taken to allow confirmation of an earlier result or for analysis of another possible analyte. It is recommended that approximately 200 mg of scalp hair should be cut off as close as possible to the skin of the posterior vertex region of the scalp and wrapped in aluminium foil for storage at room temperature (Wennig, 2000). This amounts to about a 'pencil thickness' of hair and can be made up from several regions of the posterior vertex to avoid a noticeable absence of hair. Even so, despite the potential usefulness of hair analysis in drug abuse in human sports, it is generally accepted that a negative hair result cannot exclude administration of a detected drug or its precursor and should not over-rule a positive urinary result (Sachs and Dressler, 2000). However, in a court of law, the hair analysis result is bound to cause some ambiguity to the urinary results. There are good reasons for this recommendation, since the incorporation of drugs into hair is variable and depends on a number of factors as described below. Rivier (2000) has discussed the question 'Is there a place for hair analysis in doping control?' and concluded that there are clear limitations with regard to the usefulness of hair analysis. The factors involved include:

(a) *Binding Affinity of Melanin and the Hydrophilicity and Membrane Permeability of the Drug Concerned* The latter parameter (Nakahara et al., 1995) is based on the pH gradient between blood and the acidic hair matrix. Thus, basic drugs would be expected to have a high membrane permeability, and this is borne out by Rollins et al. (1996) who showed that a single dose of codeine could be detected in hair for 8 weeks. Similarly, Henderson et al. (1996) found that the threshold dose of cocaine injected intravenously was 25–35 mg if it was to be detected in hair. On the other hand, anabolic steroids, despite their lipophilicity, have low-membrane permeabilities and, after a single i. m. dose of 50 mg nandrolone decanoate, neither the unesterified steroid nor its decanoate could be detected in the hair of a 37-year old man (Kintz et al., 2000). In contrast, his urine showed positive for nandrolone metabolites (19-norandrosterone and 19-noretiocholanolone) for at least 8 months post-injection. Similar results, by Segura et al. (2000) were obtained with men who had received single dose administration of T enanthate, propionate or undecanoate, or nandrolone and its decanoate. Kintz et al. (2000) make the important point that given current methologies, hair of athletes would be far more likely to test positive for anabolic steroid abuse if they have been chronically administered.

(b) *Influence of Hair Colour on Analytical Results* Melanin is the major (85–93%) constituent of hair (Wennig, 2000) and the principal component for binding of drugs. Both pheomelanin and eumelanin are present in hair, with black or brown hair containing greater quantities of the latter form of melanin. It is not surprising; therefore, that dark hair binds larger amounts of drugs than

do red and blonde hair (Cone, 1996). In this context, several authors (Kintz et al., 2000; Rivier, 2000; Wennig, 2000) have highlighted the very real possibility of racial bias when African–American drug abusers might be identified more frequently than Caucasians.

(c) *Stability of Drugs in Human Hair* Clearly, this factor is of crucial importance in the interpretation of drug analyses. Several recent studies have shown that dyeing and, especially, bleaching of hair can reduce the content of numerous drugs, including opiates, cocaine, cannabis, nicotine, T, and its undecanoate and nandrolone (Jurado et al., 1997; Gaillard et al., 2000). These results have important consequences; first, in the positive sense. Kidwell (1999) has suggested the possibility of incorporating dyes to reduce the binding of drugs in dark hair, and thus to minimize the inequality problem noted above. Secondly, the routine colouring or bleaching of their hair by athletes (whether for aesthetic or drug-evasion purposes), can lead to missing identification of, for example, T and other anabolic steroids (Kintz et al., 2000). Frequency of hair-cutting or the necessity for keeping hair short as, for example, in swimmers, is important and critical considerations. A particular problem occurs when athletes actually shave their heads; the only recourse the analyst then has is to ask for hair samples to be taken from other parts of the body, hopefully, without too much inconvenience. However, much more information is needed on the concentrations of anabolic steroids or their metabolites in axillary or pubic hair.

Of the research groups that have been involved in hair analysis, Deng et al. (1999) have concluded that head-hair analysis has considerable potential for detection and monitoring for abuse of steroids. In this study, seven white male steroid abusers provided head hair and urine samples. Limits of detection for various anabolic steroids ranged from 0.02 to 0.1 ng/mg hair. MethylT was detected in two hair samples (confirmed in the urine), nandrolone was found in two hair samples and confirmed in the corresponding urine samples. In contrast, although dehydromethylT (methandienone) was detected in four hair samples, its presence could not be confirmed in the urine. Clenbuterol and oxymethelone were both found in the urine samples but not in the corresponding hair samples. Finally, one abuser had high concentrations of T (0.15 ng/mg) and also 190 ng/mL in urine.

The methodology employed by this group was much the same as that by others, that is, NaOH digestion, solid phase extraction, derivatization (MO-TMS ethers) for all steroids, except stanozolol, where TMS-ethers/heptafluorobutyrates (HFBA) were utilized. Detection and quantfiication was achieved using GC-MS in EI mode (see also Chapter 6).

Kintz et al. (1999) were also successful in detecting anabolic steroids in hair, although this study included only two male bodybuilders who were suspected of taking drugs for personal reasons. Analysis of urine samples from both men showed the presence of nandrolone (19-norT), T and stanozolol. Hair samples showed the presence 19-norT (196 and 260 pg/mg), T (46 and 71 pg/mg) and stanozolol (135 and 156 pg/mg), clearly consistent with steroid abuse. The authors concluded that hair analysis can be useful adjunct to conventional drug testing in the urine of athletes. The methods used were similar to those noted above but with some changes in that

deuterated standards were added at the NaOH hair-extraction stage, liquid-liquid extraction (ethyl acetate) replaced SPE, and GC-MS of the TMS ethers.

Gaillard et al. (2000) have also carried out a comparative study in which the head hair and urine of 30 racing cyclists were examined for their amphetamine, corticosteroid and anabolic steroid content. The last of these (19-norT and T undecanoate) were determined once each (in 25 analyses) in hair compared with none in the urine (30 analyses). The high degree of purification comprised solid phase extraction of ethyl acetate extracts using aminopropyl and silica cartridges. Following MSTFA derivatization, identification, and characterization were carried out by GC coupled to a triple quadrupole MS.

When Segura et al. (2000) administered single doses of T esters intramuscularly to healthy human subjects, as either enanthate (250 mg), undecanoate (120 mg), T propionate (25 mg) plus 110 mg T enanthate (110 mg), or undecanoate (110 mg), it was not possible to detect these esterified compounds in scalp hair 3, 2, or even 1 month after administration. The authors concluded that, at the sensitivities achievable, it was not possible to determine either of the esters in hair as single dose administrations. Surprisingly, this was despite the fact that sensitivity available is at least 20 pg injected for GC-MS or GC-MS-MS.

9.7 Urinalysis – Screening by GC-MS

The radioimmunoassay screening procedures for anabolic steroids in human (Brooks et al., 1975, 1979) and horse urine were eventually replaced by analysis employing GC-MS. The introduction of benchtop quadrupole GC-MS instruments in the early 1980s offered specificity, sensitivity, and excellent data handling, together with a reduction in cost compared to previous MS instruments. Increased chromatographic resolution was obtained with the use of superior capillary columns, and increased sensitivity was achieved by using electron impact MS in the selected-ion monitoring (SIM) mode. In addition, with automated sample injection and short chromatographic run times (typically 20–30 min because of oven temperature programming), large sample throughput made GC-MS the preferred analytical tool.

9.7.1 Sample Preparation (Clean Up and Hydrolysis)

A schematic of sample preparation employing mixed-phase extractions and enzymic hydrolysis[2] of human urine is given in Fig. 9.16. Internal standards are added to the urine, for example, in the UK the WADA accredited laboratory in London routinely

[2]A separate method for isolation of certain anabolic steroids and their metabolites that were largely excreted unconjugated (free steroids) from urine was employed in the past but these steroids can be now confirmation of certain steroids, e.g. for analysis of fluoxymesterone.

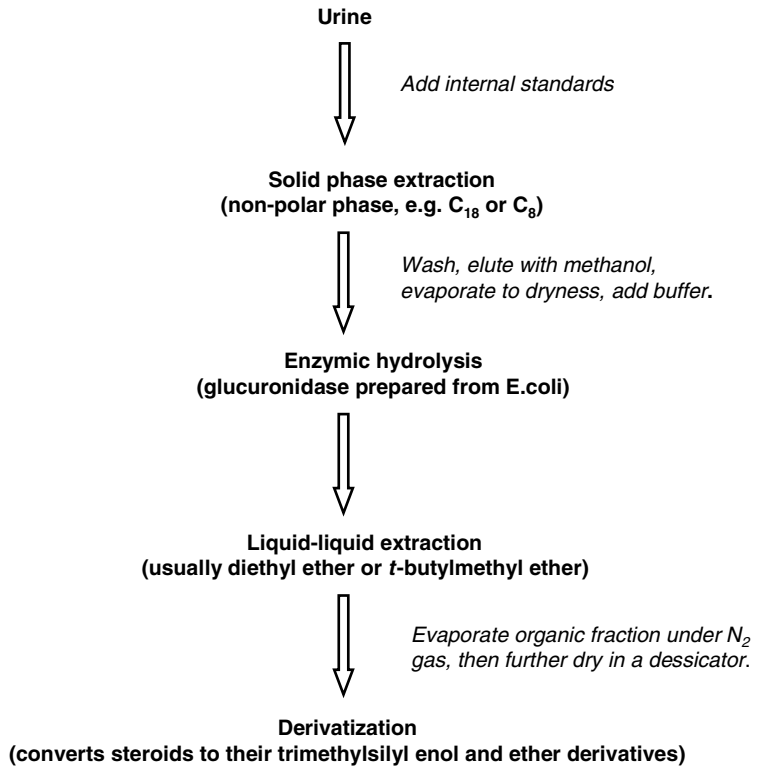


Fig. 9.16 Schematic (generic) of sample preparation for the screening of anabolic steroids and their metabolites in urine

employs a standard mixture of deuterated forms of T, E, DHT, and 5α-androstane-3α,17β-diol. This eliminates error due to sample extraction and variability in efficiency of steroid derivatisation (see below). Recently, the synthesis of a pentadeuterated form of 3′-hydroxystanozolol has been decribed (Felzmann et al., 2005), which may be particularly useful for monitoring purposes, given that the silyl derivatives of stanozolol metabolites are prone to decompose in the injection port of the GC. As many anabolic steroids or their metabolites are excreted primarily as glucuronides in the human, equivalent deuterated internal standards should ideally also be conjugated and these, with other certified anabolic steroid reference materials are now commercially available and are gradually being incorporated into routine procedures in WADA accredited laboratories, cost permitting.

Steroid glucuronides are not volatile so, prior to GC/MS analysis, the conjugate must be cleaved. Enzymatic, rather than acid, hydrolysis is chosen because, although the latter offers speed and simplicity, it can also generate non-specific interference and steroid dehydration products. In sports drug testing, much emphasis is placed on measuring trace amounts of steroid analytes and any unpredictable interference on a GC/MS chromatogram can hinder interpretation. Many WADA laboratories prefer

to perform a preliminary solid–liquid extraction to optimize the conditions for enzymatic hydrolysis, rather than perform direct hydrolysis (addition of β-glucuronidase to unextracted urine). The advantage of a solid phase clean-up procedure prior to hydrolysis is that it removes enzyme inhibitors such as lactones and ascorbic acid that are often present in urine. Perhaps even more importantly, it also helps to remove other compounds that are not removed so effectively by liquid-liquid extraction alone, the reduced presence of interferents thus helping to maintain a clean GC liner, and good chromatography and quality of mass spectrometry data. C_{18} or C_8 cartridges have superseded Amberlite XAD-2 resin or equivalent; methanol is often used to elute free and conjugated steroids. After evaporation, the dried extract is resuspended in a suitable buffer at the optimal pH for enzyme preparation, usually an enzyme extract from *E. coli*. The use of the digestive juice of *Helix pomatia* has fallen out of favor, despite it being more economical to use, because steroid artefacts can be produced, including the potential formation of T in urine (Houghton et al., 1992). A fully automated sample preparation procedure has been described that incorporates direct hydrolysis followed by liquid-liquid extraction and derivatization (Haber et al., 2001) but this automated procedure does not appear to have been adopted widely.

Following deconjugation, liquid-liquid extraction is performed with a non-polar organic solvent, usually diethyl ether or *t*-butylmethyl ether, the neutral steroids being favorably partitioned into this. The organic layer is evaporated to dryness, the extract further dried in a vacuum dessiccator containing phosphorous pentoxide and potassium hydroxide to ensure removal of all water present (which could otherwise prevent complete TMS derivatization – see Section 9.7.2) and the steroids are then derivatized.

For sample preparation of equine urine, a minor modification to the method published by Teale and Houghton (1991) allowed for steroids to be monitored in the "free + glucuronic acid conjugate group fraction" and the "sulfate conjugate group fraction". The method comprised direct enzyme hydrolysis of the urine with *E. coli*, extraction of the urine on a C_{18} Sep-Pak cartridge, elution of the aglycones with ether, elution of the sulfate conjugated steroids with ethyl acetate: methanol: sulphuric acid (solvolysis solvent) and their subsequent solvolysis, incubation of the solvolysis solvent overnight at 37°C, to cleave the sulfate conjugates. The two fractions, the aglycones and the steroids originally conjugated with sulfate were then derivatized to from the oxime/t-butyldimethysilyl derivatives for GC-MS analysis in the SIM mode, but this has been now replaced with TMS derivatization in their laboratory, as first adopted for human sports drug testing.

9.7.2 Trimethylsilyl Derivatization

Silylation is a powerful tool often used in the GC-MS, as reviewed by Halket and Zaikin (2003). For screening purposes, hydroxyl and oxo functions of anabolic steroids are converted to TMS ether and enol derivatives, respectively, to render them more volatile and thermally stable and to improve their chromatographic characteristics (Fig. 9.17). To ensure effective enolization has occurred, the peak

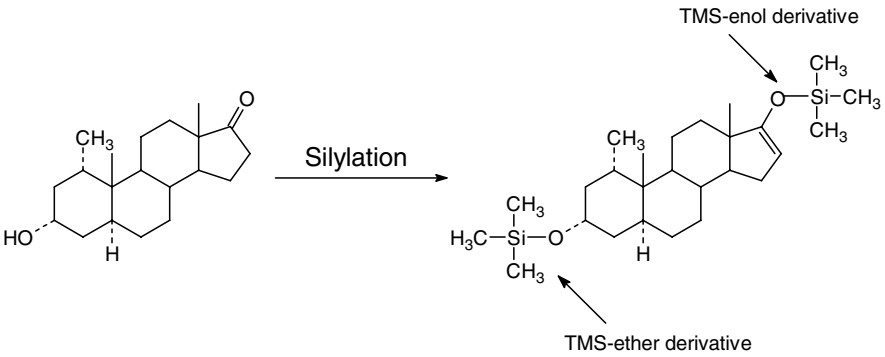


Fig. 9.17 Silylation resulting in the formation of a trimethylsilyl (TMS) enol TMS ether derivative. The example shown is a diagnostic metabolite of mesterolone

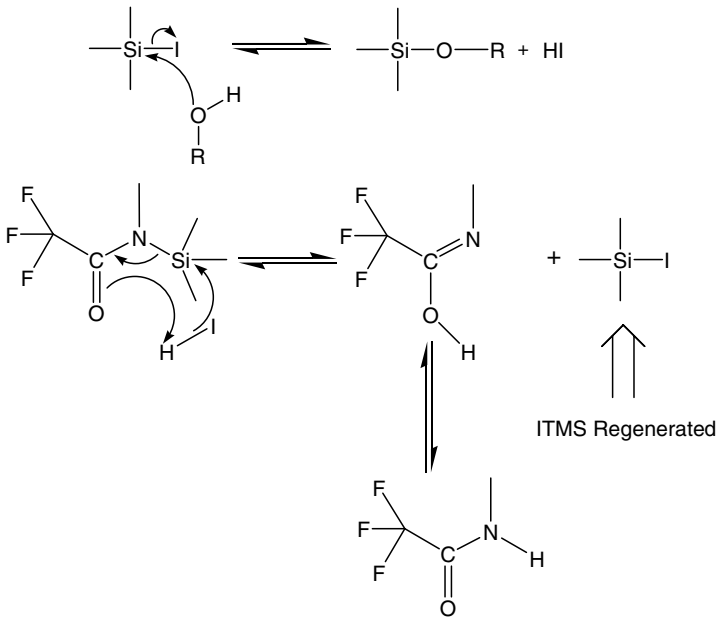


Fig. 9.18 Possible mechanism of silylation

area ratio of the bis-TMS to the mono-TMS of a mono-oxo-mono-hydroxyl steroid (such as androsterone) may be compared. The derivatization mixture used consists of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) with the powerful silylating agent iodotrimethylsilane (ITMS), such a reaction yielding isomerically pure TMS enol derivatives (Donike and Zimmermann, 1980). A possible TMS reaction mechanism is shown in Fig. 9.18 (the authors are grateful to Professors Halket

and Schänzer for helpful discussion regarding this topic). As ammonium iodide (NH_4I) is more stable under storage than ITMS, NH_4I can be reacted with MSTFA to generate fresh ITMS *in situ*. The anti-oxidant ethanethiol is included in the derivatization mixture to suppress the formation of iodine (Opfermann and Schanzer, 1997) from possible ITMS decomposition, that could otherwise add to the steroid nucleus. MSTFA:NH_4I:ethanethiol is usually used in a ratio of 1,000:2:3 (v/w/v), typically between 15 and 30 min at 60°C–80°C. Diethylsulphide is also produced during the derivatization reaction but with this short incubation time, any formation in turn of ethyl thio steroid artefacts is not significant (Van de Kerkhof et al., 2002).

Mass spectra of the TMS enol and TMS ethers of some anabolic steroids may show one characteristic ion. This can enhance sensitivity for screening purposes (e.g. 6β-hydroxy-methandienone), but it is of limited diagnostic value for confirmation. In such cases, for confirmation less powerful silylation conditions can be used (MSTFA:imidazole; 100:2, v/v) to discourage enol derivatives being formed, that is, only the hydroxyl groups undergo extensive silylation, thus facilitating the production of several characteristic ions in the source.

9.7.3 GC-MS

Screening is performed mainly by GC-MS, supplemented with GC-HRMS and/or GC-MS/MS, and most recently LC-MS/MS. With respect to gas chromatography, the TMS derivatives of anabolic steroids separate well on fused silica capillary columns (20–30 m), using cross-linked methylsilicone as the stationary phase and helium as the mobile phase. Rapid separation of components involves the use of oven temperature programming and detection with a low resolution quadrupole mass filter MS. The MS is operated in the SIM mode, up to four ions being monitored for the screening of each analyte (Donike et al., 1988; Masse et al., 1989b; Ayotte et al., 1996); examples are shown in Fig. 9.19. The limit of detection, and in some cases quantification, for most anabolic steroids can be as small as 1 μg/L (~100 pg on-column) using SIM GC/MS, but this will vary depending on the nature of the urinary matrix and the degree of fragmentation of respective analytes. The use of low resolution quadrupole mass filters, operating in SIM mode, therefore offers acceptable sensitivity at an economical price. A disadvantage of operating in SIM is that it will not detect unknown designer steroids differing in mass from ones identified for screening, even if these steroids are amenable to pertrimethylsilyltion. A complementary procedure is therefore required for a sample that does not fail the screen but nonetheless has an unusual steroid profile, most likely as evidenced by very small concentrations of natural urinary androgens, for example, T and E. Such a complementary procedure been suggested by Thevis et al. (2005) using LC triple quadrupole MS and data analysis based on common dissociation patterns of anabolic steroids (see LC-MS/MS section for more detail).

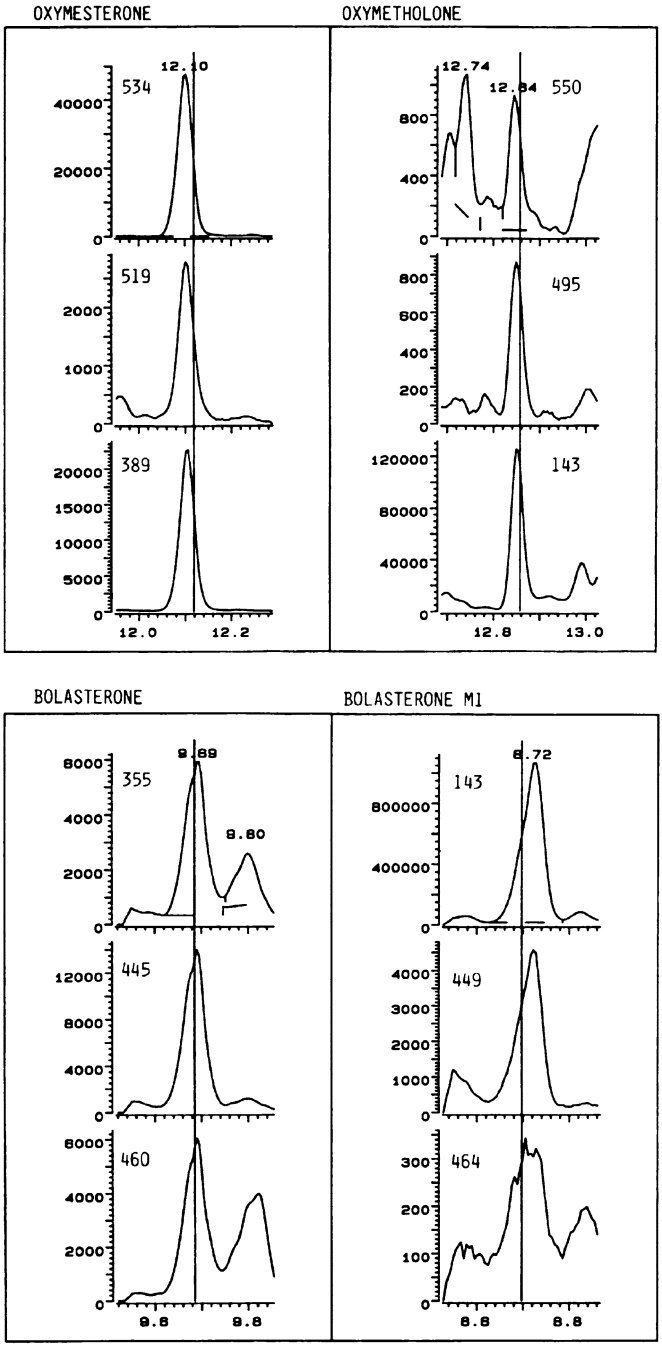


Fig. 9.19 Selected ion detection of the trimethylsilyl derivatives of oxymesterone, oxymethelone, bolasterone and bolasterone metabolite (M1) excreted in urine. In each 'mini' ion chromatogram, the abscissa is time (min) and the ordinate is abundance (arbitrary units) (From Fig. 5 of the article by Donike et al. with permission from the International Athletic Foundation, Copyright © 1988)

Although GC/MS continues to be the predominant analytical approach adopted by WADA-accredited laboratories, screening for anabolic steroids is supplemented by the application of more sophisticated MS, that is, HRMS (currently using magnetic sector instruments) or MS-MS (Bowers, 1997), and more recently still LC-MS/MS The use of GC-magnetic sector MS for screening of analytes is not a new concept; it has already been used for routine analysis of dioxins in the environment. In 1993, Horning and Donike (1994) showed the potential of HRMS for the screening of anabolic steroids, at moderate resolution in the SIM mode. Resolution in terms of MS may be defined as the ability to distinguish two ions with a small mass difference, that is, $M/\Delta M$, where M is the mass of the ion being detected and ΔM is the difference between that mass and an adjacent mass in a mass spectrum. Two peaks of similar intensity are considered to be resolved if the valley between the two peaks is equal to 10% when using magnetic sector instruments (and 50% when using quadrupoles). For example, a GC coupled to a magnetic sector intrument operating at a resolution of 5,000 can monitor an ion of mass m/z 500.0000 ± 0.0500 amu and thus distinguish it from a co-eluting chromatographic interferent with an m/z of 500.1000. An example showing the mass resolution required to separate a nandrolone metabolite from a vitamin E metabolite is given by Mueller et al. (1995).

The term screening by HRMS, as used within the sports community, is somewhat of a misnomer as these instruments are operated at moderate resolution (3,000–5,000) rather than truly high resolution, that is, a resolution >10,000. Nonetheless, this 'moderate' resolution is far superior to the unit mass resolution (±0.5 amu) obtained with quadrupole mass filters, thus offering considerable improvement in the limit of detection compared to quadrupole MS equipment, with identical sample preparation and GC conditions. Moderate resolution, rather than high resolution is chosen for screening purposes because a compromise has to be made between sufficiently eliminating other interfering ion signals compared to the ion mass of choice whilst maintaining an adequate signal for detection purposes.

In a head-to-head comparison, Kokkonen et al. (1999) concluded that GC/HRMS was 2–10 times more sensitive for detecting metabolites of methandienone compared to quadrupole GC/MS, the gain in detection sensitivity depending on the analyte (curiously, despite supposedly identical column conditions, different retention times on the SIM chromatograms are displayed in their paper between the two techniques). The superiority of GC/HRMS as a routine screening technique, especially for metabolites of stanozolol and methandienone, became apparent when there was a large increase in the number of positive samples identified by the IOC accredited laboratory in Cologne. In 1995, out of 6,700 samples tested by this laboratory, 116 positives were reported of which 41 were detected by quadrupole GC/MS, whereas an additional 75 were identified solely by their GC/HRMS screen (Schanzer et al., 1996; Horning and Schanzer, 1997). As a consequence of those findings, much greater emphasis is placed on the detection of certain anabolic agents at low concentration (minimum required performance limit [MRPL]), that is, the metabolites of the anabolic steroids, nandrolone, methandienone, 17α-methylT and stanozolol, together with the β2-agonist, clenbuterol. WADA-accredited laboratories target these specific analytes so that they can be detected at a urinary con-

centration as small as 1 ng/mL for a nandrolone metabolite (19-norandrosterone) and 2 ng/mL for the others. A corollary of a presumptive positive is that these laboratories must be capable of confirming the presence of these compounds at these low concentrations (NB 19-Norandrosterone is a 'threshold substance' and is reported at concentrations >2 ng/mL although the MRPL is 1 ng/mL).

For many WADA-accredited laboratories, the high cost of screening by HRMS, and the requirement of skilled operators to achieve the best results, currently precludes them from using such instruments. Such laboratories have chosen to use tandem GC-MS (MS/MS), as theoretically a similar enhancement in limit of detection might be expected by monitoring a selected production from collision-induced dissociation of a precursor ion. Tandem MS can be performed either in space using a sequential set of quadrupole rods or, as there are only five analytes to be targeted down to 2 ng/mL, in time using a quadrupole ion trap (QIT), the latter being a three-dimensional analog of the linear quadrupole mass filter. Currently, developments in LC-MS/MS provide approaches for the development of alternative effective screening procedures with the required sensitivity (see next section).

9.8 LC-MS Applications to Steroid Analysis in Sport

9.8.1 History

Since its introduction in the late 1970s, LC-MS has gone through various stages of development. Initial attempts used direct liquid introduction (DLI) and moving belt/wire interfaces, the latter interfaces providing the capability of generating both electron ionization (EI) and chemical ionisation (CI) data. One of the very early reports of the application of LC-MS to drug detection in sport used the moving belt interface (Houghton et al., 1981) to confirm the administration of synthetic corticosteroids to horses. The corticosteroids were isolated from urine by liquid/liquid extraction and the extracts purified by chromatography on Sephadex LH-20. The purified extracts were analyzed by LC-MS using normal phase chromatography and the moving belt interface. The mass spectrometer was operated in the negative ion CI mode. Ionization occurred through electron capture and significant fragmentation was observed primarily by loss of small neutral fragments such as water, ketene, methanol and hydrogen fluoride and different combinations of these.

Thermospray then aroused significant interest, providing a soft ionization technique that facilitated MS analysis of eluates from reverse phase systems (Blakley et al., 1980). With the development of commercial thermospray systems in 1983, the technique received widespread acceptance and was applied to a broad range of biological samples. The particle beam (PB) interface (MAGIC interface [monodisperse aerosol generation interface for chromatography]) and continuous flow fast atom bombardment (FAB) also provided approaches for the introduction of analytes into the mass spectrometer from LC systems.

As with the moving belt interface, particle beam provided the capability of generating both EI and CI data and this was used to advantage in the analysis of corticosteroids (Stanley et al., 1994) and nonsteroidal anti-inflammatory drugs (NSAIDS) (Stanley et al., 1995) in equine sports. Corticosteroids were extracted by immunoaffinity chromatography and analyzed by normal phase LC and PB-MS in the negative ion mode. PB negative ion spectra of 12 corticosteroids were obtained and the authors demonstrated the capability of the technique for the identification of corticosteroids in post administration samples. NSAIDs are the drugs most commonly occurring in reportable findings in equine sports and Stanley et al. (1995) reported the PB spectra in the EI, CI, and negative ion CI (NICI) mode for 41 drugs belonging to this class and the capability of PB-MS in confirming the identity of NSAIDs in urine extracts.

The commercial development of atmospheric pressure ionization (API) in the mid 1980s was undoubtedly, at that time, the most significant development in LC-MS and provided the analyst with expectations of a truly robust, practical and sensitive LC-MS interface. Previous workers had demonstrated the feasibility of ion evaporation from liquids (Dole et al., 1968; Horning et al., 1974; Iribarne and Thomson, 1976) and Whitehouse et al. (1985) described an electrospray source for mass spectrometers but interest in API was limited due to the lack of commercial instrumentation. The production by Sciex in the early/mid 1980s of a triple quadrupole instrument with an API source and the developments in ion spray technology (Bruins et al., 1987) rapidly changed all this. A number of groups demonstrated that the technique was robust, practical and sensitive with the ability to handle compounds of diverse polarity and molecular weight. In drug analysis the technique was rapidly accepted as the industry standard by the pharmaceutical industry in support of drug discovery and development and now API-LC-MS impinges upon many scientific disciplines. The early technical development of LC-MS have been reviewed by Covey et al (1986a), and complementary publications describing benefits of the technique to the toxicology laboratory arose around that period (e.g., Covey et al., 1986b; Bowers, 1989).

Acceptance of API-LC-MS in the field of drugs in sport was much slower than in the pharmaceutical industry primarily due to two reasons; the high degree of skill and expertise that existed in sporting laboratories in GC-MS, based around the robust, sensitive and low cost bench-top systems and, secondly, the comparative cost of these systems when compared to LC-MS systems. GC-MS was first applied as a confirmatory analysis method in sporting laboratories the mid 1970s and, as a result, significant skill was developed in this area and also, the technique evolved as the primary tool for metabolite identification in sports applications. This expertise in GC-MS applications to drug analysis also resulted in its widespread application as a generic screening procedure, initially for anabolic steroids and then more generally for basic and acidic drugs. However, LC-MS is now increasingly being used within sports laboratories to compliment GC-MS applications. Developments in API technology, MS/MS and data acquisition and data processing in the form of multiple reaction monitoring and data dependent scanning in specific time windows have

facilitated this transition. These processes allow for screening of multiple analytes in a single LC run at high sensitivity and specificity.

The ability of API-LC-MS in the analysis of compounds of diverse polarity has clearly been demonstrated in the steroid field through the analysis of the parent steroids and their Phase I and Phase II metabolites.

9.8.2 *Applications of API-LC-MS in Sport in the Analysis of Androgenic/Anabolic Steroids and their Phase I Metabolites*

Free Steroids and Aglycones Early applications of API-LC-MS to steroid analysis in sport addressed the comparative metabolism of methandrostenelone (methandienone) (Edlund et al., 1989a) and stanozolol (Muck and Henion, 1990) in man and the horse and the target analytes of choice for screening. Using a heated pneumatic nebulizer with corona discharge ionization on a triple quadrupole instrument and precursor and product ion scans Edlund et al. (1989a) identified 17-epimethandrostenolone as the analyte of choice for screening in the horse. Quantitative applications were developed using [17-methyl-$^{2}H_{3}$]-methandrostenolone as internal standard and the method detection limit was in the pg/mL range (Edlund et al., 1989b).

Muck and Henion (1990) used similar LC-MS conditions to study the metabolism of stanozolol in man and horse. Using collision induced dissociation (CID) of protonated molecular ion species $(M + H)^{+}$ and interpretation of the product ion spectra, the authors confirmed the presence of 3′-hydroxystanozolol and 4β-hydroxystanozolol in human urine following oral administration. Strong evidence was obtained for a number of other mono- and di-hydroxylated metabolites and the metabolic profile was in good agreement to that proposed by Schanzer et al. using GC-MS. Hydroxy metabolites of stanozolol were also detected in equine urine.

A more recent study has investigated the metabolism of stanozolol in the horse following intramuscular administration (McKinney et al., 2004). Using positive ion electrospray ionisation on an ion trap LC-MS, McKinney et al. showed the urinary excretion of stanozolol, 16β-hydroxystanozolol and two other monohydroxylated metabolite tentatively identified as 16α-hydroxystanozolol and 15-hydroxystanozolol. Stanozolol and its metabolites were extensively conjugated with both glucuronic acid and sulfate and the LC-MS method had a limit of detection around 100 pg/mL.

Leinonen et al. (2002) have compared three ionization techniques for the analysis of free (unconjugated) anabolic steroids, electrospray ionization, atmospheric pressure chemical ionization and atmospheric pressure photoionization. Oxandrolone, 6β-hydroxy-4-chlorodehydromethylT and 3′-hydroxystanozolol

were chosen as test compounds and overall, electrospray ionization showed the best applicability to the three steroids. The steroids could be detected at concentrations in human urine in the range 0.4–4.0 ng/mL. Leinonen et al. (2004) have extended this study to develop a method to screen for free 17α-alkyl substituted anabolic steroids by LC-MS. Using electrospray ionization and multiple reaction monitoring in the positive ion mode the method monitors nine different 17α-alkyl substituted anabolic steroids or their unconjugated metabolites in a single analysis with detection limits in the range 0.1–2.0 ng/mL. Similarly, ionization in the ion spray mode and multiple reaction monitoring have been used to detect natural androgens, T, epitestosterone, dehydroepiandrosterone, etiocholanolone and androsterone (Buiarelli et al., 2004). Leinonen et al. (2006) have recently investigated the applicability of in-vial two-phase liquid-phase microextraction (LPME) in porous hollow polypropylene for sample preparation of unconjugated anabolic steroids in urine.

An LC-MS/MS method has also been reported (Deventer et al., 2006) to screen for a limited number of aglycones of anabolic steroids in human sports. Following enzyme hydrolysis of human urine samples, the anabolic steroid residues were isolated by liquid/liquid extraction (diethyl ether). The extracts were analyzed by LC-MS/MS using an ion trap mass spectrometer and electrospray ionization. The limit of detection for the four compounds, tetrahydrogestrinone, gestrinone, 3′-hydroxystanozolol (major metabolite of stanozolol) and 17α-trenbolone (major metabolite of trenbolone), was between 1 and 10 ng/mL and the method was used to analyze (GC-MS) a positive urine sample known to contain 3′-hydroxystanozolol.

API-LC-MS with multiple reaction monitoring clearly provides a specific and sensitive approach to the detection of these parent anabolic steroids and some of their metabolites. The majority of steroids studied have keto functions at C-3 and/or C-17, hydroxyl functions at C-17 and one or more sites of unsaturation. These unsaturated hydroxyl/keto steroids tend to show a good response under API-LC-MS conditions as indicated by the sensitivities of the developed methods. However both in man and the horse, many of these steroids are extensively metabolized resulting in little or no parent drug excreted in urine. In some cases, for example, T and nandrolone (19-norT) in the horse, the major metabolites are saturated diols, isomers of 3,17-androstanediol and 3,17-estranediol (see Section 9.5.2.1), which show a very poor response under LC-MS conditions. Thus in these cases LC-MS may not be the ideal technique for control of abuse and GC-MS is the favored technique. However, in general, in drug detection in sport, GC-MS, and LC-MS are complementary.

Designer drugs and contaminated sports supplements have presented a threat to the integrity of sport over the past few years and both have received considerable press attention. The steroid, tetrahydrogestrinone (see Sections 9.3 and 9.5.2.5) was specifically designed to avoid detection by the GC-MS screening approach for anabolic steroids used in WADA accredited laboratories. This approach relies upon the formation of a single major component from the keto function by enolization

and silylation in the derivatization process prior to GC-MS analysis. The presence of the tri-ene-one system in tetrahydrogestrinone does not facilitate this derivatization process. WADA-accredited laboratories have therefore adopted LC-MS/MS for the screening of THG, as it is readily detected by this technique (Catlin et al., 2004). The metabolism in man of gestrinone, the precursor to tetrahydrogestrinone, has also been studied by LC-MS (Kim et al., 2000a, b).

Thevis et al. (2005) have also addressed the issue of the potential threat of designer steroids to the integrity of sport and developed an LC-MS/MS screening method for unknown synthetic steroids in human urine. Common structural features within steroids result in common dissociation pathways under collision induced dissociation conditions and the authors have selected common products ions to act as diagnostic markers for previously unidentified steroid drugs and metabolites. Nielen et al. (2006) have adopted a different approach to address this issue and used a combination of androgen bioassay detection and electrospray quadrupole time-of-flight mass spectometric identification. SPE extracts of enzyme hydrolyzed urine are chromatographed by gradient elution LC and directed to two identical 96 well fraction collectors by an effluent splitter. One well is analyzed by a robust reporter gene bioassay for androgen bioactivity and this directs the LC-MS (QTOF) identification activity to the corresponding well in the duplicate plate. The feasibility of the approach has been demonstrated by the screening of urine samples spiked with tetrahydrogestrinone.

For a number of years now there has been considerable publicity surrounding steroidal supplements (usually pro-hormones) and the contamination of non-steroidal nutritional supplements with these pro-hormones (Geyer et al., 2004). Several athletes with reportable findings for 19-norsteroids (nandrolone and its analogs) have cited contaminated supplements as the possible source of the drug. As a result several laboratories worldwide are developing supplement screening services to provide quality control programs for the supplement manufacturers to try to ensure the athlete is presented with contamination-free products. These assays tend to be GC-MS based but LC-MS assays have been reported for individual steroid supplements. Reilly and Crouch (2004), for example, have developed and validated a quantitative LC-MS/MS assay for 1,5α-androsten-3,17-dione, its metabolites and related endogenous hormones. A quantitative LC-MS assay has also been developed and validated for androst-4-ene-3,6,17-trione and its metabolites by Deventer et al. (2005). This is the main ingredient of the steroidal supplement, 6-OXO, which is sold as an aromatase inhibitor. Using the LC-MS method, 6α-hydroxyandrost-4-ene-3,17-dione was identified as a major metabolite and androst-4-ene-6α-ol-3,17β-diol-3-one was a minor metabolite.

Over the past few years a number of applications of LC-MS/MS to screen for anabolic steroids and a range of other drugs in equine sport have been reported. Yu et al. (2005) have developed a method for screening anabolic steroids in horse urine using positive ion electrospray and multiple reaction monitoring (MRM). Horse urine was fortified with 15 parent anabolic steroids at low ng/mL levels and all steroids were consistently detected. The method specificity, sensitivity, precision, and recovery were evaluated and the method was successfully used to analyze methenolone acetate post-administration urine samples.

The same group, Ho et al. (2006), have extended this approach to analyzse urine extracts for a number of anabolic steroids, corticosteroids, and acidic drugs. Urine samples were extracted using a strong cation exchange cartridge, C8-SCX. The acid/neutral fraction eluted from the cartridge was base washed and the organic extract, containing anabolic and corticosteroid residues, was analyzed by LC-MS in the positive ion electrospray mode using MRM. The approach allowed for the analysis of a total of 40 anabolic steroids and corticosteroids. The base wash was acidified (pH 6) and the acidic drugs isolated by liquid/liquid extraction. A second LC-MS analysis was performed and, for screening for acidic drugs, the mass spectrometer was operated in the negative ion electrospray mode again using MRM covering 50 acidic drugs.

LC-MS has also been applied to the analysis of anabolic/androgenic steroids in plasma. Guan et al. (2005) have developed a method for detection, quantification and confirmation of eight anabolic steroids (T, normethandrolone, nandrolone, boldenone, methandrostenolone, tetrahydrogestrinone, trenbolone, and stanozolol) in equine plasma using ESI in the positive ion mode. Limits of detection and quantification were in the range 25–50 pg/mL. Starcevic et al. (2003) have developed a quantitative LC-MS/MS method for T and deuterated T in human serum to support clinical research studies for the determination of pharmacokinetics, production rate and clearance of T following administration of deuterated T.

9.8.3 LC-MS Analysis of Intact Steroid Conjugates

LC-MS provides the opportunity for direct analysis of intact steroid conjugates, thus avoiding the time consuming enzymic hydrolysis processes and derivatization steps associated with GC-MS analysis of anabolic steroids. Moreover, glucuronidase extracts from *E. coli* would not cleave steroid sulfates and many such conjugates are not ideal substrates for sulphatase derived from *H. pomatia*.

Mass spectrometric confirmation of sulfate conjugation for the administered steroid, boldenone (1 dehydroT), in the horse was originally achieved through the off-line analysis of urinary extracts by fast atom bombardment (FAB) (Dumasia et al., 1983). With the development of API systems, Weidolf et al. (1988) brought the analysis on-line and developed a method for direct analysis of boldenone sulfate and related steroid sulfates in horse urine. The system used micro-bore LC interfaced to a Sciex TAG 6000E triple quadrupole mass spectrometer equipped with a home-built ion spray device. The authors demonstrated the separation of the sulfate conjugates of estrone, estradiol, nandrolone, boldenone, T and DHEA using the micro-bore LC system. Urinary extracts were analyzed in the SIM mode, by selected reaction monitoring (SRM) and in the full scan MS/MS mode. The limit of detection in the SIM mode was 10–12 pg on-column for all sulfate conjugates except DHEA. For SRM, the limit of detection ranged from 110–370 pg on-column and full scan product ion spectra required 5–10 ng of sulfate conjugates. The authors also demonstrated the capability of the system for trace level quantification. A similar approach has been adopted by the same group (Bean and Henion, 1997) for the direct determination of anabolic steroid conjugates in human urine by

LC-MS. The paper primarily addresses the determination of T and E glucuronic acid and sulfate conjugates and their quantification in human urine.

In contrast to man, the horse produces significant quantities of sulfate conjugates of both endogenous and administered steroids and Dumasia et al. (1996) have developed an LC-MS quantitative method for the quantification of T sulfate in horse urine. The urinary steroid sulfates were isolated from urine by solid phase extraction (C_{18} Cartridges) and eluted from the cartridge with methanol/water (7:3 v/v). The single quadrupole mass spectrometer was operated in the negative ion electrospray mode and calibration lines established over the range 0–400 ng/mL for T sulfate using 16,16.17α–$^{2}H_{3}$-T sulfate as internal marker, monitoring the $(M-H)^{-}$ ions *m/z* 367.3 and 370.3, respectively. The method was used to monitor the urinary excretion of T sulfate following administration of Durateston, a proprietary mix of T esters, to the horse. The authors demonstrated a good correlation between the T sulfate levels determined by LC-MS and T levels determined by GC-MS following hydrolysis of the conjugate.

Bowers and Sanaullah (1996) have developed a reverse phase chromatographic system to separate the glucuronic acid and sulfate conjugates of T and E and monitored the intact conjugates by ESI-MS. Positive ion MS and MS/MS were used to obtain structural information and quantitative data. Operation of the mass spectrometer in the negative ion mode gave better sensitivity but did not provide useful structural information. Misuse of T in human sports is monitored by the measurement of the T to E ratio and Borts and Bowers (2000) have investigated the direct measurement of the glucuronic acid and sulfate conjugates of T and E to address issues arising from unusual Phase II metabolism due to either genetic disposition (see Section 9.5.3.2) or attempts to avoid detection. Deuterated analogs of the four conjugates were used for quantification to overcome the effects of ion suppression in the analysis of extracts of complex biological matrices.

The detection of abuse of endogenous steroid hormones, for example, T, presents a significant challenge in the field of drug detection in sport and control is based upon urinary threshold concentrations of T in equine and a ratio of T to epitestosterone in man. Initially, only T presented a challenge to the sports industry but with improvements in analytical sensitivity, steroids originally regarded as "synthetic" for example, nandrolone, have now been demonstrated to be endogenous to some species. More recently, boldenone has been detected in normal urine from horses. Using LC-MS, Ho et al. (2004), have detected the sulfate conjugate of boldenone in the urine of male horses. The detection procedures involved solid-phase extraction, immunoaffinity column (IAC) purification, and then LC-MS-MS analysis on a Q-TOF instrument.

Kuuranne et al. (2000) have synthesized a number of steroid glucuronides for study by API to investigate optimization of LC-MS/MS conditions to distinguish between isomeric steroid glucuronides. Glucuronic acid conjugates of T, nandrolone, 3α-hydroxy-5α-estran-17-one, 3α-hydroxy-5β-estran-17-one, 17α-methyl-5α-androstane-3α,17β-diol and 17α-methyl-5β-androstane-3α,17β-diol were synthesized and glucuronic acid conjugates of other steroids, androsterone, E were available from other sources. The steroid glucuronides were studied under APCI and ESI condi-

tions in both the positive and negative ion modes. Positive ion ESI and APCI MS/MS provided diagnostic ions that allowed for distinction between the isomeric glucuronic acid conjugates of 19-norandrosterone and 19-noretiocholanolone and T and E. Extending the study to biological samples, this group, Kuuranne et al. (2003a) have investigated the use of liquid-phase microextraction (LPME) as a rapid, straightforward method to isolate steroid gluconides and provide extracts suitable for LC/MS/MS analysis. LPME was optimised for 13 glucuronides and compared with solid phase extraction and liquid/liquid extraction. The method was successfully applied to *in vitro* metabolic studies but, with urine samples, issues concerned with specificity were encountered.

In summary, the developments in LC-MS/MS are encouraging and the use of this technique is proven as a powerful aid to analysis for anabolic steroids, for example for the potential screening of designer steroids. Notwithstanding, currently there is no LC-MS/MS procedure that has been developed to date that can match that of GC-MS for the comprehensive screening of numerous anabolic steroids and their metabolites in urine. Primarily, this is because the chromatographic resolution of HPLC columns is far inferior. The development of ultra performance liquid chromatography-MS (UPLC-MS) may possibly help to address this issue, offering the potential advantage of a screening procedure that avoids time consuming extraction, hydrolysis and derivatization steps necessary for analysis by GC-MS.

9.9 Confirmatory Analysis

When a presumptive positive is found by screening, confirmatory analysis is performed. Paragraph 5.2.4.3 of the International Standard for Laboratories (Version 5.0) of the WADA Code states the objective of the confirmation procedure is to accumulate additional information to support an adverse analytical finding. A confirmation procedure shall have equal or greater selectivity/discrimination than the initial testing procedure. For example, this can be done by subjecting the sample to analysis by full scan MS and/or tandem MS. If the data are consistent with a positive finding, a second aliquot is taken from the 'A-sample', and the analytical procedure is repeated. When appropriate, the sample extraction procedure can be modified to obtain a cleaner and more concentrated extract, as can the derivatization procedure for characterization purposes when GC-MS is employed, although the latter is seldom necessary for confirmation of steroid identification.

Criteria for confirmation (whether by GC/MS or LC/MS/MS) are a concordance in retention time and scan data compared with those of reference urine, a minimum of three diagnostic ions being mandatory. The reference urine is prepared by spiking a reference material (standard) into negative (blank) urine that has been shown not to contain any substances of interest. When a reference material is not available for addition, the data must be compared with those of a reference collection sample, that is, elimination urine collected in a controlled study, with supporting documentation to that effect. The WADA Technical Document TD2003IDCR specifies that labora-

tories must establish criteria for identification of compounds. Examples of acceptable criteria are given for chromatography and mass spectrometry in Section 9.9.2.

A confirmatory procedure may also indicate a quantity of a prohibited substance greater than a threshold value and quantify the amount of prohibited substance in a sample. The majority of anabolic steroids are non-threshold substances, the exceptions being a 19-norandrosterone concentration >2 ng/mL (this threshold is adjusted when specific gravity exceeds 1.020), a T/E > 4, and a urinary epitestosterone >200 ng/mL to prevent manipulation of the T/E ratio. Where quantification is required, the method includes the measurement of standards and controls, and value reported needs to take the uncertainty of the measurement into account, particularly at the threshold value (which is addressed during the validation of the assay). As an adjunct, WADA accredited laboratories need to consider IRMS analysis for samples wih a T/E > 4, or where concentrations of androsterone or etiocholanolone exceed 10,000 ng/mL (equivalent to glucuronide) or dehydroepiandrosterone (equivalent to glucuronide) exceeds 100 ng/mL (see Section 9.10.5).

9.9.1 Chromatographic Separation

WADA quote the following criteria:

> For capillary gas chromatography, the retention time (RT) of the analyte shall not differ by more than one (1) percent or ±0.2 minutes (whichever is smaller) from that of the same substance in a spiked urine sample. Reference Collection sample, or Reference Material analyzed contemporaneously. In those cases where shifts in retention can be explained, for example by sample overload, the retention time criteria may be relaxed. For high performance liquid chromatography, the RT of the analyte shall not differ by more than two (2) percent or ±0.4 minutes (whichever is smaller) from that of the same substance in a spiked urine sample. Reference Collection sample, or Reference Material analyzed in the same analytical batch.

Matching the retention time to a reference standard is extremely important because some analytes can give identical spectra, for example, Goudreault and Masse (1990) found that the 17α-epimer of methenolone, a metabolite that is present in minute amounts, gives identical mass spectra to methenolone but a different retention time. The same is the case for most of the corresponding 17-epimers of 17α-methyl steroids (Schanzer et al., 1992), whether underivatized or TMS derivatized.

9.9.2 Mass Spectrometric Detection

The reader is referred to WADA technical document for examples of acceptable criteria specified for full scan mode and selected ion monitoring mode. A full or partial **scan** is the preferred approach to identification. A partial **scan** may begin at an *m*/*z* value greater than any abundant ion due to the derivatizing agent or chemical ionization reagent. WADA state the following:

> When a full or partial scan is acquired, all diagnostic ions with a relative abundance greater than 10% in the reference spectrum obtained from a positive control urine, a

Table 9.7 Maximum tolerance windows for relative ion intensities to ensure appropriate uncertainty in identification. The source is from Table 9.1 of the WADA Technical Document – TD2003IDCR

Relative abundance (% of base peak)	EI-GC/MS	CI-GC/MS; GC/MS^n; LC/MS; LC-MS^n
>50%	±10% (absolute)	±15% (absolute)
25–50%	±20% (relative)	±25% (relative)
<25%	±5% (absolute)	±10% (relative)

Reference Collection sample, or a Reference Material must be present in the spectrum of the unknown peak. In addition, the relative abundance of three diagnostic ions shall not differ by more than the amount shown in *Table* (*n.b. Table 9.7 in this chapter*) from the relative intensities of the same ions from that of a spiked urine, a Reference Collection sample, or a Reference Material. The relative abundance of the diagnostic ions may be obtained from single or averaged spectra or integration of peak areas of extracted ion profiles.

To help interpret the above, the reader is referred to the example of 19-norandrosterone, a metabolite of nandrolone, analyzed as the bis-TMS derivative. The simplest approach is to compare a full scan spectrum of a reference material with that from analysis of a urine sample where the height of the base peak (*m/z* 405) has been normalized, as in the example displayed in Fig. 9.20. The figure shows the spectra of the bis-TMS derivative of 19-norandrosterone from a reference standard spiked into urine against that of a sample collected from an athlete. The diagnostic ions are *m/z* 420 $[M]^+$, *m/z* 405 $[M–CH_3]^+$, *m/z* 315 $[M–CH_3–TMSOH]^+$, *m/z* 225 $[M–CH_3–TMSOH–TMSOH]^+$, where TMSO is the trimethylsilylether group. In addition, *m/z* 169 is interpreted as the D-ring fragment characteristic for C-17 TMS enol ether derivatives of anabolic steroids. Given the concordance in retention time between the 19-norandrosterone from the standard and the sample, and the presence of all of these ions, the next step is to compare relative abundances of three diagnostic ions. To this purpose, usually the molecular ion of *m/z* 420 is chosen together with the fragment ions *m/z* 405 (base peak) and *m/z* 315. Using the WADA criteria, the match between the two spectra is met, as demonstrated in Table 9.8.

With the acquisition of a full scan spectrum, there is decreased sensitivity compared to SIM, owing to the decreased time spent monitoring each particular ion. This can be only redressed in part by confirmation using a quadrupole ion trap, an instrument that not all laboratories employ. The concentration at which it becomes difficult to attain satisfactory full scan spectrum depends on the anabolic steroid targeted and the amount of biological interference from a particular urine sample. For 19-norandrosterone this is usually around 10 ng/mL with conventional sample work-up. Given that the threshold for adverse analytical findings for 19-norandrosterone is set at 2 ng/mL, what can be done to confirm a presumptive positive around, for example, 5 ng/mL? One way is to use SIM but to monitor a minimum of three diagnostic ions and examine whether the relative abundances of the diagnostic ions are within the maximum tolerance windows for relative ion intensities, as described above. The noise in the mini-ion chromatograms needs to be taken into account, as the signal to noise ratio for the least intense diagnostic ion must be greater than 3:1. Of course,

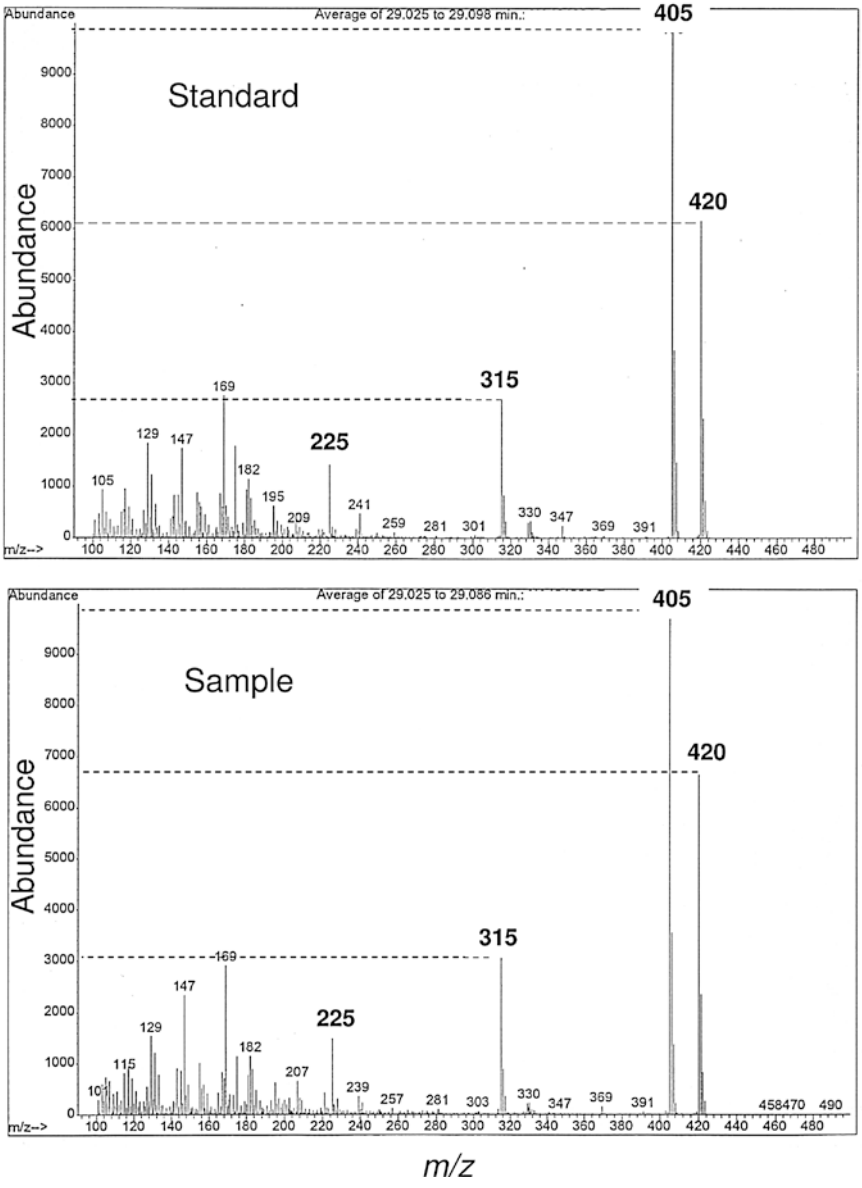


Fig. 9.20 The spectrum of the bis-TMS derivative of 19-norandrosterone from a reference standard spiked into urine compared with that from GC-MS analysis of a sample collected from an athlete

the possibility of interference in ion chromatograms due to the presence of a minor co-eluting compound becomes far greater as concentrations of 2 ng/mL are approached. The use of SIM ion ratios for confirmatory purposes of such samples is therefore limited in specificity and the alternative approach of tandem MS is preferred.

The WADA Technical document (TD2003IDCR) states that:

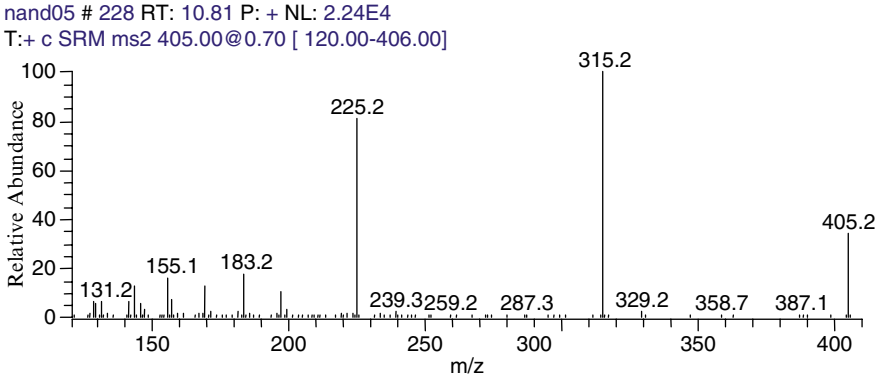
Table 9.8 Calculation of acceptance ranges based on the full scan spectra from Fig. 9.20

	Standard 50 ng/mL of 19-norandrosterone					Sample		
m/z	Relative height abundance (%)	Absolute height acceptance range (%)		Relative height acceptance range (%)		Relative height abundance (%)	Absolute height criteria	Relative height criteria
		From	To	From	To			
405	100.00	–	–	–	–	100.00	–	–
420	61.04[a]	51.04	71.04	N/A	N/A	67.62	PASS	N/A
315	28.23[b]	N/A	N/A	22.58	33.88	30.07	N/A	PASS

[a]In the spectrum from the standard, the relative height abundance for *m/z* 420 is 61.04%, which is greater than 50%, so the absolute height acceptance range must be calculated, this being 61.04% ± 15% = 46.04–76.04%. The abundance of *m/z* 420 in the spectrum corresponding to the sample is 67.62%, this being within the tolerance range, so the match is passed on absolute height criteria, that is, PASS.
[b]The relative height abundance for *m/z* 315 is 28.23%, which is between 25% and 50% of the base peak, so the relative height acceptance range must be calculated, this being ±20% **of** 28.23% = 28.23% ± 5.65% = 22.58–33.88%. The relative height abundance of *m/z* 315 in the sample is 30.07%, which is within the tolerance range, so the match is passed on relative height criteria, that is, PASS.
N/A, not applicable.

Tandem mass spectrometry data can be acquired in either the full scan or selected reaction monitoring (SRM) mode. The combination of mass selection of the precursor ion followed by a potentially unique collision-induced dissociation and mass selection or scanning of the product ion gives tandem mass spectrometry increased specificity. Collision conditions should be selected to ensure that the precursor ion is present in the MS/MS scan or SRM acquisition. In some cases, the combination of a single precursor-product ion pair may be sufficiently unique to be definitive. When monitoring one precursor ion to yield one product ion, the mass resolution of the first mass analyzer should be set to unity, When monitoring more than one product ion, the relative intensities of any of the ions shall not differ by more than the amount in Table *1* (*n.b. Table 9.7 in this chapter*) from the relative intensities of the same ions acquired from a spiked urine, Reference Collection sample, or Reference Material analyzed contemporaneously. The signal-to-noise of the least intense diagnostic ion must be greater than three-to-one (3:1). The relative abundance of a diagnostic ion shall preferably be determined from the peak area or height of integrated selected ion chromatograms. For a diagnostic ion with a relative abundance of less than 5% in the reference, the ion must be present in the unknown.

Standard:



Sample:

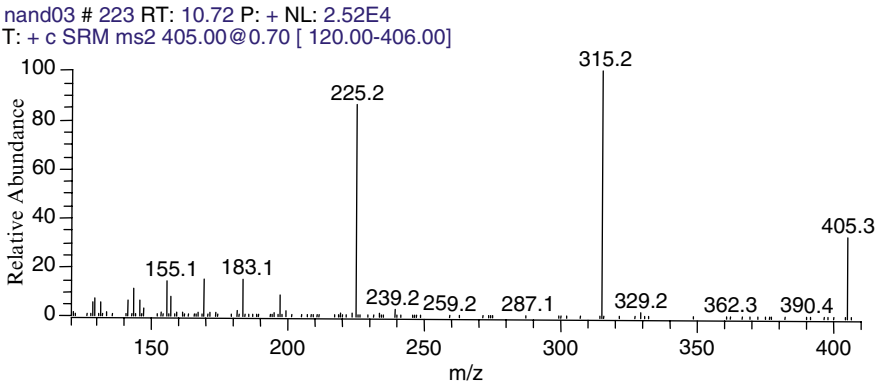


Fig. 9.21 Product ion spectrum (full scan) for a standard of 19-norandrosterone (5 ng/mL) and competitor's urine sample (6 ng/mL). The steroid was analyzed as the bis-TMS derivative

Table 9.9 Calculation of acceptance ranges based on product ion spectra from Fig. 9.21

	Standard 50 ng/mL of 19-norandrosterone					Sample		
m/z	Relative height abundance (%)	Absolute height acceptance range (%)		Relative height acceptance range (%)		Relative height abundance (%)	Absolute height criteria	Relative height criteria
		From	To	From	To			
315	100.00	–	–			100.00	–	–
225	83.30[a]	73.30	93.30	N/A	N/A	86.04	PASS	N/A
183	15.48[b]	10.48	20.48	N/A	N/A	14.80	PASS	N/A

[a]In the spectrum of the standard, the relative height abundance for *m/z* 225 is 83.30%, which is greater than 50%, so the absolute height acceptance range must be calculated, this being 83.30% ± 10% = 73.30–93.30%. The abundance of *m/z* 225 in the spectrum corresponding to the sample is 86.04%, this being within the tolerance range, so the match is passed on absolute height criteria, that is, PASS.

[b]The relative height abundance for *m/z* 183 is 15.48%, which is <25% of the base peak, so the absolute height acceptance range must be calculated, this being 15.48% ± 10% = 5.48–25.48%. The absolute height abundance of *m/z* 183 in the sample is 14.80%, which is within the tolerance range, so the match is passed on absolute height criteria, that is, PASS.

N/A, not applicable.

The following example shows the results of analysis of a urine sample containing 19-norandrosterone at a measured concentration of 6 ng/mL compared with Reference Material (standard spiked into 'blank' urine) at 5 ng/mL (Fig. 9.21). The precursor ion selected was *m*/*z* 405, as this is the most intense ion, and the product ions were acquired in full scan mode, clearly showing the precursor ion (*m*/*z* 405) and several diagnostic product ions (*m*/*z* 315, 225, 183). The relative ion intensities are calculated as before, and the data compared (Table 9.9).

9.10 Detection of Doping with 'Natural' Steroids

9.10.1 General Aspects

In the context of this section, the term 'natural' refers to steroids that are structurally identical to those produced endogenously, for example, T, DHT, DHEA, and androstenedione (Fig. 9.2). Other workers prefer the term 'endogenous' steroids, although it is a misnomer because the steroids administered are by definition from an exogenous source; hence, the term 'pseudo-endogenous' has been proposed as an alternative term.

In human sports, towards the end of the 1990s, public attention focused on the 'nandrolone problem' with the spate of adverse findings, particularly in high profile UK athletes and an inquiry was undertaken, the results of which were published and well-received within the sporting community (2003). Subsequently, a review on the significance of the metabolite, 19-norandrosterone, in athletes' urine samples has been published by Ayotte (2006). In the past, the presence of 19-norandrosterone in urine was interpreted as evidence of administration of nandrolone. Although the possibility was previously considered, only with the relatively recent application of GC/HRMS or extensive purification prior to analysis by quadrupole GC/MS, have trace amounts of 19-norandrosterone been regarded as naturally excreted by some men and non-pregnant women (see Section 9.10.2). Applying laboratory standard operating procedures, these trace amounts are far too small for full scan data to be of sufficient evidential quality to prove presence of this metabolite. Nonetheless, as the use of SIM and MRM (SRM) is permissible for confirmatory analysis and will be required for identification of trace amounts, it is important to consider the possible sources of 19-norandrosterone. In the adult female, and possibly in the male, a source could be from endogenous nandrolone production, and hence nandrolone is included in this section.

For detection of administration of natural steroids, analysis by isotope ratio mass spectrometry needs to be performed whenever possible, as stipulated by WADA, and final part of this section reviews this important approach.

9.10.2 Testosterone (T)

9.10.2.1 Detection of Administration of T by Athletes Based on the T/E Ratio

A test based on determining whether a urine concentration of T exceeds the upper limit of a reference range would be insensitive because of the wide variability in excretion associated with a single-pass urine collection. To overcome the problem, Brooks et al. (1979) introduced the concept of hormone ratio, the use of a ratio being considered to be independent of of urinary flow rates. The ratio of T to luteinizing hormone (T/LH) was originally proposed but this necessitated two separate assay procedures being performed and, in retrospect, immunoprocedures are generally accepted as not having the discriminatory power of mass-spectrometry for evidential analysis (Cowan and Kicman, 1997). In 1982, the test adopted by the IOC for detection of T administration was based on the GC/MS determination of the ratio of T to its 17α-epimer, epitestosterone (for structures, see Fig. 9.3), following glucuronide hydrolysis (Donike et al., 1983) (often referred to as the T/E ratio). The T/E decision limit was derived empirically from an observed distribution of measurements in specimens collected from a large number of individuals. In healthy men and women, the median T/E ratio approximates unity, but

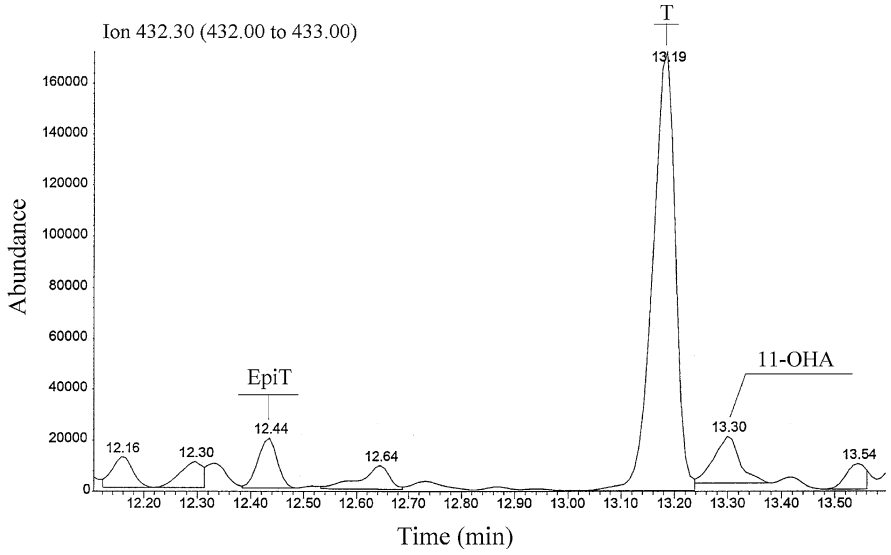


Fig. 9.22 Ion chromatogram (*m/z* 432) of the TMS enol-TMS ether derivatives of testosterone (T), epitestosterone (EpiT) in a urinary extract with a T/EpiT ratio >6. Good chromatography is important for the resolution of the adrenal steroid metabolite, 11β-hydroxyandrosterone (11-OHA), from T

supra-physiological doses of T cause an increase in the ratio as a result of increased excretion of T, the laboratory reporting threshold chosen being recently lowered by WADA from a T/E = 6 to a T/E = 4. An example of an ion chromatogram showing an augmented T/E ratio is displayed in Fig. 9.22. Large differences in T excretion between Asian and Caucasian men associated with an UGT2B17 polymorphism (see Section 9.5.3.2) may result in discordant T/E values following T administration, that is, with the same dose of T a Caucasian is more likely to exceed the urinary reporting threshold compared to an Asian man. In the future, this concern may be addressed, at least in part, by intra-individual T/E profiling (see Section 9.10.2.3).

9.10.2.2 Endogenous Sources of Epitestosterone in the Human

The T/E ratio may be augmented as a consequence of dose-dependent inhibition of testicular steroidogenesis. The testis secretes epitestosterone (as well as a proposed biosynthetic precursor, androst-5-ene-3β,17α-diol (Dehennin, 1993)), overall contributing to ~95% of the pool of urinary E glucuronide in eugonadal

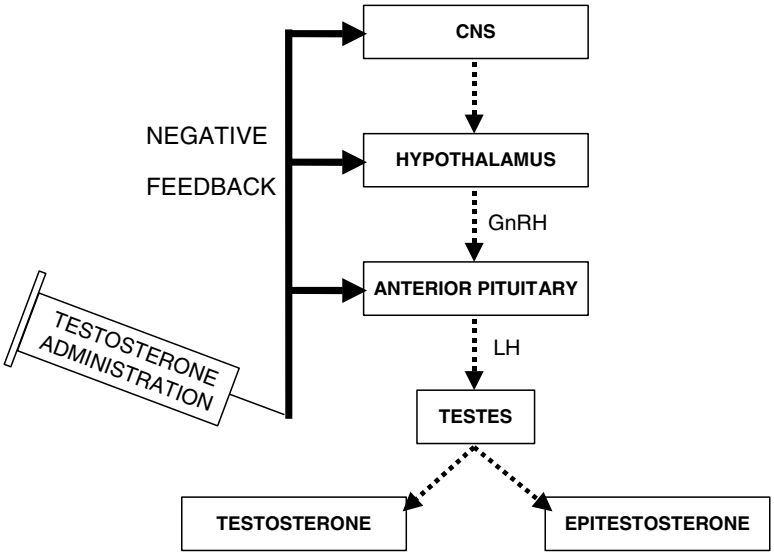


Fig. 9.23 Testosterone administered in supraphysiological doses causes disturbances to the hypogonadal-pituitary-gonadal axis. In the eugonadal male, the large negative feedback results in diminished gonadotropin releasing hormone (GnRH) and LH production, resulting in suppression of testicular steroidogenesis and hence epitestosterone production, In women (not using oral contraceptives) there is likely to be similar inhibition of epitestosterone production because of suppression of ovarian steroidogenesis

men (Kicman et al., 1999). When supraphysiological doses of T are taken, suppression of LH secretion decreases testicular production of E (Fig. 9.23) and the urinary excretion of epitestosterone glucuronide decreases (Dehennin and Matsumoto, 1993; Palonek et al., 1995; Anderson et al., 1997), for example, administration of 200 mg T enanthate weekly i.m. for 16 weeks decreases urinary epitestosterone to <10% of pretreatment values (Anderson et al., 1997). In the female, the regulation of epitestosterone is not known but, as epitestosterone has been identified in follicular fluid (Dehennin et al., 1987), some suppression of ovarian steroidogenesis of epitestosterone may occur following androgen administration. Adrenal stimulation can cause an increase in epitestosterone production but this appears to be countered in the male by a concomitant decrease in testicular steroidogenesis, probably as a result of induced hypercortisolaemia. Hence, stress-induced changes in adrenal and testicular steroidogenesis result in no change or only a small decrease in the urinary T/E ratio in eugonadal men (Kicman et al., 1999).

Only ~1% of T is excreted unchanged, apart from conjugation to glucuronic acid, compared with ~30% of EpiT. The administration of E with T simultaneously or sequentially enables an athlete to manipulate the test for T administration, if the test is based solely on the determination of the T/E ratio. Hence, an administration of these esterified steroids in a ratio of approximately 30 parts T to 1 part epitestosterone will result in a raised plasma T but an unremarkable T/E ratio, albeit that the T to LH ratio can be augmented (Kicman et al., 1990). Dehennin (1994) has proposed the determination of the concentration ratio of E (and also T) to 5-androstene-3β,17α-diol for the detection of combined T and E administration. Another approach that is used to help detect epitestosterone administration is to set a threshold for reporting for a urinary epitestosterone concentration (>200 ng/mL) but this strategy is unlikely to be of great practical value as urinary concentrations of steroids vary greatly depending on the degree of dilution of urine. The use of IRMS has been proposed for to detect co-administration of epitestosterone administration (see Section 9.10.5).

9.10.2.3 Further Investigations Following Detection of Augmented T/E Ratio

In the case of a T/E > 4, and any reliable method (e.g. IRMS – see Section 9.10.5) applied has not determined the exogenous source of the substance, further investigations may be conducted to ascertain whether a doping offence has occurred. Usually it is concluded that surreptitious T administration has happened, but occasionally the athlete may have a physiologically increased ratio (Oftebro, 1992; Kicman et al., 1993; Raynaud et al., 1993a, b; Oftebro et al., 1994; Garle et al., 1996), being a 'natural biological outlier'. In addition, the possibility of a pathological condition, for example, a T-secreting tumour accounting for an augmented ratio in a sports competitor must not be neglected,

although there is no such case report described in the scientific literature (possibly because such tumours are most likely to be of testicular origin and that these also secrete E).

With an adverse finding, investigating the T/E results from previous and subsequent tests, that is, assessing the T/E intra-individual (within-subject) variability, is useful in determining whether an offence has occurred. The application of T/E intra-individual profiling was first discussed by Donike et al. (1994). In a subsequent article (Donike et al., 1995a), the statistical test proposed by Harris (1975; Harris et al., 1980) was applied for biochemical analyses, to assess the suitability of using such data. This statistical test evaluates whether it is appropriate to use an intra-individual (subject-based) reference range, as opposed to an inter-individual (population-based) reference range, for assessing changes in biochemical status. However, to date, there are very limited data on intra-individual variation of T/E ratios presented in the peer-reviewed literature. In their article on detection of T and xenobiotics, Catlin et al. (1997) have reviewed the data on intra-individual variability. They present their criteria for determining whether T doping has occurred in men, based on T/E ratio data from drug-free males, who showed an intra-individual CV < 60% (variation from the collection of three or more samples of urine taken at monthly or greater intervals). In contrast, they report an example of a case of an athlete with an initial T/E ratio of 8.2, and after being sampled four times had a CV of 114%, indicating that T administration had occurred (Fig. 9.24). This pattern was considered to be typical of an individual who is caught and then discontinues T administration. In these authors' experience, most T users who provide three or more urine samples have a CV > 60%. However, those that have a CV < 60% and a T/E ratio between 6 and 10, are tentatively classified as 'naturally increased' (at the time of this publication by Catlin et al., the T/E threshold was 6), and Garle et al. (1996) have reported on a number of such cases that would fall within this classification. WADA in their Technical Document (TD2004EAAS) state that 'Normal

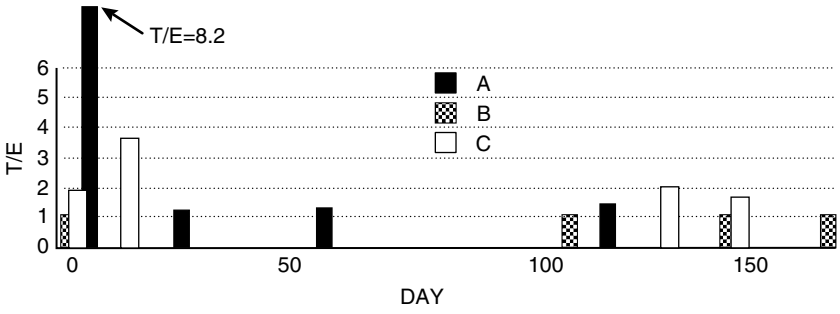


Fig. 9.24 Testosterone/epitestosterone (T/E) profiles of three athletes sampled four times over 170 days on relatively short notice (from Catlin et al., with permission from the American Association for Clinical Chemistry, Inc., copyright © 1997). The T/E of the first sample from athlete A was 8.2, followed by subsequent T/EpiTs of 1.2, 1.3, and 1.4 (mean 3.0, CV = 114%). The T/EpiTs from athlete B showed minimal variability (mean = 0.97, CV = 9.8%); athlete C revealed greater variability (mean = 2.3, CV = 40%) but gave ratios still within the laboratories norms for non-T-using controls

variation of up to 60% may be expected' and if 'using appropriate statistical evaluation is found to be significantly different, that will constitute a proof of the administration of a source of T'. In the event that previous T/E results are not available, three further unnounced tests should be carried out, preferably within a 3-month period following the report of the suspicious analytical result.

In addition to a CV > 60%, an abnormally large T/LH ratio accompanying a high T/E ratio in a urine sample collected from a man is supplementary evidence of T use (Garle et al., 1996), (in addition, there is some evidence to support that the T/LH ratio is a more sensitive retrospective marker of chronic administration of T than the T/E ratio (Perry et al., 1997). Urinary LH measurement between laboratories is yet to be standardized although some laboratories have determined their own assay reference range, and exercise-stress appears to attenuate rather than augment the ratio (Yap et al., 1996). In women, LH secretion can be considerably suppressed by hormonal contraception and therefore the determination of the T/LH ratio is not suitable.

9.10.2.4 Detection of T Administration in Equine Sport

At the time of initiation of studies to develop methods to detect the administration of anabolic steroids to horses in the mid 1970s, the proprietary veterinary anabolic steroids available were esters of T, 19-norT (nandrolone), boldenone and trenbolone; at this time only T was regarded as endogenous. For a number of years, control of misuse of T esters in female and castrated male horses was based upon a radioimmunoassay screening procedure and mass spectrometric identification of the major urinary metabolites, isomers of androstane-3,17-diol isolated from the glucuronic acid and sulfate conjugate group fractions and T also isolated from the sulfate fraction (Dumasia et al., 1986). The sensitivity of analytical techniques at the time was insufficient to detect the low urinary concentrations of endogenous T and its metabolites. However, with the improvement in sensitivity of GC-MS instrumentation and confirmation of the presence of T and its metabolites in urine samples from untreated female and castrated male horses, control shifted to absolute concentration thresholds, 0.02 μg free and conjugated T per millilitre in urine from geldings (castrated males), or 0.055 μg free and conjugated T per millilitre in urine from fillies and mares (unless in foal).

9.10.3 Nandrolone

9.10.3.1 In the Equine

Initial studies on the metabolism of nandrolone in the horse (Houghton, 1977) showed an isomer of estrane-3,17-diol to be a major urinary metabolite. Mass spectrometric identification of this metabolite in urinary extracts was used to confirm the administration of proprietary preparations of nandrolone to horses (Houghton et al., 1978); urine samples were screened for anabolic steroids by a radioimmunoassay procedure (Jondorf, 1977). These studies were performed on female and

castrated male horses. Subsequently, studies on male horses (Houghton et al., 1984) demonstrated the presence of traces of isomers of estrane-3,17-diol in extracts of urine from untreated male animals along with traces of the parent steroid, nandrolone and isomers of a second steroid–diol, 5(10)-estrene-3,17-diol. In relation to drugs in sport, this was the first indication of the possible endogenous nature of nandrolone and its related products.

The detection of major metabolite of nandrolone, 5α-estrane-3β,17α-diol, in extracts of normal urine of the male horse precluded its use in drug control for male horses and an alternative approach had to be adopted, a threshold value (Houghton et al., 1986b), the ratio of estrane-3,17-diol to 5(10)-estren-3,17-diol. Estrane-3,17-diol had been identified as the major metabolite of nandrolone, 5(10)-estren-3,17-diol was not a metabolite. Administration of nandrolone esters resulted in a marked increase in the urinary concentration of estrane-3,17-diol whereas urinary concentration of 5(10)-estren-3,17-diol, a steroid of testicular origin, was suppressed by administration of the anabolic steroid. Thus administration of nandrolone esters resulted in an increase in the ratio above normal levels (Houghton et al., 1986b). The current rules of racing define the threshold value as the mass of free and conjugated 5α-estrane-3β,17α-diol to the mass of free and conjugated 5(10)-estrene-3β,17α-diol in urine from male horses (other than geldings) at a ratio of 1. At the time of writing this threshold value is under review.

As a result of these findings, more detailed metabolism studies of nandrolone in the horse were carried out (Houghton and Dumasia, 1980; Dumasia and Houghton, 1984) and nandrolone was identified as a urinary excretion product in the form of the sulfate conjugate. Subsequent to this initial finding of nandrolone and related products in extracts of normal urine from the male horse, 19-nor neutral steroids have been detected in extracts of urine from several species, including pig (Debruyckere et al., 1990; Debruyckere and Van Peteghem, 1991), bovine (Vandenbroeck et al., 1991; De Brabander et al., 1994) and human (see next section). C_{18} neutral steroids have also been reported in follicular fluid from the horse (Short, 1960; Silberzahn et al., 1985), pig (Khalil and Walton, 1985) and human (Dehennin et al., 1987), and in testicular tissue from the horse (Dumasia et al., 1989) and pig (Ruokonen and Vihko, 1974).

The steroid profile studies on equine testicular tissue (Dumasia et al., 1989) showed the presence in the extracts of 19-norandrost-4-ene-3,17-dione, nandrolone and isomers of estrane-3,17-diol and 5(10)estrene-3,17-diol. The finding of these neutral 19-nor steroids in extracts of equine testicular tissue raised the question of the mechanism of their formation and as the male horse excretes significant amounts of conjugated estrogens, the female sex hormones, it was considered that the neutral 19-nor steroids were intermediates or by products of the aromatisation process. *In vitro* incubation studies with stallion testicular tissue and ^{2}H, ^{3}H and ^{14}C labeled steroids (dehydroepiandrosterone and androst-5-ene-3,17-diol) confirmed the conversion of the C_{19} steroid precursors to 19-hydroxylated products and 5(10)-estrene-3,17-diol and a mechanism for 19-demethylation without aromatization to form the C_{18} neutral steroids was proposed (Smith et al., 1987).

The analysis of urine samples from untreated male horses ('normal' samples) showed that, not only that nandrolone and T were detected in the sulfate conjugate group fraction, but also 19-norandrost-4-ene-3,17-dione. The finding of a 3,17-dione in the sulfate conjugate group fraction as opposed to the free steroid fraction was an interesting result that led to considerable debate as to a possible precursor, initial conclusions favored an "enol-sulfate". Attempts were made to clarify the situation by Houghton et al. (2007). Normal urine samples from the male horse were incubated at pH 3 and 37°C for 6 h. The "free" fraction, "aglycone" fraction and "solvolyzed sulfate fraction" were separated and steroids present analyzed and characterized. Nandrolone and 19-norandrost-4-ene-3,17-dione were now detected in the "free" fraction whereas T was still detected in the sulfate conjugate group fraction. This result demonstrated that (1) the precursors to nandrolone and 19-norandrost-4-ene-3,17-dione in normal urine from the male horse are labile to the incubation conditions (pH 3; 37°C; 6 h) where as T 17β-sulfate was not; (2) nandrolone detected in normal urine samples from the male horse following solvolysis is probably not derived from the 17β-sulfate and (3) the precursors to nandrolone and 19-norandrost-4-ene-3,17-dione in normal urine from the male horse prior to acid incubation steps have the same basic structure but differ in the functionality at C-17.

To identify the precursors in normal urine from the male horse that give rise to nandrolone and 19-norandrost-4-ene-3,17-dione following acid treatment, untreated urine from male horses, that is, urine that had not been subjected to any acid treatment or hydrolysis steps was crudely fractionated by HPLC to isolate the intact precursors (Houghton et al., 2007). The crude fraction was further purified by a second HPLC fractionation, and the fractions containing intact precursors were subjected to analysis by LC-MS (negative ion mode), LC-MS/MS (negative ion mode) and, following derivatization to form methyl/methoxime/trimethylsilyl derivative, analysis by GC-MS. Under LC-MS conditions, the major component in the purified extract had a base peak at *m/z* 631, assuming this to be the $(M–H)^-$ ion, this indicated a molecular weight of 632. Under MS/MS conditions the product ion spectrum showed fragments at *m/z* 587 (loss of 44 mass units), 315, and 271 (*m/z* 315–44 amu). This spectrum was interpreted (Houghton et al., 2007) as being consistent with a dimer of a compound molecular weight 316 which contained a carboxylic acid function and it was concluded that the major component in the purified urinary extract was the 19-carboxylic acid of androst-4-ene-3,17-dione (3,17-dioxo-4-androsten-19-oic acid) (Fig. 9.25).

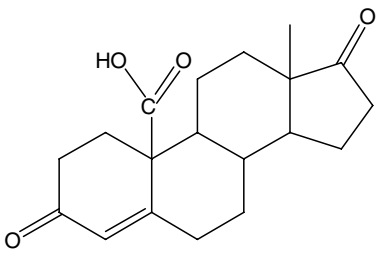


Fig. 9.25 Structure of 3, 17-dioxo-4-androsten-19-oic acid

This was confirmed by GC-MS analysis of a methyl ester/methoxime-derivatised extract of normal urine from the male horse which identified a major component with a mass spectrum corresponding to that published by Garrett et al. (1991) for the methylated bis-methoxime of the 19-carboxylic acid of androst-4-ene-3,17-dione (3,17-dioxo-4-androsten-19-oic acid). A second component was detected by GC-MS analysis of a methyl ester/methhoxime-derivatised extract, consistent with the 19-carboxylic acid of T (3-oxo-17-hydroxy-4-androsten-19-oic acid). These 19-carboxylic acids readily decarboxylate under acid conditions to produce nandrolone and 19-norandrost-4-ene-3,17-dione.

The results show that 19-norandrost-4-ene-3,17-dione and 19-norT detected in extracts of normal urine from the male horse can arise as artefacts by decarboxylation of their corresponding urinary 19-carboxylic acids as a result of acid hydrolysis (solvolysis) in the sample work-up procedure and that the two compounds may not be endogenous to the male horse. This result has interesting implication for control of misuse of proprietary anabolic preparations of nandrolone. Following administration of nandrolone esters to male horses, it has been confirmed that the parent steroid is excreted in urine as the 17β-sulfate conjugate. The studies by Houghton et al. (2007) have shown that in extracts of normal urine from the male horse, nandrolone is derived as an artefact by decarboxylation of the 19-oic acid of T. If this is the case and nandrolone-17β-sulfate is not present in "normal" urine, then the presence of nandrolone-17β-sulfate in urinary extracts provides confirmatory evidence for the administration of proprietary preparations of nandrolone to the male horse, and threshold value may no longer be necessary. Work is well advanced to develop a very sensitive LC-MS method to investigate the possible presence of trace levels of nandrolone 17β-sulfate in normal urine for the male horse (P. Grace et al., unpublished data).

9.10.3.2 In the Human

In the 1990s, preliminary reports by those working in then IOC (now WADA) accredited laboratories concluded that 19-norandrosterone was present in urine collected during pregnancy. This conclusion was based on observations of an increased SIM GC/MS signal (low resolution) and/or diagnostic product spectra following GC/MS/MS (de Boer et al., 1993; Mareck-Engelke et al., 1998; Van Eenoo et al., 1999a); and afterwards it was demonstrated that it is not uncommon for the concentration of 19-norandrosterone to exceed 5 ng/mL during pregnancy (Mareck-Engelke et al., 2002).

With the application of GC/HRMS for doping control it became apparent that small concentrations of 19-norandrosterone in urine may occur in untreated male and non-pregnant female subjects. Subsequently, three studies (Ciardi et al., 1999; Dehennin et al., 1999; Le Bizec et al., 1999) showed that the urinary concentration in untreated men is <0.6 ng/mL (two of the studies using quadrupole GC/MS following purification procedures). Full scan spectra were not presented, presumably because of the difficulty in analysing such small concentrations. Nonetheless, the study by Le Bizec et al. (1999), met the criteria for reporting low concentrations of

anabolic steroids by providing a minimum of three diagnostic ions (*m/z* 420, 405, 315; ions correspond to bis-TMS derivative of 19-norandrosterone). For these three studies the test population was small ($n = 47$ in total), not a representative racial mix and composed mainly of non-athletes. Further useful but unpublished data (to date) came from the 1996 Winter Olympics in Nagano, the preliminary results showing that of the 370 male competitors tested, only five showed urinary concentrations of 19-norandrosterone >0.1 ng/mL and no concentration exceeded 0.4 ng/mL; of the 251 female competitors tested none excreted more than 5 ng/mL (see communication from Dr. M. Ueki, Director of the WADA Accredited Laboratory in Japan, to the Expert Committee on Nandrolone, the committee being commissioned by UK Sport). Notably, it cannot be excluded that some competitors were using nandrolone, which would positively bias the data. These data support the minimum reporting concentration for WADA accredited laboratories of 2 ng/mL for 19-norandrosterone in urine from male subjects and 5 ng/mL in urine from the non-pregnant females when compared to control urine (but for women the threshold has been recently lowered – see next paragraph). Before declaring an adverse finding, WADA accredited laboratories must now determine the concentration of 19-norandrosterone when it is below 10 ng/mL and, for quantification purposes, calibrants are analyzed with deuterated internal standard, [$^2H_4$]-19-norandrosterone, so that sample values can be interpolated from a calibration plot. The estimated uncertainty must be considered when reporting. For urine samples with a specific gravity above 1.020 a correction to the threshold is made, as described in the WADA Technical Document TD2004NA; the measured concentration in the urine sample is *not* adjusted.

In 2004, the minimum reporting concentration for urine from female athletes was lowered by WADA from 5 to 2 ng/mL, that is, the same threshold as that for men. Given the above, it can be argued that the database potentially available from analysis of sports samples needs to be broadened, especially for women, and it is highly desirable that such data are brought into the public domain for peer-review. Furthermore, in untreated healthy men there is no direct evidence to date that nandrolone is produced endogenously but, even so, the urinary excretion rate of 19-norandrosterone increases under stimulation with human chorionic gonadotropin (Reznik et al., 2001). The increase was small, resulting in urinary nandrolone metabolite concentrations of <1 ng/mL as measured by GC/MS following hydrolysis with β-glucuronidase and extensive purification.

Le Bizec et al. (1999) and Saugy et al. (1999) have demonstrated that the urinary concentration of 19-norandrosterone may be increased after exercise; the study designs, however, were inappropriate to support the hypothesis that a concentration of 19-norandrosterone above the laboratory reporting threshold can arise as a result of endogenous production. Subsequent studies (all on male subjects) have shown that urine collected after exercise can have higher concentration of 19-norandrosterone (Robinson et al., 2001a; Le Bizec et al., 2002a) but this may be simply the result of dehydration rather than induction of nandrolone secretion (Schmitt et al., 2002). Le Bizec et al. (2002b) have provided preliminary results that 19-norandrosterone formed from nandrolone administration is exclusively conjugated to glucuronic acid whereas a proportion (~30%) of that produced from

an endogenous source is sulpho-conjugated. Although these results are potentially of interest, no data concerning possible variation in efficiency of hydrolysis of sulpho-conjugates and extraction are provided, the administered nandrolone was to a small number of volunteers, the dose was tiny (5 μg) and by an oral route only.

Grosse et al. (2005) have shown the *in situ* formation of 19-norsteroids by demethylation of certain C_{19} endogenous steroids in stored urine samples. The authors selected urines samples for the study from samples submitted for routine doping analysis, criteria for selection was that the concentration of 19-norandrosterone or 19-noretiocholanolone was greater than 0.5 ng/mL but less that the doping threshold (2 ng/mL). The samples were spiked with a mixture of $^2H_4$-androsterone, $^2H_5$-etiocholanolone and $^2H_4$-androsterone glucuronide. Following incubation at 37°C for 12 h the corresponding 19-nor steroids were formed as identified by high resolution mass spectrometry in the selected ion mode. 19-Noretiocholanolone (the 5β-isomer) was formed in concentrations threefold higher than 19-norandrosterone (the 5α-isomer) although the overall conversion did not exceed a relative amount of 0.1% of the initial C19 substrates. Compared with incubations at 23°C for 120 h, the studies at 37°C also resulted in a threefold higher concentration of the 19-nor steroids. *In situ* formation of the 19-nor steroids was not observed when incubations were performed with selected urine samples which did not show any detectable traces of 19-norandrosterone or 19-noretiocholanolone when analyzed post-competition. Where 19-nor steroids are detected in urine samples, the authors suggest that incubation with $^2H_4$-androsterone and/or $^2H_5$-etiocholanolone could contribute to a clarification of the potential for *in situ* formation, and this suggestion has been taken up by WADA accredited laboratories.

Analysis employing carbon isotope mass spectrometry, which can be used to distinguish between exogenous and endogenous sources of steroids in urine (see Section 9.10.5), has been applied to 19-norandrosterone by Mathurin et al. (2001) and Desroches et al. (2002), and more recently by Hebestreit et al. (2006). The latter group demonstrated that analysis of samples containing 19-norandrosterone near the chosen reporting concentration for WADA accredited laboratories can be successfully performed by extensive clean-up and concentration steps. The extraction and purification procedures incorporated solid phase extraction (C_{18}), enzymatic hydrolysis, liquid–liquid extraction and then importantly HPLC using normal (dimethylaminopropyl) and then reverse phase (C_{18}) columns. The starting volume of urine was 10 mL, the procedure concentrating 19-norandrosterone into 5 μL of methanol for splitless (3 μL) or cool on-column injection (2 μL). Analysis of samples containing 19-norandrosterone down to 2 ng/mL showed that IRMS analysis is feasible at low concentrations. The authors conclude that by applying IRMS, they can distinguish the origin of 19-norandrosterone in doping control samples, with respect to whether it is of exogenous or endogenous origin. The results are unexpected in that the authors state that the 'highest concentration for 19-norandrosterone of endogenous origin was 5.6 ng/mL', (which they consider to be an 'active urine' due to demethylation of androsterone; personal communication with Moritz Hebestreit).

Given that the reporting threshold is 2 ng/mL, these results and the authors' interpretation of their findings will, no doubt, be subjected to the utmost scientific scrutiny concordant with evidential analysis,

Nandrolone may be present in trace amounts contained within poor quality processed meat (from the injection site of animals illegally treated with nandrolone), and the athlete is advised to avoid such products; also, the offal from the boar (Le Bizec et al., 2000) and horse should likewise be avoided as these species producing significant amounts of nandrolone related compounds (Houghton et al., 2007). Apart from nandrolone, administered drugs that can be converted to 19-norandrosterone include preparations containing norethisterone, and so-called 'dietary supplements' that contain 19-norandrostenedione and/or 19-norandrostenediol. Norethisterone is a 19-nor progestogen used as a contraceptive in the female and for the treatment of menstrual dysfunction; a major metabolite is tetrahydronorethisterone but 19-norandrosterone is also excreted in small amounts. The presentation of cases where the urinary concentration of 19-norandrosterone exceeds 2 ng/mL, in the presence of tetrahydronorethisterone, are rare and are investigated on an individual basis to examine whether the finding is compatible with contraceptive medication.

The pharmacokinetic properties of nandrolone after i.m. administration of the drug as the phenylpropionate and decanoate ester, have been determined (Belkien et al., 1985; Wijnand et al., 1985; Minto et al., 1997). Formulations of esterified nandrolone are designed for depot activity, being slowly released from the arachis oil vehicle into the general circulation, where cleavage is performed by blood esterases. The mean absorption and elimination half-lives of nandrolone following i.m. injection of the decanoate ester are quoted in days (Belkien et al., 1985; Wijnand et al., 1985; Minto et al., 1997). The release rate of this esterified drug and the time of maximum concentration reached after administration varies depending on the injection site and the injection volume, for example, for a 100 mg dose in a 1 mL injection volume, a mean absorption half-life of 12.0 ± 0.9 days has been measured following administration into a deltoid site compared to 7.7 ± 0.6 days into a gluteal site. In contrast, after oral administration of the 'prohormone' steroids 19-norandrostenedione and 19-norandrostenediol, the major proportion of the dose will be converted by first- pass metabolism into inactive tetrahydro-17-oxo metabolites, conjugated with glucuronic acid, and then rapidly eliminated. Following a 50 mg dose of 19-norandrostenedione, urinary concentrations of 19-norandrosterone exceeded 100,000 ng/mL in the first urine voids (Uralets and Gillette, 1999) whereas, i.m administration of 50 mg of nandrolone decanoate resulted in concentrations of 250–750 ng/mL in the first week (Masse et al., 1985). Excessively large urinary concentrations of 19-norandrosterone are indicative of oral administration of a 'prohormone'. However, with smaller concentrations, for example, <100 ng/mL, it is impossible to distinguish the source, although 19-norandrosterone from metabolism of a precursor steroid is likely to have an apparent elimination half-life (in terms of urinary excretion rate) of only a few hours.

9.10.4 Detection of Administration of Other 'Natural' Androgens

9.10.4.1 5α-Dihydrotestosterone (DHT)

In an attempt to beat the tests for T administration, it was predicted some anabolic users would switch to administering DHT, as administration of this potent androgen would not perturb the T/E ratio (Southan et al., 1992). Subsequently, a number of samples collected at the 1994 Asian Games were found to have an abnormal urinary steroid profile, consistent with 5α-DHT administration, as declared by the accredited laboratory in Japan, and also due credit should be attributed to its director, Dr. Ueki, for his foresight as far back as the 1980s that DHT could be a drug of abuse. Tests developed to detect DHT administration in men are based on the urinary concentration of DHT/E, with secondary markers being that of the ratios of the DHT metabolite, 5α-androstane-3α,17β-diol, to that of E, 5β-androstane-3α,17β-diol and LH (Kicman et al., 1995; Donike et al., 1995b; Coutts et al., 1997) and a similar hormone ratio approach is used for women (Donike et al., 1995b). The ratio of androsterone to etiocholanolone is also augmented following administration (Donike et al., 1995b) but this appears to be a less sensitive marker than the other ratios selected (Coutts et al., 1997). No DHT findings were declared in 2005 and 2006, indicating that this steroid is now rarely, if at all misused, in sport but one cannot entirely dismiss an adverse finding by IRMS for androsterone being attributable to DHT administration.

9.10.4.2 Boldenone

Boldenone (1-dehydroT), as with nandrolone, was for many years regarded as a purely synthetic steroid. Metabolism studies for boldenone in horse (Dumasia et al., 1983; Dumasia and Houghton, 1988) showed that the major urinary metabolite was 17β-sulfate conjugate of parent steroid and, following solvolysis of the sulfate conjugate, GC-MS identification of boldenone in urinary extracts provided confirmatory evidence for the administration of boldenone to female, castrated male and male horses. However, again with the application of very sensitive mass spectrometric techniques, Dehennin et al. (2003) have reported the presence of boldenone in extracts of urine from untreated male horses. Urinary extracts were subjected to extensive purification prior to GC-MS analysis and a sensitive, quantitative GC-MS method was developed for both T and boldenone. The mean concentration of boldenone from a population of 156 male horses was 0.34 ng/mL (minimum 0.02, maximum 1.51 ng/mL). Boldenone and T concentrations were significantly correlated and *in vitro* production of boldenone was supported by an increase in boldenone and T excretion following testicular stimulation by administration of human chorionic gonadotrophin. Ho et al. (2004) have reported the direct observation of boldenone 17β-sulfate in urine form the male horse. Boldenone 17β-sulfate was isolated from urine by solid phase extraction and the extract subjected to immunoaffinity

chromatography prior to analysis by LC-MS/MS. Using these methods, boldenone was not detected in urine from untreated castrated male or female horses. To control misuse of boldenone or its esters in male horses, an absolute threshold concentration has been established – 0.015 μg free and conjugated boldenone per millilitre in urine from male horses (other than geldings).

As a corollary to the above, the presence and metabolism of boldenone in various animal species has been reviewed by De Brabander (2004).

In the human, there is a case report describing the finding of boldenone and two of its metabolites in the urine of a man who had not received boldenone. The extremely rare possibility that boldenone may be produced endogenously in the human and excreted in small but detectable amounts (low ng/mL) into urine cannot be discounted. Other possibilities may be environmental sources and/or that it is produced by microbial action. WADA have not stipulated a reporting threshold for boldenone but when an exogenous origin of the substance cannot be determined, a laboratory may conduct further investigations, including a review of previous tests or by conducting subsequent test(s). For example, if the longitudinal profile of the athlete who is subject to the subsequent tests is not 'physiologically normal', the result shall be reported as an adverse analytical finding. Presumably, if an athlete continues to produce urine containing trace amounts of boldenone, and the urinary steroid profile is otherwise unremarkable, then an interpretation is that doping has not occurred.

9.10.4.3 DHEA, Androstenedione and Androstenediol (Prohormones)

Detection methods based on abnormal urinary concentration of DHEA, androstenedione, androstenediol, or their metabolites may be successful during periods of administration. However, following cessation of administration, the decrease to basal concentrations will be rapid which means such tests will lack sensitivity in terms of retrospective detection. The application of carbon isotope ratio mass spectrometry may be useful for improving retrospective detection of administration (see next section).

With administration, changes in the T/E ratio may occur, but in men this is probably more due to hepatic metabolism of these prohormones to T glucuronide rather than an increase in circulating T. Results from preliminary studies, which have exclusively been performed with male volunteers, show that although the T/E ratio may be augmented with modest doses of DHEA or androstenedione, following administration there is a wide variability in response and the reporting threshold (at the time of these studies the threshold was a T/E = 6) is generally not exceeded. In the female, it would be expected that the T/E ratio would be a better marker of administration due to the large proportion of T produced from peripheral conversion of the administered prohormones (see Section 9.3.2.6).

For screening of DHEA administration a urinary concentration threshold of 300 μg/L of DHEA glucuronide has been proposed but, based on this threshold, a single replacement dose (50 mg) can be detected for only up to 8 h post-administration (Dehennin et al., 1998). This study, together with another where DHEA was

administered repeatedly (50 mg for 30 days; n = 7 men) (Bosy et al., 1998), reported a minimal effect on the T/E ratio. In contrast, Bowers (1999) found that one of four male subjects showed a dose-dependent increase in the T/E ratio with DHEA ingestion (50, 100 and 150 mg/day for 3 days), resulting in the T/E threshold being exceeded. In another small study (Uralets and Gillette, 2000), a single administration of 200 mg DHEA (n = 3 men) resulted in a T/E ratio > 6 in one subject being exceeded, but he had a relatively high basal T/E ratio of 4.

Lévesque and Ayotte have published their criteria for the detection of oral administration of androstenedione in the Proceedings of a Workshop (Levesque and Ayotte, 1999). The authors comment that the T/E ratio may be raised, but not systematically, the urinary concentrations of androsterone and etiocholanolone are increased to abnormal 'levels' and the presence of the characteristic metabolites 6β-hydroxyandrostenedione, 6β-hydroxyandrosterone and 6β-hydroxyetiocholanolone are also diagnostic, being present as glucuro- and sulpho conjugates (Goudreault et al., 2001). The authors' criteria may be useful for detecting presumptive positives but these criteria are unlikely to be sufficient to withstand the rigors of a strong legal challenge. All these metabolites are also produced naturally and hence the lack of decision limits for concentration of these analytes in urine (in cases where the T/E < 4) makes it difficult for the analyst to confirm an adverse finding. Van Eenoo et al. (1999b) describe in a preliminary report the results of statistical analysis of urinary concentrations of androstenedione and other endogenous steroids, in samples (n = 305) collected for doping analysis in Flanders. Using a non-parametric approach, the far-outside values for the concentration of androstenedione and ratio of androstenedione/E was 23 ng/mL and 1.2, respectively; Van Eenoo et al. regard the far-outside value as the decision limit in doping analysis (note: Laidler et al., 1994 used twice the far-outside value as a decision limit for hCG concentration in urine collected from sportsmen).

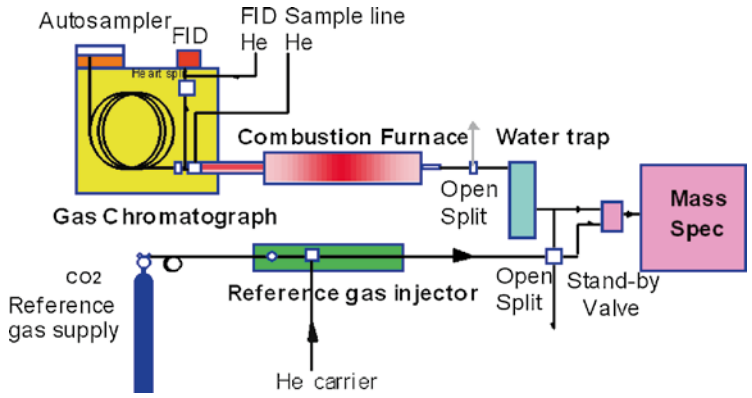


Fig. 9.26 Schematic of a GC-C-IRMS

9.10.5 *Detection of 'Natural' Androgen Administration by Determination of Carbon Isotope Ratio*

With GC/C/IRMS (Fig. 9.26), analytes from urine are separated on the GC column and are combusted catalytically to carbon dioxide and water in a furnace at high temperature. Steroids combust well, providing a uniform matrix for analysis, sample preparation requiring enzymic hydrolysis and steroid purification, prior to injection. A Nafion™ membrane or a cryogenic trap removes the water from the gas phase so that only carbon dioxide enters the mass spectrometer, between pulses of reference standard carbon dioxide obtained from a natural carbonate source and calibrated against an international standard. The three major ions monitored at *m/z* 44, 45, and 46 correspond to the isotopes $^{12}C^{16}O^{16}O$, $^{13}C^{16}O^{16}O$ and $^{12}C^{18}O^{16}O$, respectively. Measurements of ion currents at *m/z* 44 and 45 are chosen to calculate the ratio of ^{13}C to ^{12}C. The relative isotopic abundance of the analyte is then compared to the reference gas using a δ (delta) scale. As the difference from the reference gas is small, variations of the heavier isotope being of the order of 0.0001–0.05 atom % (Meier-Augenstein, 1999) values are usually expressed in parts per thousand using the 'delta per mil' (‰) notation:

$$\delta^{13}C\ \%_0 = \left[\frac{(^{13}C/^{12}C)_{sample} - (^{13}C/^{12}C)_{reference}}{(^{13}C/^{12}C)_{reference}} \right] \times 1000$$

The natural abundance of ^{13}C is ~1.11%, the human diet consisting of plant and animal sources, with varying ^{13}C isotope content relative to ^{12}C due to isotopic fractionation in biological systems. Endogenously produced steroids should thus

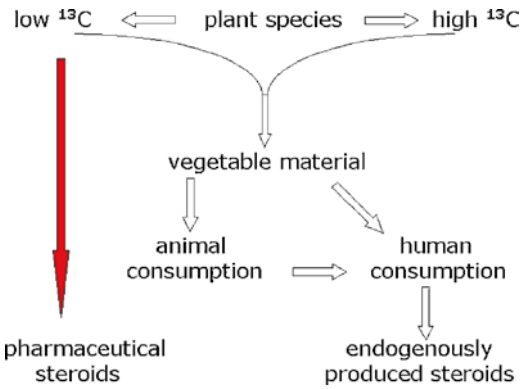


Fig. 9.27 Endogenously produced steroids have a $^{13}C/^{12}C$ content that reflects an average of that in the carbon sources ingested, whereas testosterone in pharmaceutical formulations are synthesized from soy, which has a smaller ^{13}C content

have a $^{13}C/^{12}C$ that reflects an average of that in the carbon sources ingested (Fig. 9.27), thus affecting the carbon isotopic content of the major source of cholesterol which is derived from acetate (acetyl coenzyme A), the cholesterol being then converted to steroids in the testis, ovary and adrenal glands. By contrast, T used in pharmaceutical formulations is generally chemically synthesized from stigmasterol in the soya-bean, which has a smaller ^{13}C content. Detection of misuse of T and related androgens can be therefore based on assessing whether there is a reduction in the relative carbon isotope content of targeted urinary steroids. The analytical probe used is gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS), a number of recent articles reporting its potential for detecting T administration in human sport (Becchi et al., 1994; Aguilera et al., 1996a, b, 1999, 2000, 2001; Horning et al., 1997, 1998; Machnik et al., 1997; Shackleton et al., 1997a; Ayotte et al., 2001; De la Torre et al., 2001b). Further, this technique may be useful to determine whether administration of E has occurred as a pharmacological manipulation to lower the T/E ratio in anticipation of testing (Aguilera et al., 2002)and also for the determination of doping with other androgens such as DHEA, androstenedione, and DHT (Shackleton et al., 1997b; Cawley et al., 2004, 2005).

WADA have adopted the IRMS approach as an important tool to aid the determination of natural androgen administration, as stipulated in their Technical Document (TD2004EAAS). This states that 'the isotopic ratios ($^{13}C/^{12}C$) of the relevant metabolites of such androgens should whenever possible be measured each time an elevated parameter of the steroid profile is estimated from the Screening Procedure or Confirmation Procedure and reported to the Testing Authority as having been determined. The results of the IRMS analysis and/or of the steroid profile measured by GC/MS shall be used to draw conclusions as to whether a doping violation may have been committed.' WADA recommend IRMS analysis on a urine sample with a T/E ratio greater than 4, or where concentrations (equivalent to glucuronide) of the following androgens exceed concentration limits (given in parentheses): T or E (200 ng/mL), DHEA (100 ng/mL), androsterone or etiocholanolone (10,000 ng/mL).

A 'problem' encountered with IRMS is its requirement for a larger mass of analyte injected compared to standard GC-MS. IRMS instruments require a minimum around 20–50 ng for end-point determination of the carbon isotope ratio of each steroid when injected onto a GC capillary column operating in splitless mode. For this reason, steroid metabolites are usually targeted for isotopic analysis because they are present in sufficiently large concentrations in the urine to be detected, unlike the parent compound that was administered. With respect to T, metabolism from an exogenous source appears to be similar to that of endogenous T (Fig. 9.28), where a large proportion of endogenous T being converted to androsterone and etiocholanolone and a smaller proportion to 5α- and the 5β-androstanediol (Brooks, 1975). Only a very minor proportion, about 1%, is excreted unchanged. Urinary concentrations of T and its metabolites produced endogenously are, in our experience, comparable to that found in many samples with WADA reportable T/E ratios (>4). The typical concentration of urinary T collected from a healthy adult male is approximately 40 ng/mL (Aguilera et al., 1999), whereas that of androsterone and

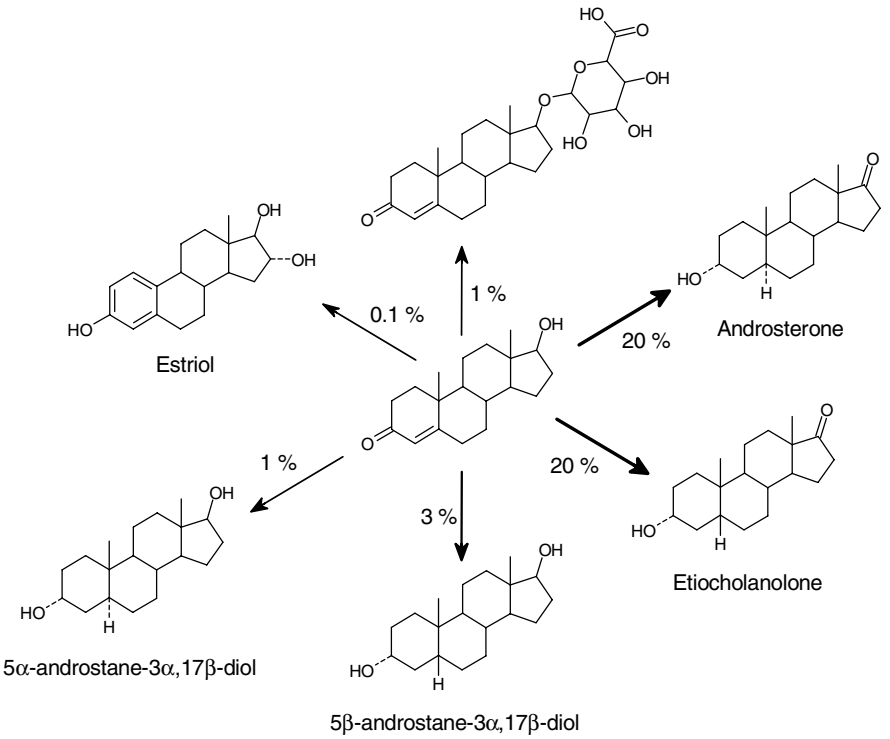


Fig. 9.28 Metabolism of endogenous testosterone, with figures attached to the arrows indicating the approximate proportion of testosterone which is metabolized by that route (Brooks, 1975). All steroids are excreted predominantly as conjugates but only the glucuronide conjugate of testosterone is shown for brevity

etiocholanolone are approximately 25- to 100-fold greater in proportion, each being present at 1,000–4,000 ng/mL (Aguilera et al., 2000).

The delta value of androsterone and etiocholanolone can be compared to an absolute cut-off value to assess whether doping with T has occurred.[3] An athlete will have failed a dope test if the IRMS values of androsterone and/or etiocholanolone in a sample are more negative than a chosen cut-off value, for example, WADA Technical Document states a value of −28%. Alternatively, a delta value that is

[3] The discriminatory value chosen will be influenced by whether the steroids are analyzed as the acetate derivatives to improve chromatographic separation or as underivatized steroids; if the former, the carbon isotopic content of the acetic anhydride used for derivatization must be considered as it will influence the isotopic content of the analytes. The addition of each acetate to the steroid will decrease the value by approximately −1 % but it will depend, of course, on the source of the acetic anhydride. As an aside, it is this reason why acetate (two carbons) is chosen over trimethylsilyl (three carbons) as a derivative, it being desirable to keep the 'isotopic influence' of the derivative on the steroid measurement to the minimum.

suspiciously negative but more positive than the chosen cut-off might require further samples collected on separate occasions also to be measured by IRMS to determine the 'usual' delta value for that individual. The problem with the approach of measuring androsterone and etiocholanolone is that the exogenous portion of these 17-oxosteroid metabolites is diluted by an endogenous source that is not suppressed with T administration. It is well recognized that the clinical assay used for urinary 17-oxo steroids was a poor index of androgenic status, since about two thirds originates from adrenal steroid metabolism (Bethune, 1975; Grant and Beastall, 1983). The adrenal steroids secreted that are metabolized to urinary androsterone and etiocholanolone glucuronides are DHEA sulfate and DHEA (which can be peripherally interconverted), and androstenedione. This adrenal contribution will attenuate the sensitivity of the IRMS test for androsterone.

Arguably, a better approach that avoids the need for multiple samples, and reduces the complication of dietary influence, is to compare the difference in IRMS ratio between a metabolite of the T and another steroid that would normally only arise from an endogenous source rather than an exogenous one. By comparison to an endogenous reference compound, each individual can act as their own marker, thus (at least in theory) eliminating between-individual differences in isotopic ratio of urinary steroids. The WADA Technical Document defines a difference of more than 3‰ as being significant. Originally, this approach was based on comparing the carbon isotope ratio of the combusted urinary metabolites of T, that is, 5α- and 5β-androstanediol, to that of a metabolite of pregnenolone, that is, a pregnanediol (5β-pregnane-3α,20α-diol) (Shackleton et al., 1997a; Aguilera et al., 1999) (Fig. 9.29). The average concentrations of 5β- and 5α-androstanediol in urine from adult men are relatively low compared to androsterone and etiocholanolone, being

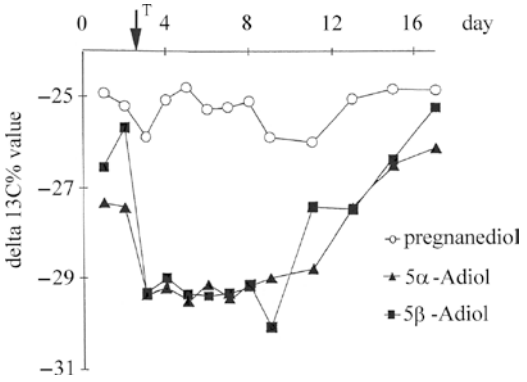


Fig. 9.29 Values of δ ^{13}C‰ of derivatised steroids (acetates) from a urinary extract of a subject before and after injection of 250 mg of testosterone heptanoate (oenanthate). The open circles represent pregnanediol, the closed rectangles 5β-androstanediol and closed triangles 5α-androstanediol (from Shackleton et al., with permission from Elsevier Science, copyright © 1997). Pregnanediol (5β-pregnane-3α,17β-diol) is a C_{21} metabolite of progesterone (a precursor of testosterone in the biosynthetic pathway) and the androstanediols are metabolites of testosterone (from an endogenous or exogenous source)

121 and 68 ng/mL, respectively. Even with raised excretion following T administration, these steroids are more difficult to analyze by IRMS unless the urinary sample volume for extraction is increased. The rather 'undesirably large' volume of 10 mL is typically taken for analysis of the androstanediols, for example, by (Aguilera et al., 1999) and (Maitre et al., 2004), compared with 2 mL of urine when androsterone is being targeted. Application of a programmable temperature vaporizer may be of benefit as it has been shown to improve the sensitivity of IRMS analysis by concentrating an underivatized metabolite of T (Mason et al., 1998). Rather than pregnanediol as the internal marker, Cawley et al. (2005) have advocated the determination of 11-ketoetiocholanolone as an endogenous reference compound.[4] It is well recognized amongst WADA accredited laboratories that there is frequently a co-eluting interferent with this 11-oxo steroid (Dr. A.T. Cawley, personal communication) but nonetheless this is a useful endogenous reference marker for comparison to androsterone because they are both C_{19} compounds.

A larger sample population is required to establish reference ranges, as well as standardization of measurements between laboratories. Also, it would be helpful to substantiate the extent that enzymes in the steroid biosynthetic and metabolic pathway differentiate between steroid substrates with different ^{13}C content, there being some evidence of kinetic isotopic fractionation in the human. Despite the above, GC/C/IRMS appears to be a useful tool in evidential analysis, as a complementary approach to proving T administration by the T/E ratio. It may also be a possible alternative in some case, as it is of interest to note that the WADA statistics for recent adverse findings show declarations for androsterone and etiocholanolone. These results indicates that the laboratories concerned found unusual steroid profiles, but could not prove the source of androsterone and etiocholanolone, and thus declared positive finding based on abnormal carbon isotope ratios of these metabolites.

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[4] The adrenal also secretes small amounts of 11β-hydroxyandrostenedione, which is metabolised to 11β-hydroxyandrosterone and 11β-hydroxyetiocholanolone, and the 11-oxo-equivalents. These C_{19} steroids are extremely useful endogenous markers for IRMS analysis as they are exclusively of adrenal origin.

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Chapter 10
Analysis of Bile Acids

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Abbreviations

APCI	atmospheric pressure chemical ionization
API	atmospheric pressure ionization
BSTFA	*N*,*O*-bis(trimethylsilyl)trifluoroacetamide
CA	cholic acid, 3α,7α,12α-trihydroxy-5β-cholanoic acid
CDCA	chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanoic acid
CI	chemical ionization
CID	collision-induced decomposition/dissociation
CRF	charge-remote fragmentation
CTX	cerebrotendinous xanthomatosis
Da	Dalton
DCA	deoxycholic acid, 3α,12α-dihydroxy-5β-cholanoic acid

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DEAP	diethylaminohydroxypropyl
DEHS	diethylhydrogensilyl
DHCA	3α,7α-dihydroxy-5β-cholestanoic acid
DMAE	dimethylaminoethyl
DMES	dimethylethylsilyl
ECD	electron capture detector
EI	electron impact ionization
EIA	enzyme immunoassay
ELSD	evaporative light scattering mass detector
ESI	electrospray ionization
ESI-keV-CID	ESI-CID under keV collision energy conditions
ESI-MS	electrospray ionization-mass spectrometry
FAB-MS	fast atom bombardment mass spectrometry
FID	flame ionization detector
G	glycine
GC-MS	gas chromatography-mass spectrometry
GC	gas–liquid chromatography
Glc	glucose
GlcA	glucuronic acid
GlcNAc	*N*-acetylglucosamine
G/T ratio	ratio of glycine conjugates to taurine conjugates
HMDS	hexamethyldisilazane
HSD	hydroxysteroid dehydrogenase
LC	liquid chromatography
LCA	lithocholic acid, 3α-hydroxy-5β-cholanoic acid
LC-MS	liquid chromatography-mass spectrometry
Me	methyl (ester)
MO	methyl oxime
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MU	methylene unit
NICI	negative ion chemical ionization
N/P ratio	ratio of negative/positive ions
OA	orthogonal acceleration
ODS	octadecylsilane
PHP	piperidinohydroxypropyl
PMS	phenazine methosulphate
RI	Kovats retention index
RIA	radioimmunoassay
SIM	single (selected) ion monitoring
SLO	Smith–Lemli–Opitz syndrome
SRM	single reaction monitoring
T	taurine
TBDMS	t-butyldimethylsilyl
TEAS	triethylamine sulphate
TEAP	triethylaminohydroxypropyl

THCA	3α,7α,12α-trihydroxy-5β-cholestanoic acid
TOF	time of flight
TSIM	trimethylsilylimidazole
TIC	total ion current
TMS	trimethylsilyl
UDCA	ursodeoxycholic acid, 3α,7β-dihydroxy-5β-cholanoic acid
UDP	uridine diphosphate
UPLC	ultra-performance liquid chromatography
UV	ultraviolet

10.1 Introduction

Bile acids constitute a large family of steroids in vertebrates, normally formed from cholesterol and carrying a carboxyl group in a side-chain of variable length. Bile alcohols, also formed from cholesterol, have similar structures as bile acids, except for the absence of a carboxyl group in the steroid skeleton. The conversion of cholesterol to bile acids and/or bile alcohols is of major importance for maintenance of cholesterol homeostasis, both from quantitative and regulatory points of view (Chiang, 2004; Kalaany and Mangelsdorf, 2006; Moore, Kato, Xie, et al., 2006; Scotti, Gilardi, Godio, et al., 2007). Appropriately conjugated bile acids and bile alcohols (also referred to as bile salts) are secreted in bile and serve vital functions in the absorption of lipids and lipid-soluble compounds (Hofmann, 2007). Reliable analytical methods are required for studies of the functions and pathophysiological importance of the variety of bile acids and bile alcohols present in living organisms. When combined with genetic and proteomic studies, analysis of these small molecules (in today's terminology: metabolomics, steroidomics, sterolomics, cholanoidomics, etc.) will lead to a deeper understanding of the integrated metabolic processes in lipid metabolism.

The literature on bile acid analysis is very extensive. One important reason for this situation is the desire to achieve greater simplicity and speed in specific applications. Methods that provide the most detailed information about bile acid composition are presently time-consuming and consequently outside the scope of most routine laboratories. Where simpler methods are described, they generally suffer from a number of limitations, which are often not pointed out or realized. Thus, claims of excellence, simplicity, versatility and speed are often exaggerated and reflect the limited requirements of the particular application. It is important to be aware of the potential complexity of bile acids in biological materials and to realize that knowledge about the structures of bile acids in the mixture to be analysed may be incomplete. This introduction provides a brief summary of chemical structures that have been reported to occur under different physiological and pathological conditions, in different species, at different anatomical sites and in different biological fluids. The choice of an analytical method should preferably be based on some knowledge of the anticipated structures and concentrations of the bile acids/bile alcohols in the samples to be analysed. While this may seem a simple enough task there are many examples of inappropriate analytical methods being used for the particular purpose, or on specific types of samples.

10.1.1 Structures of Naturally Occurring Bile Acids and Bile Alcohols

When bile acids and bile alcohols are formed, a number of transformations of the cholesterol skeleton take place (Russell and Setchell, 1992; Une and Hoshita, 1994; Russell, 2003; Norlin and Wikvall, 2007). The reactions in mammalian bile acid biosynthesis have been elucidated and it is probable that analogous reactions take place in the synthesis of bile alcohols in primitive species. Several of the enzymes involved have broad substrate specificities, so that the order of the reactions may not be fixed, and several biosynthetic sequences might operate simultaneously and in different subcellular compartments. Thus, the number of potential intermediates is large and these intermediates may also be released from their site of formation and appear in biological fluids in free, hydroxylated or conjugated forms. The terms bile acids and bile alcohols are sometimes difficult to demarcate but the intermediates in the biosynthesis of the compounds and the possible metabolites of the intermediates are included in our definitions. Because of their structural similarity, bile acids and bile alcohols can be analysed by similar or analogous methods. This is why bile alcohols are frequently mentioned in this chapter, which focuses on bile acid analysis.

The present view is that the two major pathways of bile acid synthesis are initiated by a hydroxylation of cholesterol, either in the endoplasmic reticulum at the C-7α position, or in the mitochondria at the C-27 position (Axelson and Sjövall, 1990; Javitt, 2000). The order of subsequent reactions is less clearly defined and there are probably marked species differences. The 7α-hydroxylation of cholesterol only occurs in hepatocytes while 27-hydroxylation occurs in many cell types and tissues, as does the subsequent 7α-hydroxylation of side-chain oxygenated substrates. Thus, bile acids may be formed in many tissues via pathways starting with 27-hydroxylation. The importance of the demonstrated extrahepatic formation of C_{27} bile acids is not clear (Zhang, Larsson and Sjövall, 1995; Zhang, Akwa, el-Etr, et al., 1997), but products and intermediates in this pathway could have roles as regulators of cellular metabolism.

For biosynthetic reasons the predominant naturally occurring bile acids and alcohols possess a 7α-hydroxy group, and have a 3α-hydroxy-5β(H) configuration (*normal*, A/B ring junction *cis*). There are three major types of side-chain at position C-17: (1) five carbon atom length ending with a carboxyl group; (2 and 3) eight carbon atom length ending with either a primary alcohol or a carboxyl group. Alan Hofmann has proposed the term “default cholanoids” for these three families (Hofmann, Schteingart and Hagey, 1995). This term reflects the common biosynthetic reactions and occurrence in bile but is chemically inconsistent as two of the families have a cholestane skeleton. The nomenclature of bile acids and bile alcohols in the biomedical literature is often confusing and inconsistent (Hofmann, Sjövall, Kurz, et al., 1992). Since the application of a strict systematic nomenclature (IUPAC) leads to awkward names for the biologist, guidelines for a semi-systematic nomenclature have been proposed (Hofmann, Sjövall, Kurz, et al., 1992).

Less common bile acids and bile alcohols have other configurations at C-3, C-5 and C-7 and carry side-chains other than those with five or eight carbon atoms.

Naturally occurring compounds with a 3β-hydroxyl or 7β-hydroxyl group may originate in the liver (or other tissues) or may be bacterial metabolites, and the same is true of the compounds having a 5α-configuration (*allo*, A/B ring junction is *trans*). Side-chains may vary in length from one to ten carbon atoms. Naturally occurring bile acids can have 1–10 carbon atoms while the side-chain of bile alcohols can vary from four to eight carbon atoms (Une and Hoshita, 1994). Norcholic acid is a naturally occurring bile acid in man (Almé, Bremmelgaard, Sjövall, et al., 1977) and is increased in patients with cerebrotendinous xanthomatosis where many nor- and bisnor bile acids are formed, probably from bile alcohols with hydroxyl group(s) in the side-chain (Kuramoto, Furukawa, Nishina, et al., 1990). The main metabolic origin of C_{20} acids (etianic (21-norpregnanoic) acids) and C_{21} acids (pregnanoic acids) is probably steroid hormones. However, appreciable amounts of such acids, carrying hydroxyl groups in the same positions as corticosterone metabolites (11β and 15β) in female rats, have been shown to be formed from plant sterols and cholesterol in the rat liver (Lund, Boberg, Byström, et al., 1991). Whether termed bile acids or not, these acids will be included in multicomponent analyses of bile acids in biological materials. In steroid metabolism, bacterial 16α-dehydroxylation leads to formation of metabolites with a 17α-oriented side-chain (Eriksson, Gustafsson and Sjövall, 1968). While bile acids and alcohols with this configuration have not been described in humans, 16α-hydroxylated bile acids have been found in several animal species (Haslewood, 1978; Hagey, 1992).

Bile acids and bile alcohols usually carry additional hydroxyl groups, mostly introduced by hepatic cytochromes P450, and also epimerized by oxidoreductions in the host, or by the action of enzymes from intestinal microflora. The third most common hydroxylation is at the C-12α position. The C_{24} bile acids rarely carry more than four hydroxyl groups, whereas the C_{27} bile alcohols can carry as many as seven hydroxyl groups. The following positions can be potentially hydroxylated in naturally occurring bile acids or bile alcohols: 1α, 1β, 2β, 3α, 3β, 4α, 4β, 5β, 6α, 6β, 7α, 7β, 12α, 12β, 15α, 15β, 16α, 19, 21, 22*R*, 23*R* and S, 24*R* and S, 25, 26, 27 (i.e. 25*R* and *S*) (for references see below and Haslewood, 1978; Hagey, 1992; Une and Hoshita, 1994). It is important to remember that both free and conjugated bile acids may undergo hydroxylation (Elliott, 1985). In the rat liver, the taurine conjugate is often the preferred substrate. The possibility exists that different conjugated forms of the same unconjugated bile acid have multiple metabolic origins due to involvement of separate hydroxylating enzymes for the different forms.

In the course of oxidoreductions, ketonic bile acids and bile alcohols are also formed. The most common positions for the oxo group are C-3, C-7, C-12, (and C-24 in bile alcohols). Bile acids and bile alcohols may also be unsaturated. The biosynthetic sequences lead to natural occurrences of compounds unsaturated at C-5,6 and C-4,5, but double bonds in positions C-1,2, (Wahlén, Egestad, Strandvik, et al., 1989; Kimura, Suzuki, Murai, et al., 1997; Kimura, Mahara, Inoue, et al., 1999), C-2,3, C-3,4 (Kelsey, Molina, Huang, et al., 1980; Robben, Janssen, Merckx, et al., 1989) C-6,7 and C-22,23 (Thompson, Davis and Morris, 1993; Setchell, Yamashita, Rodrigues, et al., 1995; Rodrigues, Kren, Steer, et al., 1996) have been reported for naturally occurring C_{24} bile acids and in positions C-23,24 and C-25,26 in bile acids and bile alcohols with a longer side-chain (Une and Hoshita, 1994).

The bile acids and bile alcohols undergo an enterohepatic circulation (Hofmann, 2007) in which the intestinal flora plays an important role in modifying the mixture and presenting the liver with metabolites which can be further modified by hydroxylations, reductions and conjugating reactions. The terms primary and secondary bile acids were introduced by Bergström, Danielsson and Samuelsson (1960) for bile acids originally formed from cholesterol in the liver and their bacterial metabolites, respectively. The term tertiary bile acid was then used occasionally for hepatic metabolites of the latter. With the present state of knowledge these terms, especially tertiary, should be avoided, because 7-dehydroxylation and oxidoreductions, originally thought to be specific to bacteria, may also occur in the liver. The metabolism of bile acids during the enterohepatic circulation results in an increased complexity of the naturally occurring bile acid mixture, which gets further complicated by conjugating and deconjugating reactions.

The bacterial changes of bile acid structures include oxidation of hydroxyl groups and reduction of oxo groups, often resulting in epimerization of the original hydroxyl groups. Bacterial 7α-dehydroxylation is a very important reaction. Other hydroxyl groups may also be removed, exemplified by the elimination of sulphuric acid from a 3-sulphate ester yielding saturated or unsaturated products (Kelsey, Molina, Huang, et al., 1980; Robben, Janssen, Merckx, et al., 1989). Bile alcohols inhibit bile acid 7-dehydroxylation (Lindqvist, Midtvedt, Skrede, et al., 1990) and are therefore not 7-dehydroxylated. Conjugated bile acids and bile alcohols can be hydrolysed by bacterial enzymes, and fatty acyl esters may be formed at C-3 during the microbial metabolism in the large intestine (Norman, 1964; Norman and Palmer, 1964; Kelsey, Molina, Huang, et al., 1980; Korpela, Fotsis and Adlercreutz, 1986). The findings of these old studies are often forgotten but they are important to consider in optimizing the design of analytical methods. Polydeoxycholic acid is another ester form of quantitative importance in faeces (Benson, Haskins, Eckers, et al., 1993).

The complexity of the bile acid profiles is increased in liver diseases when both the synthetic and metabolic pathways may be affected. Genetic enzyme deficiencies result in blocks of the normal biosynthetic pathways and introduction of compensatory pathways for metabolism of accumulating intermediates. (Setchell and O'Connell, 2007; Setchell, Bove and Heubi, 2008). The deficiencies can also be secondary to liver disease not primarily affecting bile acid formation but resulting in changes of enzyme activities and formation of abnormal bile acid structures.

10.1.2 Modes of Conjugation of Bile Acids and Bile Alcohols

Bile acids and bile alcohols, with the rare exception of patients with genetic defects in bile acid conjugation (Setchell, Heubi, O'Connell, et al., 1997; Carlton, Harris, Puffenberger, et al., 2003; Setchell and Heubi, 2006; Heubi, Setchell and Bove, 2007), are found in bile predominantly in the conjugated form. Bile alcohols are esterified with sulphuric acid in the side-chain and bile acids are amidated with glycine or taurine at the carboxyl group. The latter mode of conjugation is both

species- and structure-dependent. For example, C_{27} bile acids are conjugated with taurine (Hofmann, Schteingart and Hagey, 1995). In rare cases a taurine derivative (*N*-methyltaurine or cysteinolic acid) or β-alanine is the amidating moiety (Hofmann, Schteingart and Hagey, 1995). A glycyl-taurine conjugate has been found in the rabbit (Hagey, Schteingart, Rossi, et al., 1998). Conjugation with other amino acids has been reported but has not been confirmed by other studies (Yanagisawa, Akashi, Miyazaki, et al., 1984). However, a recent paper describes mass spectrometric identification of lithocholic acid covalently attached to lysine or arginine residues in a number of proteins from the liver of bile duct-ligated rats (Ikegawa, Yamamoto, Ito, et al., 2008). Bile acids conjugated with 2-fluoro-β-alanine are found in patients given fluoropyrimidine drugs (Zhang, Barnes and Diasio, 1991).

The first step of the amino-acid conjugation reaction is the conversion of the bile acids into coenzyme A (CoA) thioesters (Polokoff and Bell, 1977). Bile acid adenylates and CoA thioesters can be analysed by liquid chromatography (LC)/electrospray ionization (ESI) mass spectrometry (MS) (see Section 10.4.2). The bile acid-CoA thioesters are then converted into their glycine or taurine conjugates through the action of the bile acid-CoA/amino acid *N*-acyltransferase (Czuba and Vessey, 1980; Czuba and Vessey, 1982). Five common human bile acids (cholic (CA), chenodeoxycholic (CDCA), deoxycholic (DCA), lithocholic (LCA), and ursodeoxycholic (UDCA) acids) are good substrates for this enzyme, which shows similar activity for each of them. Because human hepatocytes contain relatively low levels of taurine, the ratio of the levels of the glycine-conjugated bile acids to those of the taurine-conjugated bile acids (G/T ratio) in human bile is usually around 3 (Sjövall, 1959). Administration of taurine (which increases taurine levels in hepatocytes), the presence of chronic liver disease, or a decrease in the total amount of bile acids results in a lower G/T ratio.

In addition to the side-chain conjugates, with important functions in bile formation and lipid absorption, a variety of other naturally occurring conjugates can be present. It is not always clear if their formation is fortuitous, due to broad substrate specificities of the enzymes involved, or if they have a functional meaning. Conjugation does not always have a detoxifying effect. Some glucuronides induce cholestasis, and sulphates may have a biological activity, as exemplified by petromyzonol sulphate and related derivatives which are pheromones (and some have antibacterial properties) in the sea lamprey (Sorensen, Fine, Dvornikovs, et al., 2005).

In 1971, Palmer and Bolt found a sulphate derivative of lithocholic acid in human bile that accounted for 40–75% of the total lithocholic acid (Palmer and Bolt, 1971). Bile acid sulphates also exist in serum and urine, and their levels significantly increase due to diseases of the hepatobiliary system, especially cholestasis (Stiehl, 1974). Sulphation of bile acids is catalysed by hydroxysteroid sulphotransferase (Chen, Bolt and Admirand, 1977; Radominska, Comer, Zimniak, et al., 1990), which transfers a sulphate group from 3′-phosphoadenosine 5′-phosphosulphate to a hydroxyl group on the bile acid. Although nonamidated, glycine-conjugated, and taurine-conjugated bile acids are all converted into the corresponding sulphate conjugates by this enzyme, amino-acid conjugates are better substrates than nonamidated bile acids. Therefore, many bile acid sulphates exist

as double conjugates, i.e. sulphates of glycine- or taurine-conjugated bile acids (Goto, Myint, Sato, et al., 2007). Sulphation makes bile acids more water-soluble and usually reduces their liver toxicity. Bile acid sulphates are easily excreted due to high renal clearance and low absorption efficiency in intestinal epidermal cells. This characteristic is important as a biological defense mechanism for the elimination of lipophilic bile acids. Although the hydroxyl group at C-3 is the most common position of sulphation, other positions may also be sulphated. For example, cholic acid 7α-sulphate is a major bile acid in the large intestine of the mouse (Parmentier, Mertens and Eyssen, 1975). Bile alcohols in lower species are sulphated at a hydroxyl group in the side-chain to provide the bile salt needed for lipid absorption (Haslewood, 1978; Une and Hoshita, 1994).

In 1974, Back et al. reported the existence of bile acids coupled with glucuronic acid in the urine of jaundiced patients (Back, Spaczynski and Gerok, 1974). The concentrations of these conjugates were lower in the urine and serum of healthy subjects compared with jaundiced ones. However, the concentration changes of bile acid glucuronides in diseases of the hepatobiliary system are smaller than those of bile acid sulphates. Glucuronidation of bile acids is catalysed by UDP-glucuronosyltransferases of the UGT2B subfamily (Matern, Matern, Schelzig, et al., 1980; Radominska, Little, Lester, et al., 1994). Based on analyses of urinary bile acid glucuronides, conjugation was originally found to be selective for a hydroxyl group at C-6 (Almé and Sjövall, 1980) and coupled with drug-induced increase of 6-hydroxylation (Wietholtz, Marschall, Sjövall, et al., 1996). However, glucuronidation can also occur at C-3 and at the carboxyl group of the side-chain. The extent and sites of glucuronidation depend on the length of the side-chain and the stereochemistry at C-3 and C-5 (Radominska-Pyrek, Zimniak, Chari, et al., 1986). Goto et al. reported that rat hepatic microsomal fractions mainly produce ester-type glucuronides conjugated via the C-24 carboxyl group (Goto, Murao, Nakada, et al., 1998); these chemically active carboxyl-linked glucuronides can react with amino groups on protein molecules to generate protein adducts (Ikegawa, Murao, Nagata, et al., 1999). Analyses of C-3-glucuronides (Ikegawa, Murao, Motoyama, et al., 1996) and C-24-glucuronides (Ikegawa, Okuyama, Oohashi, et al., 1999) in human urine showed similar levels of the two types, with only small differences between the concentrations in healthy subjects and patients with hepatobiliary disease. This might be due to an inhibition of the liver bile acid acyl glucuronosyltransferase by bile acids and their amino-acid conjugates and carboxyl-linked glucuronides (Mano, Nishimura, Narui, et al., 2002). In addition, bile acids in the liver inhibit the acyl glucuronidation of drugs, such as nonsteroidal antiinflammatory drugs, resulting in the production of protein adducts (Mano, Goto, Nikaido, et al., 2003).

Marschall et al. identified bile acid glucosides in the urine of healthy subjects and reported that the majority of these molecules were also amino-acid conjugates (Marschall, Egestad, Matern, et al., 1987). They also found that 7β-hydroxylated bile acids in urine were conjugated with *N*-acetylglucosamine (GlcNAc) (Marschall, Green, Egestad, et al., 1988; Marschall, Matern, Wietholtz, et al., 1992). Tandem mass spectrometric studies showed that the 7β-hydroxyl was the site of conjugation both *in vitro* and *in vivo* (Marschall, Griffiths, Götze, et al., 1994; Marschall,

Griffiths, Zhang, et al., 1994). This confirmed the finding by Niwa et al., who developed a method using high-performance liquid chromatography with fluorescence detection for the analysis of bile acid *N*-acetylglucosaminides (Niwa, Fujita, Goto, et al., 1992). Their chromatographic data showed that the site of GlcNAc conjugation of ursodeoxycholic acid in urine from a patient with primary biliary cirrhosis was the 7β-hydroxyl group (Niwa, Fujita, Goto, et al., 1993).

Recently, bile acid acyl galactosides were identified in the urine of healthy human subjects; this conjugation was specific for the 12α-hydroxylated cholic and deoxycholic acids (Goto, Shibata, Sasaki, et al., 2005). Interestingly, shark repellents isolated from certain species of fish and closely related to bile alcohols are conjugated with galactose (the mosesins) or *N*-acetylglucosamine (the pavoninins) at the 7α-hydroxy group (Williams and Gong, 2004). Finally, esterification of the 3-hydroxyl group of bile acids (especially the 3β isomers) with fatty acids may be regarded as a special form of conjugation occurring in the intestinal microflora (Norman, 1964; Norman and Palmer, 1964; Kelsey, Molina, Huang, et al., 1980). Analytical methods frequently neglect the quantitative importance of such nonpolar conjugates.

Conjugation may occur at different sites in the same molecule, resulting in double and occasionally triple conjugates. Combinations of aminoacyl amidation with sulphation, glucuronidation, glucosidation, or *N*-acetylglucosamidination are commonly observed for urinary bile acids. Disulphates and double conjugates with sulphuric and glucuronic acids also occur (Meng, Griffiths, Nazer, et al., 1997). Since conjugation and excretion are structure-dependent processes, large differences between bile acid profiles of different groups of conjugates are usually found (exemplified in Section 10.2; Fig. 10.1) (Meng, Reyes, Palma, et al., 1997). Genetic defects involving defective amidation of bile acids will also lead to major differences in the bile acid profiles, with unconjugated bile acids being found in abundance and sulphation and glucuronidation taking the place of the usual conjugation with amino acids (Setchell, Heubi, O'Connell, et al., 1997; Carlton, Harris, Puffenberger, et al., 2003; Setchell and Heubi, 2006; Heubi, Setchell and Bove, 2007). These differences will obviously influence the results of different analytical methods that may or may not account for all types of conjugates.

10.1.3 Occurrence of Bile Acids and Bile Alcohols

From the foregoing sections it is apparent that the bile acid/bile alcohol profiles of biological fluids or materials can be extremely complex. Under normal conditions the mixture of major components in an individual species does not show such a complexity. However, the possible presence of structurally unusual or minor components with known or unknown biological activities may be of pathophysiological importance, exemplified by ursodeoxycholic acid and 3-oxo-Δ^4 bile acids, and should not be forgotten while making the choice of an analytical method. A number of factors determining the composition of the bile acid/alcohol mixtures should be considered, including species, age, sex, state of health and other physiological

and experimental conditions. A recent widening of the bile acid field of great interest and analytical importance is the finding that 3-oxo-cholest-4-en-26-oic acid plays an important regulatory role as ligand for a nuclear receptor in the invertebrate *Caenorhabditis elegans* (Motola, Cummins, Rottiers, et al., 2006). Common bile acids like cholic and deoxycholic acids have also been shown to be formed from cholesterol in some marine microorganisms (Maneerat, Nitoda, Kanzaki, et al., 2005).

Comparative studies of structure and occurrence of bile acids and bile alcohols have been made for more than 100 years. Species differences were noted early and were systematically studied in particular by Haslewood and colleagues in London and by Shimizu, Kazuno, Hoshita and their colleagues in Japan. Differences in biliary bile acids and bile alcohols were suggested to provide information regarding evolutionary relationships between animal species and to reflect the evolution of the series of enzymes needed to convert cholesterol into the "modern" C_{24} bile acid conjugates. Hagey and Hofmann have continued this line of research and reviewed the field (Hagey, 1992; Hofmann, Schteingart and Hagey, 1995). Une and Hoshita (1994) have published a comprehensive review of the structure and occurrence of bile alcohols and higher and short-chain bile acids. It is obviously important to consider the bile acid/alcohol composition in the particular species to be analysed. As mentioned in Section 10.1.2, the mode and sites of conjugation are also species-dependent, e.g. sulphation in the mouse is at C-7 rather than at C-3 as in humans (Parmentier, Mertens and Eyssen, 1975). Sex differences in biliary bile acid profiles with regard to hydroxylation, stereochemistry of the steroid skeleton and conjugation patterns also occur as exemplified in the rat (Eyssen, Smets, Parmentier, et al., 1977; Eriksson, Taylor and Sjövall, 1978). It is important to consider such differences when selecting an analytical method for investigating the regulation of bile acid synthesis and metabolism.

From an analytical point of view, the most important sites of hydroxylation in mammalian C_{24} bile acids, besides 3α, 7α and 12α, are the carbon positions 1β, 2β, 3β, 4β, 5β, 6α, 6β, 7β, 22*S*, and 23*R*. The possibility of C-15- or C-16-hydroxylation should also be considered (Hagey, Schteingart, Ton-Nu, et al., 2002; Kakiyama, Iida, Goto, et al., 2006). Major biliary bile acids in the rat and mouse possess a 6β-hydroxyl group and in the pig a 6α-hydroxyl group. In the rat and mouse, these 6-hydroxy acids also occur as 22,23-unsaturated species (Thompson, Davis and Morris, 1993; Rodrigues, Kren, Steer, et al., 1996), resulting in a complex bile acid mixture in bile. In humans, bile acids with a hydroxyl group in one or several of the above positions are found, usually as minor components in healthy subjects, but in increased amounts in patients with hepatobiliary disease and patients treated with exogenous bile acids. Furthermore, because of differences in metabolic origin, modes of conjugation and hepatic and renal handling, the less common bile acids may be enriched in certain compartments or excreta. The forms in which different bile acids occur in different organs and fluids are often not well defined, influencing the reliability of analytical procedures. The situation for bile alcohols is similarly complex.

When enzymes involved in the biosynthesis of bile acids are deficient for genetic or other reasons, very complex mixtures of intermediates and metabolites of intermediates accumulate, which can occur in different forms of conjugation. As an

example, when testing an analytical procedure based on group separation followed by LC-MS/MS, about 150 different conjugated bile acids and bile alcohols were detected in urine from an infant with an acquired deficiency of 3-oxo-Δ^4-steroid 5β-reductase (Yang, Griffiths, Nazer, et al., 1997). An electrospray mass spectrum of the same sample only showed about 20 significant peaks.

There also occur age-dependent differences in bile acid composition. The bile acid composition in humans shows significant differences between the foetal (Colombo, Zuliani, Ronchi, et al., 1987; Setchell, Dumaswala, Colombo, et al., 1988), neonatal, and adult periods, both with regard to type of conjugation and extent of hydroxylations. Bile acids hydroxylated in the 1β, 2β, 4β, or 6α-positions are important constituents in meconium, are also present in urine excreted in the neonatal period (Clayton, Muller and Lawson, 1982; Elliott, 1985; Back, 1988; Dumaswala, Setchell, Zimmer-Nechemias, et al., 1989), but are absent or present at very low levels in healthy adults (Almé, Bremmelgaard, Sjövall, et al., 1977). The bile acids in foetal bile of pigs change during the progress of gestation (Kuramoto, Miyamoto, Konishi, et al., 2000). Age-dependent differences in rabbits and bovids are probably connected to the development of the intestinal microflora (Hagey, Gavrilkina and Hofmann, 1997; Hagey, Schteingart, Rossi, et al., 1998). In humans, establishment of the intestinal microflora after birth also leads to dynamic changes in bile acid composition over the first year of life, particularly with increased formation of bacterial metabolites of the primary bile acids (Setchell, Street and Sjövall, 1988).

10.2 Sample Preparation Procedures

10.2.1 Extraction Procedures

Most analytical methods, at least those that aim at multicomponent analysis and reliable specificity in the presence of a complex matrix, require a sample pre-treatment. With the development of electrospray ionization mass spectrometry, the sensitivity of measurements makes it possible to simplify the sample preparation but an extraction step is needed for tissue samples and usually for liquid samples when it improves the specificity of the measurements and widens the dynamic range of the analysis.

Previous reviews have covered different aspects of the extraction of bile acids and bile alcohols from biological fluids and tissues in detail (Sjövall and Setchell, 1988). This section will discuss a few points of relevance for non-destructive and hopefully quantitative yields of known steroid structures. Losses of presently unknown forms of the compounds cannot be excluded by any method. In general, it is difficult to evaluate extraction recoveries. Internal standards added to biological fluids or tissue homogenates may not equilibrate with the endogenous compounds (Setchell, Lawson, Tanida, et al., 1983). *In vivo* labelling of compounds to be measured is sometimes possible and when a radioactive standard has been equilibrated

with the pool of endogenous compound, its recovery should give the most reliable evaluation of extraction recovery. Such studies were formerly made, for example, with faeces (Hellström and Sjövall, 1962), and later by Hedenborg et al. with muscle, fat and skin (Hedenborg, Norlander and Norman, 1986). In most multicomponent analyses only one or a few representative compounds are added as internal standards and recoveries of the other components of the mixture are assumed to be the same. One should keep in mind that this assumption might not be correct.

The collection of samples for analysis constitutes another potential source of errors. For example, the excretion of bile acids in faeces is very irregular and bile acids are not even equally distributed in single stools from the same defecation (Setchell, Ives, Cashmore, et al., 1987). Representative values for daily faecal bile acid excretion can only be obtained by analysis of aliquots of thoroughly homogenized 4–5-day collections of faeces.

10.2.1.1 Solvent Extraction

In most cases extraction with ethanol should give satisfactory recoveries of bile acids and alcohols from tissues and fluids. Addition of 0.1% ammonium hydroxide or sodium hydroxide to pH 11 has been recommended to further decrease protein binding but this might cleave labile conjugates. Quantitative yields were obtained in extractions of rat liver with ethanol in the absence of ammonium or sodium hydroxide (determined by *in vivo* labelling; Masui and Sjövall, unpublished results, 1987). The ratio of solvent/sample should be kept high (10:1–20:1) and biological fluids should be added drop by drop to the ethanol volume held in an ultrasonic bath to obtain maximal contact surface and distribution in the solvent. Tissue homogenates may be extracted first with ethanol (held in an ultrasonic bath) and then with chloroform (or methylene chloride)/methanol at 1:1 (v/v) when fatty acid ester derivatives of bile acids are suspected to be present or lipids are suspected to trap compounds of interest. However, the high sensitivity of present analytical methods permits analysis of very small sample sizes so that pure ethanol in sufficient excess is likely to give quantitative yields of most bile acid derivatives. Since solvent volumes are still small in this case, contamination problems are minimized.

Extraction with methanol is not recommended both because of poor extraction efficiency and possible formation of artefacts. Thus, 3-oxo-5β-cholanoic acids in methanol were converted to 3-dimethyl ketals at pH below about 6.4 (Fantin, Fogagnolo, Medici, et al., 1992).

Tissues may contain non-covalent protein-bile acid complexes that have to be dissociated for quantitative bile acid extraction. In a recent study of bile acids in brain, the cytosolic fraction required the presence of 7.3 M guanidine hydrochloride in Tris-HCl buffer, pH 8.6, with dropwise addition to a sevenfold excess of ethanol to extract the major bile acid, chenodeoxycholic acid (Mano, Goto, Uchida, et al., 2004). Pure ethanol did not extract this bile acid. The effect of the guanidine hydrochloride may be analogous to that of triethylamine sulphate in solid-phase extractions of plasma (see below).

Rat liver bile acids have also been quantitatively (determined by [^{14}C]-labelling *in vivo*) extracted by homogenization of rat liver in a micellar solution of decyltrimethylammonium bromide. Following centrifugation, the supernatant is diluted to give submicellar levels of the detergent and the solubilized bile acids are extracted as ion pairs and purified by solid phase methods (a combination of Lipidex 1000 and ODS-silica; see below) (Masui, Egestad and Sjövall, 1988). This method avoids use of organic solvents and should be further studied for use in combination with electrospray mass spectrometry. The behaviour of polar double conjugates in this system is not known.

10.2.1.2 Solid Phase Extractions

At present, the most commonly used methods for extraction of bile acids and bile alcohols from biological fluids are based on solid-phase sorbtion. The procedures and their problems have been discussed in Sjövall and Setchell (1988). This extraction has several objectives: to remove inorganic salts, macromolecules and small polar metabolites. It can also serve to remove nonpolar lipids. The method first described utilized an Amberlite A-26 anion exchanger, then the presumed neutral polymer Amberlite XAD-2 was introduced, followed about 10 years later by octadecylsilane-bonded (ODS) silica. It is important to remember that multiple mechanisms are responsible for the sorption process and that the chemical properties of a sorbent may be unexpected (e.g. presence of low levels of ion exchanging sites on sorbents claimed to be neutral, resulting in low yields for low levels of bile acids). Problems with poor reproducibility and recoveries with different solid phase procedures have been described in several studies (Wahlén, Tamasawa, Ichimiya, et al., 1994; Rodrigues and Setchell, 1996). A wide variety of solid sorbents is available and many have not been evaluated for extraction of bile acids. The combination of ionic and nonpolar binding to the sorbent can make the elution of amphipathic compounds difficult. Thus, dependent on the nature and condition of the fluid to be extracted the yields of different chemical and physical forms of the bile acids and alcohols in the fluid may vary and be unsatisfactory. In serum and plasma, bile acids are bound to proteins and may be incorporated into lipoproteins and be inaccessible for transfer to the sorbent phase.

Problems with extraction of bile acids from plasma are exemplified by the studies of Nuber, Maucher and Stange (1990). These authors were unable to reproduce published procedures using Amberlite XAD-2 or ODS-silica (Nuber, Maucher and Stange, 1990). Instead they performed a size exclusion chromatography on Sephadex G-75 and had excellent recoveries. The sample was diluted with 0.1 M sodium hydroxide (as previously done with Amberlite XAD-2 and Bond-Elut C_{18}) and applied to the gel column. Unfortunately the method uses large solvent volumes, and does not desalt the sample as needed for analyses by electrospray mass spectrometry. In a more recent study Rodrigues and Setchell (1996) compared different reversed-phase bonded silica cartridges under different extraction conditions and found large variations in the recovery of common bile acids from serum (Rodrigues and Setchell,

1996). They concluded that performance characteristics should always be critically evaluated when manufacturer and batch of sorbent are changed. This is not a simple matter when extraction yields are dependent on bile acid structure and mode of conjugation (Wahlén, Tamasawa, Ichimiya, et al., 1994). It should also be mentioned that the chemical structure of the ODS-silica and the conditions of the extraction (e.g. pH) determine the potential release of covalently bound stationary phase from the sorbent and influence the subsequent chromatographic analysis, for example, by producing interfering peaks (Sjövall and Setchell, 1988).

The variable results with solid sorbent extractions can be explained by several mechanisms. The commercial Amberlite XAD-2 changed its chemical properties between 1972 and 1990 and the structures of reversed-phase sorbents from different manufacturers are not the same. The sodium hydroxide used in many procedures is damaging to the ODS-silica. Detailed studies of the extraction of free and conjugated steroids by XAD-2 and ODS-silica were performed by Axelson and Sahlberg (1981) and showed that several factors were involved, including ion exchange and protein binding (Axelson and Sahlberg, 1981). Following these and other studies in the Karolinska laboratory, the following general extraction procedure for hormonal neutral steroids, bile acids and bile alcohols and their conjugates is used in this laboratory (our evaluation has mostly been done using Sep-Pak C_{18} (Waters) materials but are likely to be valid for Bond Elut (Varian) and possibly other brands of ODS-silica).

The sample is diluted with one volume of 0.5 M triethylamine sulphate (TEAS) (preparation: add 8 mL of conc. sulphuric acid to 100 mL water (drop by drop), then add 40 mL triethylamine, adjust pH to 7.0–7.4, finally add 140 mL water and store in a dark place at 4°C). The sample/TEAS mixture is heated for 5 min at 60–64°C and passed through the bed of ODS-silica, preferably but not necessarily held at about 60–64°C with the help of a jacketed column. The size of the ODS-silica bed is determined by the volume of the sample, about 300 mg for 2–5 mL serum. Smaller beds, now applicable because of the higher sensitivity of the mass spectrometric methods, can be prepared in Pasteur pipettes. Before use, the ODS-silica bed is washed with methylene chloride/methanol, 1:1 (v/v) (to remove contaminating lipids), methanol and 0.25 M TEAS. Following application of the sample the ODS-silica bed is washed with water to remove proteins, TEAS and other salts and polar organic compounds. The steroids as well as the bile acids and their conjugates are eluted with methanol or ethanol (depending on the particular application or bile acids to be analysed, the ODS-silica can be washed and eluted with other methanol or ethanol/water concentrations; very nonpolar compounds require addition of 2-propanol or methylene chloride to the alcohol for quantitative elution). The functions of TEAS in this procedure are probably multiple: coverage of ion exchanging sites in the sorbent, formation of ion pairs with polar conjugates, denaturation of lipoproteins, and (together with the heating) inhibition of nonpolar protein binding. It should also be mentioned that recoveries of certain conjugated bile acids from urine, i.e. in the absence of protein binding, are improved when this method is used instead of conventional extraction of neat urine without pH correction (Wahlén, Tamasawa, Ichimiya, et al., 1994). The introduction of electrospray mass spectrometry (or the

previous FAB or SIMS (secondary ion mass spectrometry) methods) has made it possible to directly analyse extracts of urine and plasma and thus to evaluate the efficiency of different extraction procedures directly by monitoring endogenous bile acids and bile alcohol conjugates.

Another important aspect of solid-phase extraction is the need for a homogenous *solution* of the compounds to be extracted. Thus, lipids like cholesterol are poorly or not at all extracted from an aqueous mixture. This is probably due to the poor miscibility of water, lipid and sorbent surface. Cholesterol and many oxysterols in serum are only partially or not at all extracted by ODS-silica whether TEAS is present or not. In this case a solvent extraction can be performed with ethanol and the ethanol phase applied to an ODS-silica bed. The effluent is collected, diluted with water (without formation of a precipitate) and again passed through the ODS-silica bed. This effluent is again diluted with water and passed through the bed. The process (recycling sorbent extraction) is repeated with the most suitable number of steps and dilutions depending on the nature and polarity of the compounds to be analysed. When very polar bile acid derivatives are to be analysed the methanol (or ethanol) content should be 5% or less in the final step. Very nonpolar constituents may require addition of 2-propanol or methylene chloride to the eluting alcohol. Examples have been described (Axelson and Sjövall, 1985; Björkhem, Andersson, Ellis, et al., 2001; Liere, Pianos, Eychenne, et al., 2004). In a recent paper Persson et al. described the simultaneous extraction of common bile acids and lipids with high recoveries from intestinal fluid using ODS-silica with addition of 30% methanol to the intestinal fluid at pH 5 (Persson, Löfgren, Hansson, et al., 2007). The bile acids were eluted separate from the other lipids with 75% methanol/water. The composition of the intestinal fluid, e.g. the presence of solubilizing phospholipids, probably determines the methanol concentration needed for extraction of cholesterol. Thus, sorption of sterols from a faecal extract required a methanol concentration of at least 50% (Axelson and Sjövall, 1985).

As mentioned, the original solid phase extraction methods had desalting as a major aim. With the development of liquid chromatography–mass spectrometry (LC-MS) methods where desalting is achieved on a precolumn, delipidation has become equally important. In this case a solvent extract in, for example, ethanol is diluted with water to an extent compatible with passage (no retention) of all bile acid derivatives of interest through the sorbent bed. Less polar lipids are retained by the sorbent. This reduces the solubility limitations inherent in gradient LC separations of bile acid mixtures containing highly polar components, reduces the load of sample on a capillary LC column and reduces interferences in the mass spectra. A method for analysis of neurosteroids in rat brain illustrates these principles (Liu, Sjövall and Griffiths, 2003).

10.2.1.3 Gel Phase Extractions

A method of greater value than reflected by the literature is the extraction using hydroxyalkylated derivatives of Sephadex LH-20 (e.g. Lipidex 1000 (10% (w/w)

alkyl chains), Lipidex 5000 (50% alkyl chains) (Canberra-Packard) and hydroxyalkoxypropyl-Dextran (Sigma)). Column beds of these gels can be prepared in water and used to extract nonpolar lipids and moderately polar bile acids and bile alcohols from aqueous solutions or suspensions that are passed through the beds. The extracted compounds are then eluted with a suitable solvent, e.g. methanol or aqueous methanol, depending on the polarity of the compounds to be isolated. These extractions are comparable to solvent extractions from water and have the advantage of not giving emulsion problems. Contrary to the case with the solid sorbents discussed above, the extractions are determined solely by the polarity of the compounds to be extracted. Thus, polar conjugates of bile acids and alcohols are extracted poorly or not at all. Since the gels are tightly cross-linked, proteins are not admitted, and steroid ligands are extracted when protein binding is minimized (e.g. by elevated temperature). Extraction with Lipidex 1000 is useful as an alternative to solvent extraction of free bile acids after enzymatic hydrolysis (Dyfverman and Sjövall, 1983; Setchell, Lawson, Tanida, et al., 1983; Nuber, Maucher and Stange, 1990). Conjugated bile acids can be extracted as ion pairs by Lipidex 1000 from urine, plasma and bile to which phosphate buffer pH 7 and decyltrimethylammonium bromide have been added to final concentrations of 0.25 and 0.03 M, respectively (Dyfverman and Sjövall, 1983). The Lipidex column beds can be used repeatedly for long periods of time (months) and are washed with suitable solvents between use. In contrast to ODS-silica, the Lipidex gels will extract nonpolar lipids from aqueous suspensions and the combination of a Lipidex gel bed and an ODS-silica bed in series will extract sterols and bile acids of a wide polarity range, usually without need for a recycling procedure (Setchell, Lawson, Tanida, et al., 1983). As is the case for ODS-silica, Lipidex 1000 or 5000 will not extract nonpolar lipids, or oxysterols quantitatively from plasma because of protein binding and presence in lipoproteins. However, Tamasawa et al. found that when Lipidex 5000 containing absorbed 2-propanol (about 2:1, w/w) is shaken with plasma diluted with an equal volume of 0.5 M phosphate buffer these lipids are quantitatively recovered in the Lipidex gel phase (Tamasawa N, Ichimiya H, Axelson M and Sjövall, J., unpublished results 1989). Axelson made a modification of this method for use with ODS-silica to extract and purify sterol mixtures and identified a new dehydrocholesterol in human plasma (Axelson, 1991).

10.2.1.4 Combined Extractions

The possibility of combining solvent, sorbent and gel extractions offers great flexibility in the choice of method depending on the particular application and the types of bile acids to be analysed. Solvent extracts can be subjected to sorbent extraction via adjustment of the solvent composition of the former (e.g. dilution (or evaporation) of ethanol extracts with water followed by single step or recycling sorbent procedures). In this way different sample types can be purified by the same column methods with differences only in the initial extraction step.

Another possibility is to evaporate a tissue extract in the presence of Lipidex 1000 as described for analysis of steroids in testis (Andersson and Sjövall, 1983).

The extracted components will be transferred into the gel, which can then be slurried into a chromatography column for elution with appropriate solvent mixtures to achieve sample purification. In such procedures an ODS-silica bed is attached to the end of the Lipidex column to trap polar steroid/bile acid conjugates and retain undesired phospholipids.

10.2.2 Group Separations

Depending on the nature of the bile acids to be included in an analysis, their structures and absolute and relative concentrations, the complexity of the bile acid mixture and the matrix in which it is distributed, a group separation may be required. Simple analyses of major bile acids in bile or plasma/serum will not require this step. A group separation will increase the dynamic range and sensitivity of the analysis and remove interfering substances. Group separations are usually based on charge or polarity of the bile acids but can also be done on the basis of specific functional groups. Separations of groups differing in charge should be based on ion exchange (or other charge-dependent principle) and not on polarity since this varies with pH, substituents in the neutral part of the molecules and the structure of the sorbent surface. Molecules carrying the same or no charged substituent but differing in polarity can be group separated by partition (reversed or straight phase) or adsorption chromatography. Different group separation methods have been reviewed in detail by Sjövall and Setchell (1988) and the section below is limited to presently used validated methods applicable to studies of metabolic profiles of bile acids and bile alcohols.

10.2.2.1 Ion Exchange

The first reliable methods were based on the use of ion exchangers prepared by attaching ionic groups to a Sephadex LH-20 matrix (Ellingboe, Nyström and Sjövall, 1970; Almé and Nyström, 1971; Almé, Bremmelgaard, Sjövall, et al., 1977). At that time, available ion exchangers based on cross-linked polystyrenes had very strong adsorbtive properties necessitating forceful and damaging conditions for elution and reduced yields of low levels of the bile acids.

Three types of ion exchanging substituents have been most extensively used for group separations of bile acids and steroid conjugates: diethylaminohydroxypropyl- (DEAP-LH-20) (Almé, Bremmelgaard, Sjövall, et al., 1977), later becoming commercially available as Lipidex-DEAP (Canberra Packard); piperidinohydroxypropyl- (PHP-LH-20) (Goto, Hasegawa, Kato, et al., 1978) (Shimadzu); and triethylaminohydroxypropyl-Sephadex LH-20 (TEAP-LH-20, (Axelson, Sahlberg and Sjövall, 1981; Sjövall and Axelson, 1984). These are easily synthesized from Sephadex LH-20 via reactions with epichlorohydrin and the amine (Almé, Bremmelgaard, Sjövall, et al., 1977) (DEAP-LH-20); (Goto, Hasegawa, Kato, et al., 1978) (PHP-LH-20); (Curstedt, 1977; Axelson, Sahlberg and Sjövall, 1981) (TEAP-LH-20)).

The ion exchangers are reusable after washing and conversion into the appropriate form. They are stable for years in their acetate form in methanol at 4–8°C. Column beds of these materials can be prepared and used in a variety of solvents, e.g. 70–90% aqueous methanol or ethanol, and mixtures of aqueous methanol or ethanol with chloroform (not in their base form), methylene chloride or 2-propanol. It is very important that the sample to be separated is well dissolved when applied to the column bed. Thus, the origin and nature of the biological extract determine the choice of solvent to be used. The ion exchangers are used in acetate, bicarbonate or base form. The acetate form is preferred because the base form of the strong anion exchanger TEAP-LH-20 (and probably PHP-LH-20) can catalyse formation of artefacts from labile bile acids and steroids. The pH of the sample applied should be around 6–7 and the ionic strength low. This is mostly the case because the sample has usually been desalted in a previous extraction step. Enough ion exchanger should be used to exceed the anionic content of the sample. For this estimate it is important to realize that the capacity of ion exchangers decreases with increasing molecular size of the analytes (because of molecular sieving effects by the cross-linked matrix). A common size for a column bed for bile acid analysis is 60 × 4 mm when the chloride capacity of the ion exchanger is about 1 meq/g. The volumes of eluant needed for each group will then be around 5–10 mL. Microcolumns can also be prepared in Teflon tubing for use with small samples.

Ion exchange is used to separate different conjugated forms of bile acids and bile alcohols. Retention increases with the number of charged groups and is inversely proportional to the pK. Unconjugated bile alcohols are not retained; unconjugated bile acids require 0.1–0.15 M acetic acid in the solvent to be eluted. This is a very simple and rapid method to separate bile acids and bile alcohols after cleavage of their conjugates. The bile acids become purified and more easily derivatized for gas chromatography–mass spectrometry (GC-MS) analysis. The physical separation of bile acids and bile alcohols is most valuable because mass spectra of derivatives of bile alcohols and bile acid methyl esters are very similar since the side chain of a C_{27} bile alcohol is 2 Da lighter than the side chain of a bile acid methyl ester with the same nuclear substituents. Thus, it can be difficult to tell whether a peak in a GC-MS analysis is due to a bile acid lacking two hydrogen atoms or to a bile alcohol. Illustrative examples of spectral similarity can be found in Axelson, Mörk and Sjövall (1988) (see Section 10.4.1.1). The physical separation on an ion exchanger eliminates the problem. Some groups have solved this problem by using butyl ester derivatives that separate bile acids from bile alcohols in the GC and GC-MS analyses (see Section 10.3.2).

Bile acid conjugates are eluted in the order glycine conjugates, taurine conjugates, monosulphates and disulphates by ammonium acetate buffers of increasing ionic strength or pH or both, in a suitable solvent that does not have to be the same as the solvent for sample application. This offers great flexibility in the purification. Bile alcohol sulphates and taurine-conjugated bile acids have similar mobilities. In the ammonium acetate systems bile alcohol glucuronides have a mobility similar to that of glycine conjugated bile acids, and bile acid glucuronides have different mobilities depending on whether they are ester glucuronides, i.e. singly charged, or ether glucuronides (doubly charged). The latter have mobilities between those of

taurine and sulphated conjugated bile acids. Sulphates and different types of double conjugates can be recovered with ammonium acetate at pH 9.6. Elution schemes can be designed to obtain more detailed separations of groups. For example, following elution of non-glucuronidated non-sulphated bile acids with 0.1 M sodium acetate in aqueous methanol, ether glucuronides can be eluted as a group with 0.1 M formic acid in the solvent (which protonates the carboxyl groups and prevents interaction with the ion exchanger) (Setchell, Almé, Axelson, et al., 1976) followed by sulphates eluted with 0.3 M sodium acetate in the solvent (Stiehl, Raedsch, Rudolph, et al., 1982). Finally, ion exchange separations can aid in the isolation of bile acids that can exist in lactone form (Une, Tsujimura, Kihira, et al., 1989).

The introduction of electrospray ionization mass spectrometry (ESI-MS) makes it possible to monitor elution of the various groups of conjugated steroids and bile acids and the group separation system can thus be optimized for particular needs. A few references to work by Japanese and Swedish groups serve to illustrate the combination of group separations with GC-MS and LC analyses (Goto, Kato, Saruta, et al., 1981; Matoba, Une and Hoshita, 1986; Axelson, Mörk and Sjövall, 1988; Goto, Miura, Inada, et al., 1988; Marschall, Green, Egestad, et al., 1988; Une, Tsujimura, Kihira, et al., 1989; Wahlén, Egestad, Strandvik, et al., 1989; Axelson, Mörk and Everson, 1991; Ichimiya, Egestad, Nazer, et al., 1991; Ikegawa, Hirabayashi, Yoshimura, et al., 1992; Niwa, Fujita, Goto, et al., 1993; Marschall, Griffiths, Götze, et al., 1994; Ikegawa, Yoshimura, Ito, et al., 1995; Ikegawa, Murao, Motoyama, et al., 1996; Meng and Sjövall, 1997; Meng, Griffiths, Nazer, et al., 1997; Meng, Reyes, Palma, et al., 1997; Goto, Murao, Nakada, et al., 1998; Axelson, Ellis, Mörk, et al., 2000; Alvelius, Hjalmarson, Griffiths, et al., 2001; Goto, Myint, Sato, et al., 2007). Several of these references contain complete descriptions of methods for analysis of bile acid profiles in different biological materials.

The importance of separating groups of conjugates prior to LC-MS in analyses of urinary bile acid and bile alcohol metabolomes in cholestatic liver disease is illustrated by the capillary LC-MS study of Yang, Griffiths, Nazer, et al. (1997), and that by Meng, Reyes, Palma, et al. of women with intrahepatic cholestasis of pregnancy treated with ursodeoxycholic acid (Meng, Reyes, Palma, et al., 1997) referred to in Sections 10.1.3 and 10.4.2. The resolution and dynamic range of the analysis is greatly increased by preliminary fractionation. The need for a group fractionation is analogous to that for peptide analyses in proteomics. Shown in Fig. 10.1 is the GC-MS profile of bile acids from the fraction containing bile acids conjugated with glycine or taurine and the profile of bile acids doubly conjugated with either of these amino acids and glucuronic acid. As seen, these profiles are completely different, both qualitatively and quantitatively. The pathophysiological importance of such differences is not yet known but from an analytical point of view they are important.

Other ion exchangers have also been used to effect group separations of bile acids. Bond-Elut SAX, a strong anion exchanger from Varian, to separate unconjugated, glycine-conjugated, taurine-conjugated, and sulphated bile acids (Scalia, 1990; Scalia and Pazzi, 1990; Scalia, 1995). Korpela, Fotsis and Adlercreutz (1986) used DEAE-Sephadex in a method for analysis of faecal bile acids. The polar nature of this ion exchanger makes it less suitable for ion exchange in nonpolar

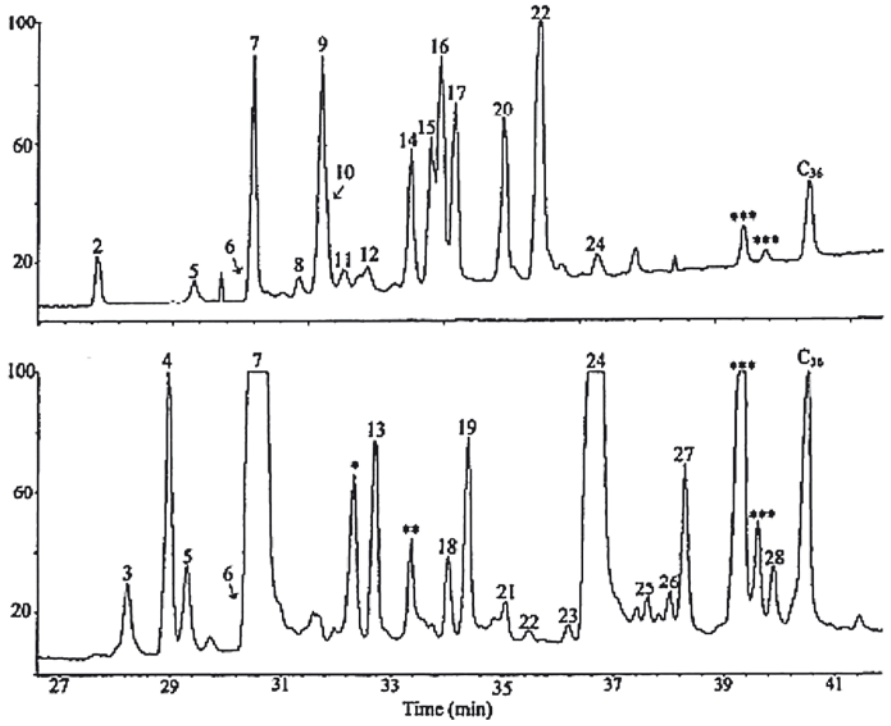


Fig. 10.1 Comparison of the total ion current chromatograms obtained in the GC-MS analyses of bile acids in the fraction of glycine or taurine conjugated bile acids (*top* chromatogram) and bile acids in the fraction of bile acids doubly conjugated with glycine or taurine and glucuronic acid (*bottom* chromatogram) from the urine of a patient with intrahepatic cholestasis of pregnancy given ursodeoxycholic acid (the injected aliquot of the glycine/taurine conjugated fraction was smaller than that of the doubly conjugated fraction). The numbered peaks correspond to derivatives of 28 different bile acids whose structures are given in Meng, Reyes, Palma, et al. (1997), from which the figure is with permission from Elsevier

solvents. Considering the availability of a variety of ion exchangers built on different matrices, it should be possible to develop faster, simpler and miniaturized methods for improved group separation to be combined with capillary LC-MS and GC-MS analyses. Automation of such procedures will also be possible (Uusijärvi, Egestad and Sjövall, 1989), using present advances in microfluidic systems.

10.2.2.2 Adsorption and Partition Chromatography

Thin-layer chromatography was once used extensively both for analytical purposes and for group separations based on polarity and mode of conjugation. It has a number of drawbacks for group separations as discussed by Sjövall and Setchell (1988) and by Scalia (1995) and cannot be recommended for that purpose. It may be useful in

synthetic work for rapid checks of product mixtures and for screening of bile samples. Also, it is useful for detection of radiolabelled bile acids by autoradiography (Roda, Piazza and Baraldini, 1998). The method has largely been replaced by LC.

Besides its use for analysis of individual bile acid profiles, LC (see Section 10.3.1) can serve to isolate and concentrate groups of bile acids present at low levels, thus increasing the dynamic range of an analysis. This group separation/enrichment can also be achieved in a low-pressure/low resolution system using silicic acid columns, silica cartridges (Street, Trafford and Makin, 1985), or Lipidex 1000 or 5000 operated in the straight-phase mode. These methods have been extensively reviewed by Sjövall and Setchell (1988). As an example, Bremmelgaard and Sjövall (1980) in a study of bile acid metabolism in humans used Lipidex 5000 to separate methyl esters of mono-, di-, tri-, and tetrahydroxylated bile acids into four fractions by stepwise elution of the column with hexane/chloroform 1:4 (mono), 3:7 (di), 1:1 (tri) and methanol (tetra) (Bremmelgaard and Sjövall, 1980). Marschall et al. used this system with a 60 × 4 mm Lipidex column for group enrichment of methyl esters of otherwise unconjugated bile acid glucosides and *N*-acetylglucosaminides from human urine processed by group separation on Lipidex-DEAP as described above (Marschall, Green, Egestad, et al., 1988).

As mentioned above, group separations of conjugates differing by number and strength of charges should be based on ion exchange. However, the separation of sodium or potassium salts of conjugated bile acids and their sulphates on Sephadex LH-20 in nonaqueous chloroform/methanol mixtures (Sjövall and Vihko, 1966; Almé, Bremmelgaard, Sjövall, et al., 1977) is an exception for special applications. Makino et al. used this method in pioneering studies of sulphated bile acids in human plasma and urine (Makino, Shinozaki, Nakagawa, et al., 1974). Haslewood and coworkers found that it was a simple and quick method to obtain a purified bile salt fraction from bile of different species (Anderson, Haslewood, Oldham, et al., 1974). It is also a useful method for the purification of larger samples, e.g. synthetic mixtures. Sodium or potassium chloride, respectively, is added to the solvent and the system can be regarded as an ion-pair chromatography of the respective salts. Potassium salts are more retarded than sodium salts. The nonaqueous condition is imperative; if more than a certain percentage of water is present, the salts dissociate and no separation is obtained. The separations depend on the number of sulphate/sulphonic acid groups in the molecules. The order of elution is: glycine- < taurine- < monosulphates of glycine- < monosulphates of taurine- < disulphates of glycine- < disulphates of taurine- < trisulphates of glycine- < trisulphates of taurine-conjugates. The methanol content of the solvent has to be increased for elution of conjugates with an increasing number of sulphate/sulphonic acid groups.

10.2.2.3 Group Isolation of Ketonic Bile Acids and Sterols

Ketonic bile acids and sterols occur as intermediates and metabolites in bile acid metabolism and have important pathophysiological effects, e.g. in inherited and acquired liver diseases (Sjövall, 1995) and possibly in colon cancer (Setchell, Street and Sjövall, 1988).

Recently C_{27} bile acids, and alcohols with a 3-oxo-Δ^4-structure were found to be ligands for a nuclear receptor in *C. elegans* regulating dauer formation and reproduction (Motola, Cummins, Rottiers, et al., 2006). Thus, it is important to be able to analyse ketonic bile acids and sterols. They often occur at low concentrations and a method for their selective enrichment is useful. This is achieved by conversion of the oxo groups into oximes, which can then be selectively sorbed as cations on a strong lipophilic cation exchanger, sulphohydroxypropyl Sephadex-LH-20 (SP-LH-20) (Axelson and Sjövall, 1979). The method has been applied to analyses of ketonic bile acids and sterols in faeces (Axelson and Sjövall, 1985).

10.2.3 Cleavage of Conjugated Bile Acids

The introduction of new ionization methods, particularly ESI, that permit direct analysis of conjugated bile acids by mass spectrometry and LC-MS has diminished the need for cleavage of conjugates prior to analysis. Many sample types, e.g. bile and serum, can now be analysed directly by these methods (see Section 10.4.2). However, when the bile acid mixture is complex, present LC methods may not provide the separations required and ESI-MS/MS may not give sufficient structural information and specificity, especially when high-energy CID is not available. In these cases conjugates have to be cleaved and the liberated bile acids or alcohols analysed by GC-MS (see Sections 10.3.2 and 10.4.1). Drawbacks of cleavage reactions are that they may be incomplete or give rise to artefacts. In many applications, ESI-MS may help to detect such problems. ESI spectra can provide information about the composition of bile acids in the untreated (unadulterated) original sample and can reveal changes that take place after enzymatic or chemical reactions. For example, using ESI-MS before and after a reaction to hydrolyse conjugated bile acids will indicate whether the reaction is complete, incomplete, or if unwanted/unexpected reactions have occurred. GC-MS spectra should be consistent with the information obtained from the ESI spectra. If this is not the case, the reactions used to convert the conjugated bile acids into derivatives for GC-MS have produced unwanted products and need to be modified. An early example was the discovery of the inherited deficiency in bile acid synthesis caused by mutations in the *HSD3B7* gene (Cheng, Jacquemin, Gerhardt, et al., 2003), where 3β,7α-dihydroxychol-5-enoic acid structures were destroyed by the method used to cleave bile acid sulphates. This resulted in complex GC-MS peak patterns that were difficult to interpret, yet the FAB spectra clearly showed deprotonated molecules compatible with the presence of sulphated and unsaturated di- and trihydroxy bile acids and their glycine conjugates (Clayton, Leonard, Lawson, et al., 1987).

10.2.3.1 Cleavage of Glycine and Taurine Conjugates

The amide bond can be cleaved by a large number of microbial enzymes (Coleman and Hudson, 1995; Dean, Cervellati, Casanova, et al., 2002; Kumar, Brannigan,

Prabhune, et al., 2006). A commercially available preparation from *Clostridium perfringens* consists of a mixture of enzymes as suggested in the original studies by Nair and coworkers (Coleman and Hudson, 1995). These preparations hydrolyse glycine conjugates more efficiently than taurine conjugates. Different conditions have been described, exemplified in the papers by Karlaganis and Paumgartner (1979) and Huijghebaert and Hofmann (1986). Several reports indicate the importance of a long incubation time (e.g. 16 h). Studies of synthetic bile acid conjugates show that increasing steric hindrance near the amide bond, or acidic, or basic amino acid residues greatly decrease rates of hydrolysis and that the negative charge on the terminal group of the amino acid moiety is required for activity (Huijghebaert and Hofmann, 1986). The results of specificity studies are likely to vary considerably depending on the origin and nature of the enzyme preparations. Marschall recently told us that a commercial batch of enzyme hydrolysed cholylglycine, but not cholyltaurine (H.-U. Marschall, personal communication, 2008). Other bile acids were not tested.

Glycine- and taurine-conjugated bile acids may be additionally conjugated with sulphuric acid, glucuronic acid, other sugars, fatty acids and probably as yet unidentified groups. Lepage et al. showed that a crude extract of enzyme hydrolysed glycine and taurine conjugates of mono-, di-, and trihydroxy bile acids sulphated in the 3 or 7 positions provided that the incubation time was 16 h (Lepage, Roy and Weber, 1981). The sulphate ester bonds remained stable under these conditions. Our experience of work with biological mixtures indicates that the amide bond of various doubly or triply conjugated bile acids often becomes hydrolysed after 16 h. However, the efficiency of the enzymatic hydrolysis in such cases remains undefined. Analyses of biological materials with ESI-MS before and after treatment with the enzyme(s) may help to clarify the limitations of the enzyme hydrolysis. The sites of the additional conjugating groups (at C-3, C-7, C-12 or others) are likely to be important.

The nature of oxygen substituents in the conjugated bile acid is important for the efficiency of hydrolysis. The time for complete hydrolysis increases with increasing number of hydroxyl groups and presence of oxo groups (Beher, Stradnieks, Beher, et al., 1978; Batta, Salen and Shefer, 1984).

The length of the bile acid side chain is very important (Batta, Salen, Cheng, et al., 1979; Batta, Salen and Shefer, 1984). Glycine and taurine conjugates of C_{23}, C_{25} and C_{27} acids are either not, or very slowly, hydrolysed by the *C. perfringens* enzyme. However, active enzymes are present in the intestinal microflora, and 3α,7α,12α-trihydroxy-5β-cholestan-26-oyltaurine is hydrolysed, with retention of the configuration at C-25, by anaerobic incubation with a rat faecal suspension (Batta, Salen, Cheng, et al., 1979).

The peptide bond can also be cleaved by alkaline hydrolysis but the vigorous conditions required result in various modifications of other functional groups in the bile acid skeleton particularly in the presence of alcohol (Sjövall and Setchell, 1988). If bile acid sulphates or glycosides are present they are destroyed by the conditions. Thus, alkaline hydrolysis should only be used in selected cases and when no other method is available. Alternative conditions may lead to revision of this recommendation. Thus, Dayal and Ertel (1998) have shown that glycine and taurine conjugates of C_{24} and C_{27} bile acids are hydrolysed (with retention of configuration at C-25) in 1 M

sodium or lithium hydroxides in propylene glycol for 3–4 min in a commercial microwave oven (Dayal and Ertel, 1998). Further work will be needed to validate this technology for work in an analytical microscale and to establish the stability of naturally occurring bile acids with labile or reactive functional groups or structures.

10.2.3.2 Cleavage of Sulphates

Sulphates can be cleaved by enzymes or by solvolysis. Enzymes (e.g. from *Helix pomatia*) do not have a sufficiently broad specificity to hydrolyse all kinds of naturally occurring bile acid sulphates and are not recommended for general use in analytical applications. Eyssen and coworkers have published detailed studies of the occurrence and structures of bile acid sulphates and their hydrolysis by sulphatases in the intestinal microflora (Huijghebaert, Parmentier and Eyssen, 1984, and later publications by this group). These sulphatases hydrolyse 3-sulphates but not 7- or 12-sulphates and in some cases not 3β-sulphates (Robben, Parmentier and Eyssen, 1986). These limitations are of both analytical and biological importance since sulphoconjugation of cholic acid at C-7 is a major reaction in mice. In humans, 3β-hydroxy bile acids can occur in sulphated form. For this reason, enzymatic methods are less useful in multicomponent analyses of bile acids.

A large number of solvolytic procedures have been described, many of which give incomplete solvolysis or produce artefacts (Cohen, Budai and Javitt, 1981; Princen, Meijer and Kuipers, 1990). Conditions for simultaneous solvolysis and methylation may be useful in some specific applications, but are not for general use. Solvolysis in solvent mixtures containing acetone or dimethoxypropane will result in formation of acetonides of vicinal *cis*-glycols (e.g. in β-muricholic and hyocholic acids), which can then be further transformed if subsequent derivatization reactions are performed (Janssen, Toppet, Compernolle, et al., 1989). Presence of ethyl acetate results in ester formation. The convenient method of Hirano et al. was not tested on 7- and 12-sulphates (Hirano, Miyazaki, Higashidate, et al., 1987). However, complete hydrolysis of the 3,7-disulphate of ursodeoxycholic acid by this method was recently demonstrated and confirmed by FAB-MS analysis (Setchell, unpublished observations, 2008). The original method however cannot be used as described, with direct evaporation of the trifluoroacetic acid solution, when acid-labile bile acids are present. In such cases the reaction mixture is neutralized with aqueous sodium bicarbonate and the solvolysed bile acids recovered by solid phase extraction (Ichimiya, Egestad, Nazer, et al., 1991). In a careful study of conditions for solvolysis of sulphates of free and aminoacyl amidated bile acids, Princen et al. emphasize the importance of complete dissolution of the sample and describe quantitative solvolysis when the sample was sonicated in 0.12 mL water followed by 3.88 mL 1,4-dioxane containing 1 mmol/L HCl incubated at 37°C overnight (Princen, Meijer and Kuipers, 1990). Most common sulphates were solvolysed in 2 h but the longer time was required for cleavage of 7- and 12-sulphates and disulphates. The reaction mixture was evaporated without an extraction step, but neutralization and solid-phase extraction, as above, is probably necessary when acid-labile bile acids are present.

10.2.3.3 Cleavage of Glycosides

Naturally occurring bile acid glycosides carry the sugar moiety either in ether linkage to a hydroxyl group or in ester linkage at C-24. The position of glycosylation depends on the structures of both the bile acid and the sugar moieties. When the sugar is attached in ester linkage it is readily hydrolysed in 5 min in 0.3 M aqueous sodium hydroxide (Goto, Shibata, Sasaki, et al., 2005) and the liberated bile acid can be analysed by LC-MS/MS or, after derivatization, GC-MS. The liberated sugar moiety can also be analysed after derivatization by either of these methods. Bile acid acyl galactosides were identified in this way in the urine of healthy humans (Goto, Shibata, Sasaki, et al., 2005).

Bile acid ether glucuronides, linked at C-6 or C-3, have for a long time been hydrolysed with β-glucuronidase from *Helix pomatia* (Momose, Maruyama, Iida, et al., 1997). Other ether glycosides may be more difficult to hydrolyse. Momose et al. performed detailed studies of the hydrolysis of a number of the most common bile acid glycosides with a number of commercially available β-glycosidases (Momose, Maruyama, Iida, et al., 1997). While the *Helix pomatia* glucuronidase gave essentially quantitative hydrolysis, the rates of hydrolysis of glucosides and *N*-acetylglucosaminides varied greatly with the source of enzyme and the bile acid glycoside structure. Indirectly, this study indicates that bile acid glycosides are best analysed intact by LC-MS/MS, which also permits localization of the sugar moiety, while enzymatic hydrolysis is presently needed for definitive identification of the bile acid (and sugar) moiety.

10.3 High-Performance Separations

The separation of bile acids by different chromatographic techniques has been reviewed elsewhere (e.g. Roda, Piazza, Baraldini 1998; Scalia, 1995; Batta and Salen, 1999; Shimada, Mitamura and Higashi, 2001). This section is limited to some principles for the separations.

10.3.1 Liquid Chromatography

Bile acids have several polar functional groups, such as hydroxyl groups on the steroid nucleus and anionic groups located at the end of the side chain. Thus, it is difficult to obtain well-shaped peaks with a normal-phase LC system. To prevent the tailing and/or distortion of peaks in a chromatogram, an organic acid is usually added into the mobile phase. Under normal-phase conditions, bile acids are eluted in order of increasing number of hydroxyl groups. Shaw and Elliot (1976) first described the separation of bile acids from human bile. The bile acids were first separated into four fractions, followed by the chromatographic separation of each

fraction. This method, however, required the in-line combination of five columns, and did not separate chenodeoxycholate and deoxycholate. The separation of compounds with the polarity of bile acids is better achieved with reversed-phase partition chromatography (Parris, 1977). An 8:2 (v/v) mixture of methanol and water at pH 2 (adjusted by adding phosphoric acid) was used as the mobile phase with a Zorbax-ODS column. UV detection at 210 nm of 50 ng of bile acid was possible but this method failed to separate chenodeoxy- and deoxycholic acids.

Several factors influence the chromatographic behavior of bile acids on an ODS-silica column (Shimada, Hasegawa, Goto, et al., 1978; Goto, Kato, Saruta, et al., 1980). Because bile acids are anionic compounds, the pH of the mobile phase markedly influences the retention of bile acids (Fig. 10.2). Although unconjugated, glycine-conjugated, and taurine-conjugated chenodeoxycholic acids (Fig. 10.3) are each retained with similar retention factors in the pH range from 7.0 to 8.0, the retention factor of unconjugated chenodeoxycholic acid dramatically increases when the pH decreases from 6.5 to 5.0. The retention factor of glycine-conjugated chenodeoxycholic acid is also markedly changed following a pH change from 4.5 to 3.5. Acidic conditions can separate bile acids according to the acidic groups at the end of the side chain. These chromatographic behaviors depend on the acidity

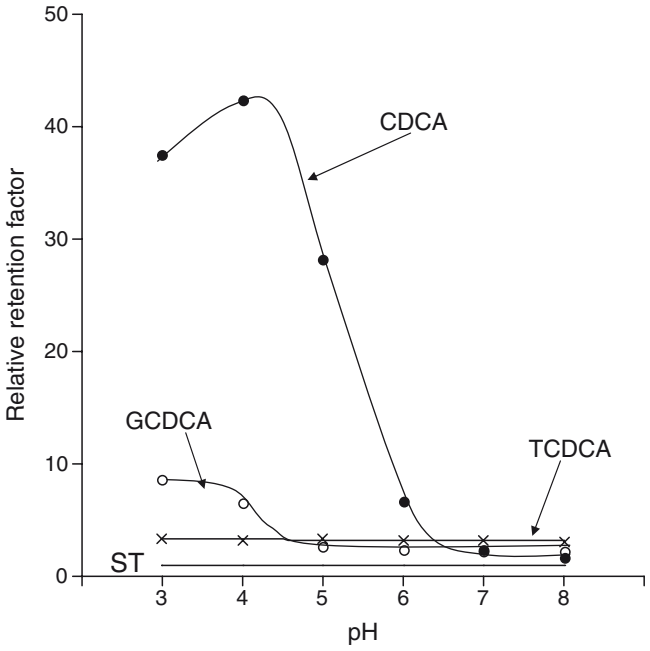


Fig. 10.2 Effects of the pH of the mobile phase on the retention factors of nonamidated (CDCA), glycine-conjugated (GCDCA), and taurine-conjugated (TCDCA) chenodeoxycholic acid relative to that of taurocholic acid (ST). Conditions: column, Capcell Pak C18 UG120 (5 μm, 4.6 mm i.d. × 150 mm); mobile phase, 20 mM ammonium phosphate buffer/acetonitrile; flow rate, 1.0 mL/min; detection, UV 205 nm

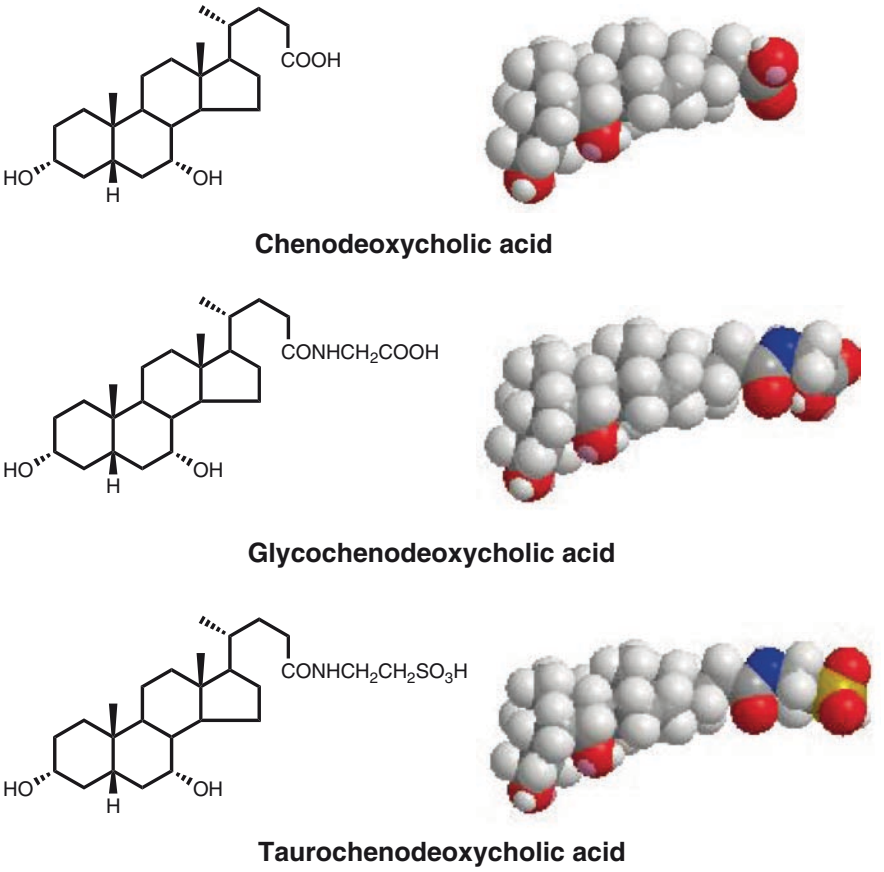


Fig. 10.3 Structures of nonamidated, glycine-conjugated, and taurine-conjugated chenodeoxycholic acid

of anionic groups on the side chain of the bile acids; the pK_a values are approximately 6.0, 4.5, and 1.5 for unconjugated, glycine-conjugated, and taurine-conjugated bile acids, respectively. Elution of unconjugated bile acids, however, takes a longer time than that of glycine- and taurine-conjugated bile acids, and it is difficult to completely separate chenodeoxycholic acid and deoxycholic acid.

Bile acids with an equatorial hydroxyl group at the 7β or 12β position are retained less effectively than the corresponding epimers with axial hydroxyl groups (Table 10.1). The retention time of ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholanoic acid (Fig. 10.4)) is usually almost half of that of chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholanoic acid) and is less than or equal to that of the trihydroxylated cholic acid. Although the 3β-hydroxyl group is an axial hydroxyl group, it produces effects similar to those of the 7β and 12β hydroxyl groups. The presence of a hydroxyl group on the β-side of a bile acid reduces the retention time. The retention behaviors of keto bile

Table 10.1 Relative retention factors of 5β-cholanoic acids

	Relative retention factor	
	pH 5.0	pH 7.5
3α-OH	1.00	1.00
3β-OH	0.93	0.77
3α,7α-$(OH)_2$	1.00	1.00
3α,7β-$(OH)_2$	0.42	0.34
3β,7α-$(OH)_2$	0.65	0.46
3β,7β-$(OH)_2$	0.42	0.28
3α,12α-$(OH)_2$	1.00	1.00
3α,12β-$(OH)_2$	0.57	0.57
3β,12α-$(OH)_2$	0.64	0.64
3β,12β-$(OH)_2$	0.61	0.61
3α,7α,12α-$(OH)_3$	1.00	1.00
3α,7β,12α-$(OH)_3$	0.24	0.27
3α,7α,12β-$(OH)_3$	0.27	0.23
3α,7β,12β-$(OH)_3$	0.08	0.02
3β,7α,12α-$(OH)_3$	0.04	0.04
3β,7β,12α-$(OH)_3$	0.14	0.10
3β,7β,12β-$(OH)_3$	0.11	0.06

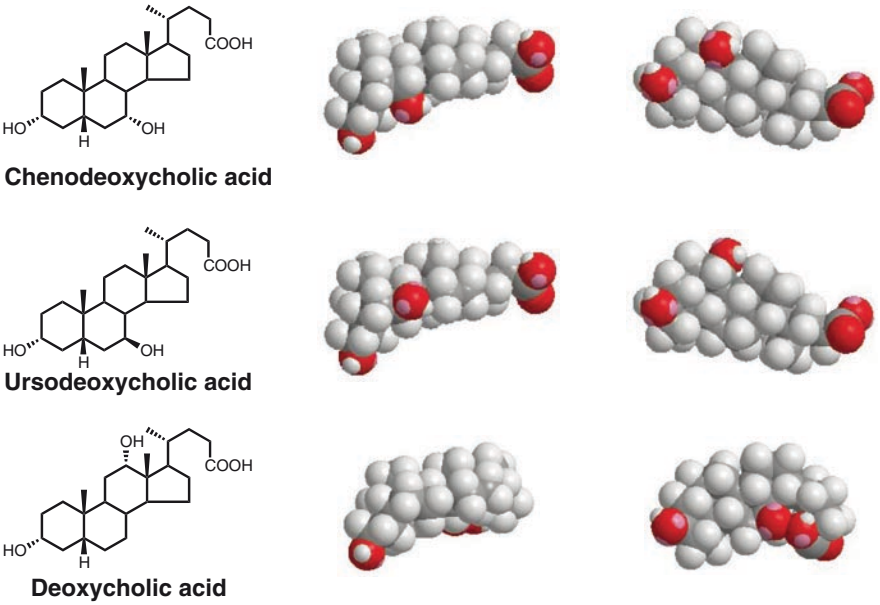


Fig. 10.4 Structures of chenodeoxycholic acid, ursodeoxycholic acid, and deoxycholic acid viewed from the side and from below

acids are similarly influenced by the positions and number of carbonyl groups. The retention behavior of a compound in reversed-phase chromatography depends on its solubilities in and partitioning between the mobile and stationary phases. The polarity

of a molecule controls the solubility in the mobile phase, whereas its hydrophobic surface plays an important role in the interaction with the stationary phase. Bile acids have a hydrophobic region on the β-side of the steroid nucleus, an area that is constant among the common bile acids (see Fig. 10.4). Therefore, increasing the number of hydroxyl groups on the α-side of the molecule increases the polarity of the molecule and enhances its solubility in the aqueous mobile phase (Table 10.1). On the other hand, the presence of a β-hydroxyl or carbonyl group reduces the hydrophobic area of the β-side, resulting in a decrease in the retention time.

As mentioned above, normal phase chromatography cannot usually separate the two positional isomers chenodeoxycholic acid and deoxycholic acid due to their similar polarities. On the other hand, reversed-phase chromatography can separate these molecules when a neutral or slightly alkaline mobile phase is used (Goto, Kato, Kaneko, et al., 1981). Deoxycholic acid has a 12α-hydroxyl group. This hydroxyl group can form an intramolecular hydrogen bond with the carboxyl group at the C-24 position, whereas a 7α-hydroxyl group is not able to form a similar bond. Therefore, deoxycholic acid is characterized by a larger retention factor than that of chenodeoxycholic acid at pH 6.0 or above (Fig. 10.5). Although the separation of bile acids while discriminating between the conjugated forms is somewhat difficult under neutral or slightly alkaline pH conditions, the simultaneous separation of bile acids can be achieved using a preliminary fractionation into

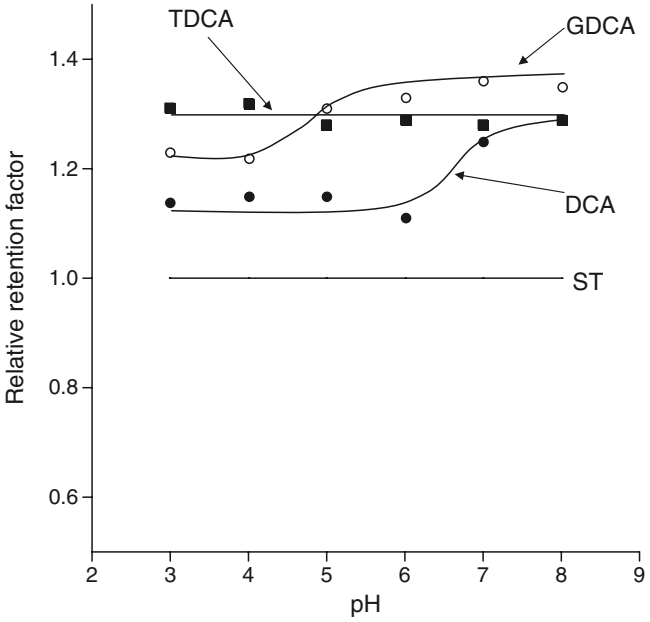


Fig. 10.5 Effects of the pH of the mobile phase on the retention factors of nonamidated (DCA), glycine-conjugated (GDCA), and taurine-conjugated (TDCA) deoxycholic acid relative to those of corresponding conjugates of chenodeoxycholic acids (ST). Conditions are the same as those described in Fig. 10.2

unconjugated, glycine-conjugated and taurine-conjugated molecules (Goto, Kato, Kaneko, et al., 1981) (see Section 10.2.2.1).

Based on these principles, numerous systems have been used for analysis of the common bile acids in biological materials. In most systems pH is controlled using phosphate buffers. This is not compatible with online connection to an electrospray ionization mass spectrometer or an evaporative light-scattering mass detector (ELSD). In those cases a volatile buffer is used, usually ammonium acetate.

Examples of LC systems for analysis of bile acids in bile without derivatization can be found in papers by Rossi, Converse and Hofmann (1987) and by Hagey, Hofmann and colleagues cited in other parts of this chapter. Extensive tables of retention indices for glycine- and taurine-conjugated bile acids from different vertebrates have been published (Moschetta, Xu, Hagey, et al., 2005). Other systems are exemplified in papers, for example, by Scalia and Pazzi (1994) using UV detection and by Roda, Cerrè, Simoni, et al. (1992); Torchia, Labonte and Agellon (2001) and Kakiyama, Iida, Goto, et al. (2006) using evaporative light-scattering detection. In the latter study, detection by ESI mass spectrometry led to the identification of a novel 15α-hydroxylated bile acid in the bile of swans, tree ducks and geese. Sakakura et al. presented a detailed study of the separation of 26 bile acids in rat bile and serum by LC using enzymatic detection (Sakakura, Suzuki, Kimura, et al., 1993) (see below and Section 10.5). This method could be used for analysis of 30 bile acids in rat liver tissue with only one extractive delipidation and one solid phase extraction prior to LC (Sakakura, Kimura, Takeda, et al., 1998).

As discussed below, UV-detection systems are too insensitive for analysis of bile acids in most biological materials except for bile and serum from cholestatic subjects. Serum bile acid levels in healthy subjects are too low for analysis by this method. Contrary to claims in different studies, bile acids in urine cannot be reliably analysed by LC with nonspecific detection methods. This is both because of the considerable complexity of the bile acid mixture and the low concentrations of many components. The only valid detection system in such cases at present is ESI-MS (see Section 10.4.2).

Because bile acid sulphates and glucuronides are very polar, nonvolatile, and thermolabile, LC and liquid chromatography–mass spectrometry coupled with a reversed-phase column are suitable for analyses of these molecules. A sulphate or glucuronate group at the C-3α position is located on the α-side of the steroid nucleus, and their presence controls the polarity of the molecule and affects its solubility in the aqueous mobile phase. Because such sulphate and glucuronate groups have no direct effect on the hydrophobic area of the β-side, the separations of bile acid 3-sulphates (Fig. 10.6a) and 3-glucuronides (Fig. 10.6b) are similar to those of the corresponding unconjugated molecules. Moreover, the retention factors of these bile acids depend on the acidity of the side-chain anionic groups and the pH of the mobile phase (Goto, Kato and Nambara, 1980; Goto, Kato, Kaneko, et al., 1981; Goto, Suzaki, Chikai, et al., 1985) (see Fig. 10.6a, b). As mentioned in the introduction (Section 10.1.2), bile acids may be glucuronidated at the C-24 carboxyl group (Goto, Murao, Nakada, et al., 1998). Because all bile acid acyl glucuronides have the same anionic glucuronate group at the end of the side chain, the chromatographic

behaviors of these molecules are very similar (Ikegawa, Ishikawa, Oiwa, et al., 1999). The retention factors of bile acid acyl glucuronides relative to that of taurodeoxycholic acid are influenced by the pH of the mobile phase; the retention factors increase for pH values less than 3.5, a shift that is determined by the pK_a value of the carboxyl group on the glucuronate group.

Takeuchi and coworkers first reported the separation of bile acids using micro-LC in 1983 (Ishii, Murata and Takeuchi, 1983; Takeuchi, Saito and Ishii, 1983). Fifteen bile acids (CA, CDCA, DCA, LCA, and UDCA and their glycine and taurine conjugates) were separated using a single-pump exponential gradient elution system coupled with a mixing vessel at a flow rate of approximately 1–2 μL/min in combination with an immobilized 3α-hydroxysteroid dehydrogenase (HSD) system. The authors also connected the micro-LC system to continuous-flow fast atom bombardment mass spectrometry (Ito, Takeuchi, Ishii, et al., 1986). Separation of bile acids by micellar electrokinetic capillary electrophoresis has also been described

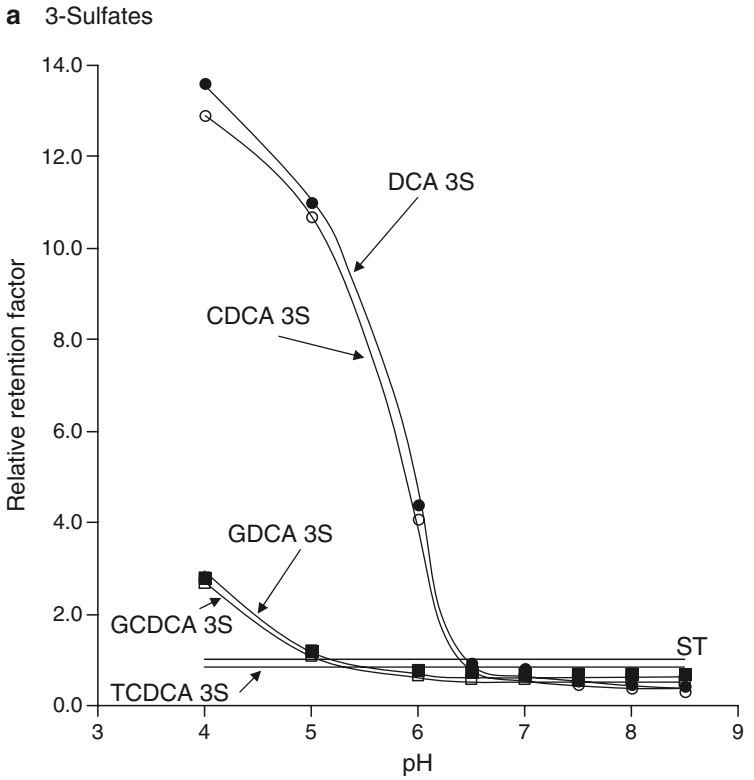


Fig. 10.6 Effects of the pH of the mobile phase on the relative retention factors of bile acid 3-sulphates (**a**) and bile acid 3-glucuronides (**b**). (**a**) Conditions: column, Symmetry C_{18} (5 μm, 4.6 mm i.d. × 150 mm); mobile phase, 20 mM ammonium phosphate buffer/acetonitrile; flow rate, 1.0 mL/min; detection, UV 205 nm

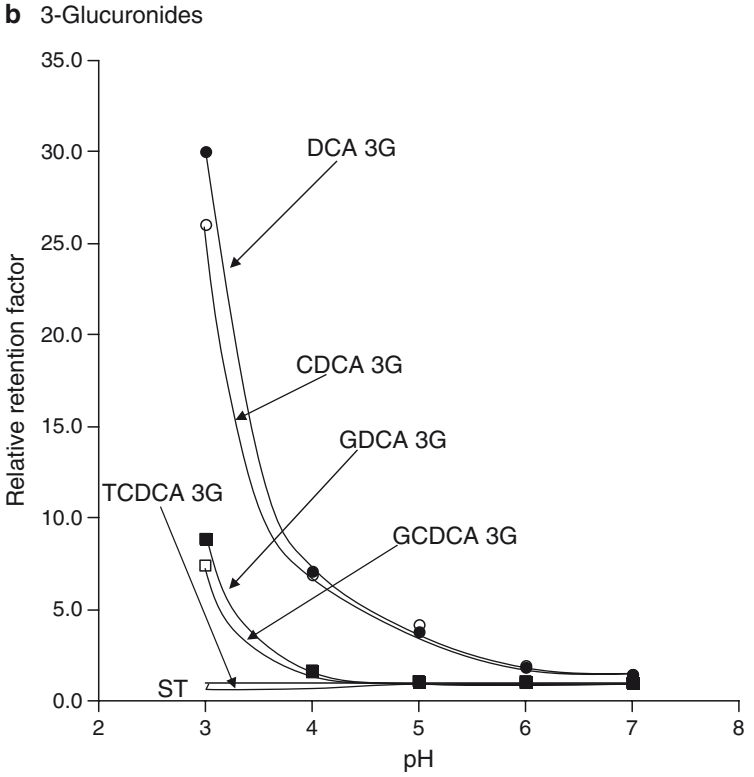


Fig. 10.6 (continued) (**b**) Conditions: column, Develosil ODS-HG-5 (5 μm, 1.0 mm i.d. × 150 mm); mobile phase, 20 mM ammonium acetate buffer/methanol; flow rate, 0.05 mL/min; detection, electrospray ionization mass spectrometry

and compared with separations by capillary column LC (Lee, New and Ong, 1997). UV-detection is a limiting factor. A promising method for separation of bile acids by capillary electrochromatography has been described, using solvent without nonvolatile components permitting detection by ESI-MS (see Section 10.4.2); column efficiencies of 610,000 plates/m were obtained with short analysis times and a detection limit of about 40 fmol (Que, Konse, Baker, et al., 2000).

Swobodnik, Klüppelberg, Wechsler, et al. (1985) and Legrand-Defretin, Juste, Henry, et al. (1991) described the analysis of bile acids in human and pig bile using reversed-phase ion-pair LC and an acetonitrile/water mobile phase modified with tetrabutylammonium counter ions (Swobodnik, Klüppelberg, Wechsler, et al., 1985; Legrand-Defretin, Juste, Henry, et al., 1991). Although this method does not require strict pH control of the mobile phase, it is not superior to conventional reversed-phase LC apart from increased separation selectivity for taurine conjugates from glycine conjugates. Alternatively, β-cyclodextrin has been added to the mobile phase for analyses of bile acids on reversed-phase columns (Shimada, Komine and Mitamura, 1991). The formation of host-guest complexes of bile acids

and β-cyclodextrin causes faster separation. The 12α-hydroxyl group of 12α-hydroxylated bile acids, however, interferes with the formation of the host-guest complexes, resulting in weak retention (Momose, Yamaguchi, Iida, et al., 1998). β-Cyclodextrin/2-hydroxypropyl-β-cyclodextrin mixtures have been added to the solvent to improve separations of plasma bile acids by micellar electrokinetic chromatography (Tripodi, Lucangioli, Scioscia, et al., 2003). Sensitivity with UV detection at 185 nm appears to be a limiting factor. A drawback with the systems in which nonvolatile components have to be added to the mobile phase is that they are not compatible with ESI-MS detection.

The side-chain of bile acids absorbs UV light at 190–200 nm. Due to the absorption of the peptide bond, amino acid-conjugated bile acids in bile can be analysed using an LC-UV method after suitable pretreatment; the limits of detection for glycine and taurine conjugates are approximately 50 and 150 ng, respectively (about 0.1–0.3 nmol). In the absence of carbonyl groups, the absorbance of unconjugated bile acids is 20–30 times lower. Therefore, it is difficult to analyse bile acids in biological fluids other than bile (and serum from patients with cholestatic liver disease) using a direct UV detection method. Detection by ELSD has also been employed in LC analyses of bile acids (Roda, Cerrè, Simoni, et al., 1992). This methodology has a detection limit of 0.08 nmol (Torchia, Labonte and Agellon, 2001), and allows a gradient elution with aqueous methanol. For comparison, the concentrations of individual bile acids in serum from healthy subjects are in the 0.1–1 μM range (Björkhem and Falk, 1983; Stellaard and Paumgartner, 1987). Thus, the sensitivity of the ELSD system may still be a limiting factor for analysis of small sample volumes. In a recent study, a charged aerosol detector was used in LC analyses of bile acids in human gastric and duodenal aspirates. A volatile methanol/formate buffer system could be used as mobile phase with limits of quantification in the range of 0.2–1.8 μM (Vertzoni, Archontaki and Reppas, 2008).

To improve detectability, various precolumn derivatizations of the carboxyl group on the side chain of unconjugated and glycine-conjugated bile acids have been proposed. These derivatizations have been thought to be useful in analyses of faeces, since bile acids are considered to be primarily unconjugated in healthy adult subjects. However, this is by no means a valid assumption and varies, for example, with age, health, diet and disease. 1-*p*-Nitrobenzyl-3-*p*-tolyltriazene (Okuyama, Uemura and Hirata, 1976), or *O*-*p*-nitrobenzyl-*N*,*N*′-diisopropylisourea (Shaikh, Pontzer, Molina, et al., 1978) can convert unconjugated bile acids into their *p*-nitrobenzyl esters. The derivatized bile acids are then monitored at UV 254 nm using a normal-phase column, such as the MicroPak-NH_2 and Partisil-10 columns with mixtures of isooctane/dichloromethane and isopropanol/isooctane as the mobile phases, respectively. With these methods, approximately 2 nmol of bile acids can be detected. The use of α-bromoacetophenone as a derivatization reagent results in bile acid phenacyl esters, which can also be detected by monitoring at UV 254 nm (Stellaard, Hachey and Klein, 1978). Moreover, *p*-bromophenacyl bromide (Mingrone and Greco, 1980; Iida, Ohnuki, Chang, et al., 1985), *m*-methoxyphenacyl bromide (Iida, Ohnuki, Chang, et al., 1985), *N*-chloromethyl-4-nitrophthalimide (Iida, Ohnuki, Chang, et al., 1985), and 9-chloromethylanthracene (Iida, Ohnuki, Chang, et al., 1985) have been used as

UV-sensitive labelling reagents to esterify the side-chain carboxyl groups of bile acids. These LC-UV methods, however, are not sensitive enough for analysis of bile acids in serum from healthy humans or rats.

Several techniques for precolumn fluorescent labelling of the carboxyl group on the side chain of bile acids have been reported. Bile acids derivatized with 4-bromomethyl-7-methoxycoumarin can be monitored at 410 nm as an emission wavelength following excitation at 360 nm (Okuyama, Uemura and Hirata, 1979; Wang, Stacey and Earl, 1990; Güldütuna, You, Kurts, et al., 1993; Gatti, Roda, Cerre, et al., 1997). Gatti, Roda, Cerre, et al. (1997) derivatized with 2-bromoacetyl-6-methoxynaphtalene for analysis of serum bile acids with a detection limit of 1–2 pmol, and Budai and Javitt with 4-bromomethyl-6,7-dimethoxycoumarin for analysis of free and glycine conjugated bile acids and their 3-sulphates (Budai and Javitt, 1997). An extensive table of retention times of reference compounds is presented in the latter reference. Reaction with 1-bromoacetylpyrene produces fluorescent derivatives of bile acids with a detection limit of 5–10 pmol (Kamada, Maeda and Tsuji, 1983) and has been applied to analysis of human faecal bile acids (Ikawa, Miyake, Mura, et al., 1987). Kurosawa et al. derivatized with the novel 3-(4-bromomethylphenyl)-7-diethylaminocoumarin and achieved a detection limit of 15 fmol at a signal/noise ratio of 3 in the separation of 24-hydroxylated stereoisomeric C_{27} bile acids and related acids (Kurosawa, Sato, Sato, et al., 1997). Their method permitted analysis of these acids in rat liver homogenates.

It is clearly a drawback that taurine conjugated bile acids are not included in analyses based on derivatization of the carboxyl group and require chemical or enzymatic deconjugation prior to derivatization. Also, in several cases the methods do not produce sufficient separation of chenodeoxycholic and deoxycholic acid conjugates. Their utility for analysis of the complex mixture of bile acids in urine is limited.

The majority of bile acids have an equatorial 3α-hydroxyl group, which is more reactive than the more sterically hindered axial secondary 7α- and 12α-hydroxyl groups. The equatorial 7β-hydroxyl group of ursodeoxycholic acid is similar to the 3α-hydroxyl group, but the steric hindrance associated with this position is different from that associated with the 3α-hydroxyl group. The pre-column labelling reagent 1-anthroyl nitrile selectively reacts with the 3α-hydroxyl-group of bile acids (and with the 3β-hydroxyl of 5α-steroids but not 5β-steroids because of steric hindrance) in the presence of quinuclidine in acetonitrile, resulting in only 3-(1-anthroyl) derivatives (Goto, Goto, Shamsa, et al., 1983; Goto, Saito, Chikai, et al., 1983) (Fig. 10.7). Because this derivatization at the C-3α position does not affect the chromatographic behaviors of the molecule as much as sulphation or glucuronidation at this position, the derivatized bile acids can be efficiently separated on an ODS-silica column and monitored by fluorescence detection (excitation: 370 nm; emission: 470 nm) with a detection limit of 20 fmol (Fig. 10.8). This procedure is also applicable to the quantification of 7- and 12-sulphates of bile acids in human urine (Goto, Chikai and Nambara, 1986), and taurine-conjugated 7-sulphated bile acids in urine from patients with hepatobiliary disease, such as primary biliary cirrhosis (Goto, Chikai and Nambara, 1987).

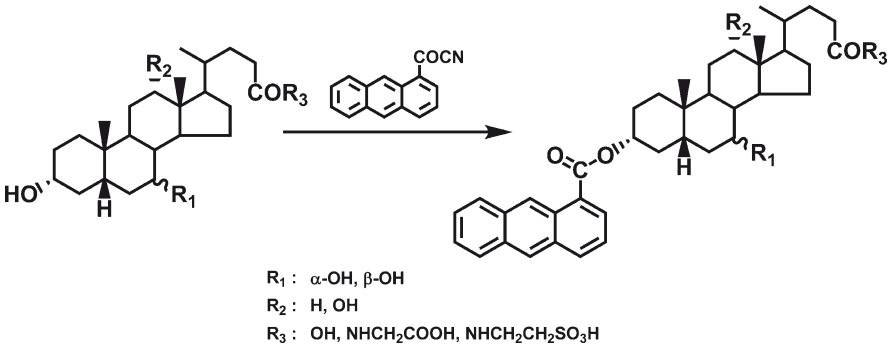


Fig. 10.7 Derivatization of bile acid using 1-anthroylnitrile

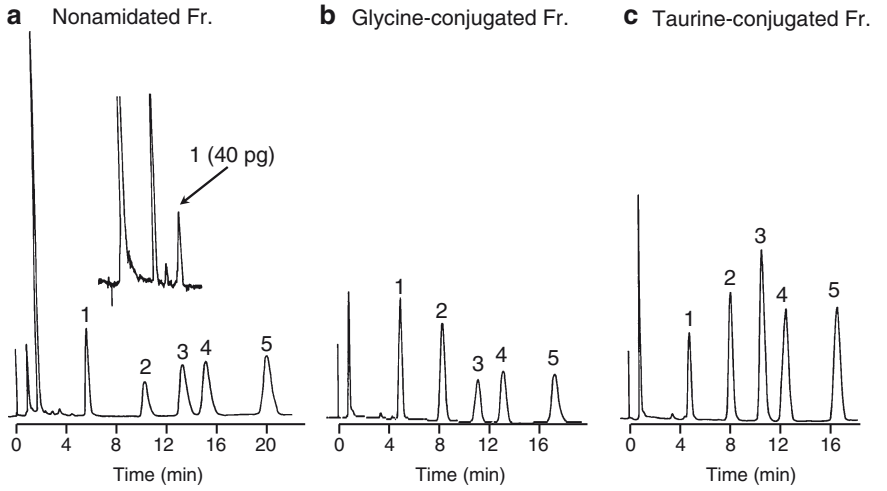


Fig. 10.8 Chromatograms of 3-(1-anthroyl) derivatives of nonamidated (**a**), glycine-conjugated (**b**), and taurine-conjugated bile acids (**c**). Conditions: column, Cosmosil $5C_{18}$ (5 μm, 4.6 mm i.d. × 150 mm); mobile phase, 0.3% potassium phosphate buffer (pH 6.0)/methanol at a 1:5 v/v ratio; flow rate, 1.8 mL/min; detection, excitation wavelength: 370 nm, emission wavelength: 470 nm

Another method of derivatization at C-3 is to convert the 3α-hydroxy group into an oxo group using 3α-hydroxysteroid dehydrogenase (3α-HSD) and NAD^+ under slightly alkaline conditions. This method, which converts NAD^+ into NADH resulting in UV absorption and fluorescence, was first developed to determine the levels of total serum bile acids (see Section 10.5). Today, online LC systems, which are capable of detecting 10–20 pmol of bile acids using an immobilized enzyme, are widely used for clinical analysis of serum bile acids (Okuyama, Kokubun, Higashidate, et al., 1979).

It is not clear if C-2 or C-4 hydroxylated bile acids, present in the neonatal period and under pathological conditions, are measured by this method (see also Section 10.5 for use of other enzymes).

Alternatively, the 3-oxo group can be subjected to a precolumn reaction with *O*-(2-anthrylmethyl) hydroxylamine to produce stable oxime derivatives, which possess a detection limit of 20 fmol (Goto, Saisho and Nambara, 1989). Although oxime derivatives produce two peaks corresponding to the *syn* and *anti* isomers, these two isomers can be eluted as one peak using a phenyl column. This method has contributed to analyses of glycine- and taurine-conjugated 3-dehydrocholic acid and 3-dehydrochenodeoxycholic acid in sera from patients with primary biliary cirrhosis (Goto, Saisho and Nambara, 1991). This reaction should also be useful for analysis of bile acids with 3-oxo-Δ^4 or 3-oxo-Δ^1 structures that occur in the neonatal period and in liver disease. The 3-oxo group can also be reacted with dansyl hydrazine to produce fluorescent derivatives for analysis of serum bile acids with a detection limit of about 0.5 pmol (Kawasaki, Maeda and Tsuji, 1983; Goldsmith, Gruca, O'Halloran, et al., 1994). Obviously, bile acids conjugated at C-3 will not be analysed by these methods without prior cleavage of the conjugates.

10.3.2 Gas–Liquid Chromatography

Gas–liquid chromatography (GC) using a flame ionization detector (FID) has been extensively used for the identification and quantification of individual bile acids in biological fluids. Using capillary columns, the method offers high resolution and a compatibility with electron impact ionization mass spectrometry that is in most cases a necessity for validation of specificity (see Section 10.4.1). Analysis of bile acids with GC, however, requires extensive sample clean-up, cleavage of conjugates (see Section 10.2.3) and conversion of the free acids into volatile and thermally stable derivatives (Blau and King, 1979; Blau and Halket, 1993). Many reviews of the subject have been published during the past 45 years discussing methods of derivatization also Sjövall, Lawson and Setchell (1985); Lawson and Setchell (1988); Setchell and Lawson (1989). In the earliest methods the carboxyl group was protected by methylation and hydroxyl groups were left free, but it was soon realized that protection of hydroxyl groups improved peak shapes and quantification Eneroth and Sjövall (1971); Kuksis (1976). Methylation continues to be the most common method of protecting the carboxyl group but other approaches have been used for specific purposes (see below). Hydroxyl groups were originally protected by acetylation. Trifluoroacetylation and trimethylsilylation were then introduced by E.C. Horning and coworkers and alkylsilylation became the preferred derivatization method (Horning, Brooks and Vanden Heuvel, 1968).

Methylation is best achieved with fresh diazomethane in ether added to a methanol solution of the sample. This method gives the highest specificity for carboxyl groups and side-reactions are few. Formation of methyl ethers is generally not a problem and is further prevented by low reaction temperature, limited concentrations of methanol,

and short reaction times. Oxo groups may react to a minor extent with diazomethane. Other methods have been described which give side-products from bile acids with allylic hydroxyl groups (methanol-HCl) or vicinal hydroxyl groups (dimethoxypropane). The drawback of using diazomethane is its toxicity in preparation and use, and this reagent requires care in handling. The reagent trimethylsilyldiazomethane in toluene is commercially available and appears useful for preparation of bile acid methyl (Me) esters (Alvelius, Hjalmarson, Griffiths, et al., 2001).

Butyl esters have served as an alternative to methyl esters. Tsaconas et al. prepared iso-butyl ester-trimethylsilyl (TMS) ethers for analysis of sterols and bile acids in hepatocyte incubations (Tsaconas, Padieu, Maume, et al., 1986). The advantage of the isobutyl esterification is that the retention times are prolonged and bile acid iso-butyl ester-TMS ether derivatives elute later than the corresponding methyl ester-TMS ethers and are separated from C_{27}-sterol TMS ethers. In this way, prior group separation of bile acids and sterols can be omitted. However, plant sterols still interfere and therefore other investigators have analysed faecal and plasma bile acids as n-butyl ester-TMS or acetate derivatives, which have longer retention times than plant sterol derivatives (Child, Aloe and Mee, 1987; Batta, Salen, Rapole, et al., 1998; Batta, Salen, Rapole, et al., 1999; Batta, Salen, Batta, et al., 2002). A drawback with these methods is the potential formation of artifacts and losses of labile and other bile acid derivatives during the derivatization with n-butanol-HCl at 60°C for 4 h. On the other hand, bile acids fatty acylated at C-3, as occurring, for example, in faeces, might be cleaved by this procedure.

Protection of hydroxyl groups is mostly achieved by alkylsilylations of the esterified bile acids. Perfluoroacyl derivatives are more thermolabile but give high responses with electron capture detection. Acetylation is used by some groups but is both chemically and mass spectrometrically less satisfactory than alkylsilylations. In selected cases, depending on the nature of the bile acid sample, acetates can provide better separations, but have lower sensitivity (Batta, Salen, Batta, et al., 1997; Batta and Salen, 1999), possibly due to adsorptive losses or thermal lability. Protection of carboxyl and hydroxyl groups is generally performed in two steps, but a simple one-step reaction has been used to convert bile acids into hexafluoroisopropyl ester-trifluoroacetyl derivatives using a mixture of anhydrous trifluoroacetic acid and hexafluoroisopropanol (Imai, Tamura, Mashige, et al., 1976).

Trimethylsilyl (TMS) ethers (Fig. 10.9) can be prepared in many different ways. An excellent review on silylation reactions has been published (Evershed, 1993). Hydroxyl groups differ markedly in their rates of silylation, depending upon their nature (primary, secondary, tertiary, equatorial, axial) and differing steric environments. Thus, varying conditions and studying reaction rates can be helpful in

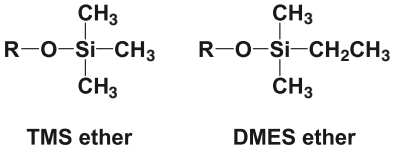


Fig. 10.9 Structures of trimethylsilyl (TMS) ether and dimethylethylsilyl (DMES) ether derivatives of bile acid R

elucidating the identity of unknown GC peaks. This is particularly valuable with mass spectrometric detection, exemplified in studies by Meng et al. of urinary metabolites of ursodeoxycholic acid in pregnant women (Meng, Reyes, Palma, et al., 1997). It should be mentioned that very minor peaks of ethyl and/or TMS esters can be seen as artifacts when methyl ester (Me)-TMS ether derivatives are prepared and analysed.

A common and mild method to prepare TMS ethers is to react the sample with a mixture of distilled dry pyridine, hexamethyldisilazane (HMDS), and trimethylchlorosilane (TMCS), 3:2:1 or 9:3:1 (by vol) with, or without heating. Tertiary hydroxyls (e.g. at C-5) are not, or only partly, derivatized under these conditions. Another volatile reagent is *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Tsaconas, Padieu, Maume, et al., 1986). Trimethylsilylimidazole (TSIM) (Street, Trafford and Makin, 1989) is a forceful reagent but due to its low volatility it has to be removed by solid or gel phase extraction (see below) prior to GC. TMCS can be added to the above reagents to catalyse derivatization of slow-reacting hydroxyls. However, if oxo groups are present in the bile acid, these catalysed reactions can yield enol-TMS ethers and multiple products. This artifact can be avoided by converting the oxo group into an oxime, usually a methyloxime (MO). To do this, the sample is dissolved in 50 μL pyridine with 5 mg methoxyammonium chloride and heated for 30 min at 60°C. Then 50 μL TSIM is added and heating continued at 100°C for 2 h (Thenot and Horning, 1972a). Crowded oxo-hydroxy groups require longer reaction times (3 h and 6 h, respectively, Thenot and Horning, 1972b). The reaction mixture is purified and the derivatives isolated by passage through a small bed of Lipidex 5000 in hexane/pyridine/HMDS 97:2:1 (by vol). The first few milliliters of effluent are taken to dryness under a stream of nitrogen and reconstituted in a suitable volume of solvent (e.g. hexane) prior to GC (Axelson and Sjövall, 1977).

Formation of other alkylsilyl ethers is achieved by analogous reactions to TMS ethers. The reactivities of the reagents will depend on the nature of the alkyl chains, the nature of the hydroxyl groups, and the extent of steric hindrance. The differences in reactivities can be utilized for preparation of mixed TMS/dimethylalkylsilyl ethers. The most commonly used dimethylalkylsilyl derivative is the dimethylethylsilyl (DMES) ether in which one methyl group of a TMS ether is exchanged for an ethyl group (Fig. 10.9). It is prepared using dimethylethylsilylimidazole (DMESIM) (Miyazaki, Ishibashi and Yamashita, 1978) and its advantages are discussed below. *t*-Butyldimethylsilyl (TBDMS) ethers prepared with TBDMS-imidazole are formed only with unhindered hydroxyls.

Numerous GC stationary phases have been used in the analysis of bile acid derivatives. These can be divided into non-selective and selective. The most commonly used liquid phase is a non-selective, nonpolar dimethyl polysiloxane, coated and crosslinked in fused-silica capillary columns. Other non-polar phases are the phenyl methyl polysiloxanes. Selective, more polar phases are useful for separations of isomeric bile acids but several of the sufficiently thermostable phases used in past decades are no longer commercially available. Examples of different phases are given in the tables of MU or RI values in the papers referred to below.

Due to the complex mixtures of bile acids in many sample types, analyses of metabolic profiles (metabolomes) are best carried out on a non-selective nonpolar phase. Inevitably there will be overlaps of some isomers but a change of stationary phase will only result in a different pattern of overlap. When a mass spectrometer is used as the detector it is important to consider background (column bleed), and the less polar stationary phases produce less background.

Using a non-selective nonpolar stationary phase, the retention time of a bile acid derivative is determined mainly by the size and shape of the molecule. For comparison with literature data and calculations of effects of functional groups, retention times are best expressed as methylene units (MU) or Kovats retention indices (RI, corresponds to = 100 × MU). These are obtained by relating the retention times to those of a homologous series of *n*-hydrocarbons. Using a fused-silica capillary column with a dimethyl polysiloxane phase the MU values determined at 270°C for the Me-TMS derivatives of LCA, DCA, CDCA, UDCA and CA are 31.18, 31.83, 32.05, 32.46, and 32.24, respectively (Table 10.2). Thus, the derivative of one of the dihydroxy isomers (UDCA) has a longer retention time than that of the CA derivative (Fukunaga, Hatta, Ishibashi, et al., 1980) because of the orientation of the silylated 7β-hydroxyl. By contrast, the corresponding Me-DMES ether derivatives have MU values of 32.37, 33.93, 34.23, 34.70, and 35.54, i.e. they are eluted according to the number of hydroxyl groups (and molecular weight). The differences in MU values (ΔMU) between the TMS and DMES ethers are 1.19 for the LCA, 2.10 for the DCA, 2.18 for the CDCA, 2.24 for the UDCA, and 3.30 for the CA derivatives, respectively. This shows that, compared with the corresponding TMS ether derivatives, the Me-DMES ether derivatives not only allow the bile acids to be separated into the mono-, di-, and trihydroxylated groups, but also greatly improve the separation of the individual bile acids. In addition, the ΔMU between DMES and TMS ethers are useful for estimating the number of hydroxyl groups in an unknown bile acid using GC. However, as seen from the MU values of the derivatives of dihydroxy acids mentioned above, the molecular shape of the

Table 10.2 Methylene unit (MU) values of methyl ester (Me)-TMS and Me-DMES ether derivatives of bile acids

GLC stationary phase PEG-20			
	TMS	DMES	ΔMU
LCA	37.27	38.29	1.02
DCA	35.98	37.71	1.73
CDCA	36.24	38.12	1.88
UDCA	37.52	39.32	1.80
CA	34.55	37.58	3.03
GLC stationary phase SE-30 (dimethyl polysiloxane)			
LCA	31.18	32.37	1.19
DCA	31.83	33.93	2.10
CDCA	32.05	34.23	2.18
UDCA	32.46	34.70	2.24
CA	32.24	35.54	3.30

isomers also has an important effect on the separations, and overlaps between groups of compounds with different molecular weights may occur depending on the molecular shapes.

RI or MU values are obviously very important for partial characterization of peaks in GC analyses of bile acid derivatives. Extensive tables of such values can be found in the literature both for different derivatives and for different stationary phases (e.g. Iida, Chang, Matsumoto, et al., 1983; Iida, Momose, Tamura, et al., 1987; Lawson and Setchell, 1988b; Dumaswala, Setchell, Zimmer-Nechemias, et al., 1989; Shoda, Osuga, Mahara, et al., 1989; Iida, Komatsubara, Chang, et al., 1990; Iida, Komatsubara, Chang, et al., 1991a,b; Batta, Aggarwal, Tint, et al., 1995; Batta, Salen, Batta, et al., 1997; Meng, Reyes, Palma, et al., 1997; Meng and Sjövall, 1997; Batta and Salen, 1999). RI/MU values for derivatives of bile alcohols and oxysterols have also been published (e.g. Batta, Aggarwal, Mirchandani, et al., 1992; Iida, Hikosaka, Goto, et al., 2001; Shan, Pang, Li, et al., 2003), and these are also valuable when evaluating contributions of different functional groups to the RI/MU values in analyses of bile acids. The absolute RI and MU values for individual compounds will vary between studies depending on differences in column manufacture and analytical conditions (e.g. temperature), but much less so than the absolute retention times or retention times expressed relative to that of a single reference bile acid derivative. However, differences in the values for pairs of compounds differing by a particular functional group (i.e. ΔRI or ΔMU) are usually similar between different studies and important structural information can be gleaned from the RI/MU value of a particular bile acid.

Reactions containing *N*,*O*-bis(diethylhydrogensilyl)trifluoroacetamide in pyridine (60 min at room temperature) convert bile acid methyl esters into diethylhydrogensilyl (DEHS) ethers (Goto, Teraya, Nambara, et al., 1991) (Fig. 10.10). This derivatization results in larger changes in MU values than the corresponding dimethylethylsilyl ether derivatizations; the $\Delta[MU]_{DEHS}$ values of the methyl esters are 1.80 ± 0.26 for monohydroxylated bile acids, 3.32 ± 0.24 for dihydroxylated bile acids, and 4.84 ± 0.25 for trihydroxylated bile acids. These results suggest that the number of hydroxyl groups on the steroid nucleus of a bile acid can be deduced from the ΔMU value following DEHS ether derivatization. In addition, this derivatization allows a discrimination of bile acids that possess either a diequatorial *trans*-glycol or axial-equatorial *cis*-glycol structure at C-3,4 and a diequatorial *trans*-glycol structure at C-6,7 from other bile acids that have isolated hydroxyl groups and/or a diaxial *trans*-glycol group at C-3,4 or C-6,7 based on their markedly smaller

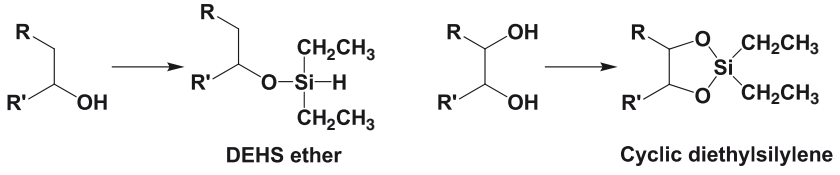


Fig. 10.10 Production of diethylhydrogensilyl (DEHS) ether and the cyclic diethylsilylene derivative

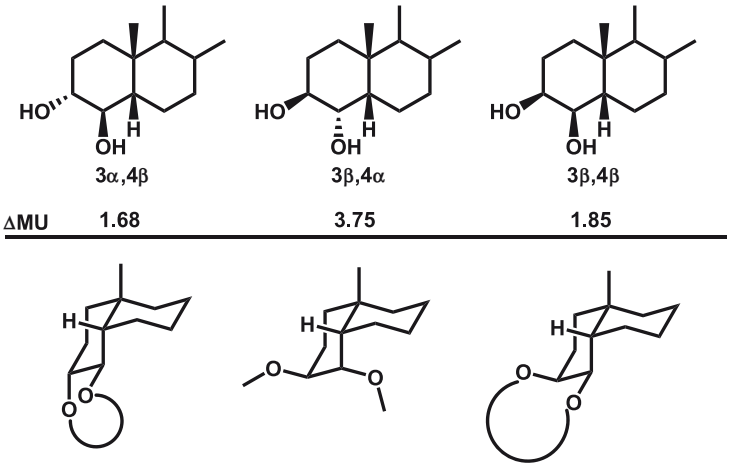


Fig. 10.11 Structures of the A and B ring of 5β-steroids with 3,4-vicinal glycols and the ΔMU values following treatment with *N,O*-bis(diethylhydrogensilyl)trifluoroacetamide as compared with the TMS ether derivatives. The bottom part of the figure shows schematic structures and conformations of the derivatives (cf. Fig. 10.10)

ΔMU values (Goto, Teraya, Nambara, et al., 1991). Derivatization with *N,O*-bis(diethylhydrogensilyl)trifluoroacetamide converts vicinal glycols, except for those with a diaxial *trans* configuration, into cyclic diethylsilylene derivatives (Fig. 10.11) whereas the isolated hydroxyl groups on bile acids are converted into diethylhydrogensilyl ethers. This derivatization method was applied to the separation of C-4- and C-6-hydroxylated bile acids in urine from a newborn infant (Goto, Hasegawa, Nambara, et al., 1992). Acetonide and alkylboronate derivatives have also been used in the GC characterization of bile acids with a vicinal glycol structure (Brooks, Barrett and Cole, 1984; Iida, Komatsubara, Chang, et al., 1991b).

Bile acids containing an oxo group are usually analysed without derivatization of the group. When several oxo groups are present (causing adsorptive losses), or when vigorous conditions of silylation of hindered hydroxyl groups are required (see above) the oxo group(s) is/are converted to an oxime, usually the methyloxime. This may be done as a first step in the analysis (Kimura, Suzuki, Murai, et al., 1997), or after the methylation of the carboxyl group. The peak shift (compared to the silyl ether derivative) caused by derivatization of the oxo group is helpful in confirming the presence of the oxo group(s) and may also result in better separation from potentially co-eluting compounds. Hydroxyl groups are converted into TMS or DMES (Eguchi, Miyazaki and Nakayama, 1990) derivatives. Depending on the positions of the carbonyl group(s), the *syn* and *anti* isomers of the MO derivatives are eluted either together or separately. Most 3-oxo bile acids, including the 7α-ol-3-one, 7β-ol-3-one, 7α,12α-diol-3-one, 3,7-dione, and 3,7,12-trione forms, are separated into the two isomers of the 3-methoxyloxime using a nonselective dimethylpolysiloxane phase, whereas the 3-one, 12α-ol-3-one (broad), and 3,12-dione (broad) forms

produce single peaks. The ethyl ester-MO-DMES ether derivatives of monooxo bile acids are eluted in order of the number of hydroxyl groups in the bile acid.

Conjugated bile acids can also be analysed by GC provided that they do not contain groups that cannot be derivatized, for example, to TMS ethers. Thus, glycine conjugates and glycosidic conjugates can be subjected to GC following derivatization (Marschall, Green, Egestad, et al., 1988; Street, Trafford and Makin, 1989; Iida, Tamura, Chang, et al., 1991; Iida, Tazawa, Tamaru, et al., 1995). However, these have long retention times in the range MU 40–46 and may undergo thermal degradation depending on instrumental (injector or column) conditions. With the advent of LC-MS/MS methods for bile acids (see Section 10.4.2), GC analysis of derivatized conjugated bile acids will have limited practical value.

A flame ionization detector responds in proportion to the carbon-hydrogen content of the molecule. Thus, in analyses with an FID, Me-TMS ether derivatives of bile acids produce about the same peak area per unit mass as the parent underivatized methyl esters. However, this does not take into account the variable (non-linear in the low ng–pg range) adsorptive losses on the column caused by underivatized hydroxyl or oxo groups. In the presence of such groups response factors have to be determined for quantitative analyses. Electron capture detectors (ECDs) have also been used for quantitative gas chromatographic analysis of bile acids. Since bile acids do not have any electron-accepting functional groups, there is a need to derivatize with electron capturing reagents (e.g. trifluoroacetate and pentafluorobenzyl esters) when this method of detection is employed. Such derivatives can permit high sensitivity (pmol level) to be achieved (Kanno, Tominaga, Fujii, et al., 1971). However, ECDs have now been replaced by mass spectrometric detection methods that offer the ultimate sensitivity and specificity (see Section 10.4.1).

10.4 Mass Spectrometry

10.4.1 Gas Chromatography–Mass Spectrometry

10.4.1.1 GC-MS – Qualitative Analysis

While capillary column GC offers much in the way of high resolution compared with other chromatographic techniques commonly used for bile acid analysis, the major drawback of a stand-alone GC technique remains the relative lack of specificity and the uncertainty of accurately assigning bile acid structures based on retention indices alone. While this may not be a major disadvantage in the analysis of pure and simple mixtures of bile acids, it can be a critical deficiency when analyzing more complex biologics, where there is usually a diverse array of positional and stereo-isomers of bile acids, intermediates, and their metabolites, many of which may be difficult to completely separate. Artifacts, plasticizers, and impurities from solvents and reagents may also elute with similar or identical retention times to those of specific bile acids, and the use of FID or ECD is insufficient for the

characterization of the structures of peaks in chromatograms. For this reason, a mass spectrometer coupled to a GC provides the most powerful analytical tool in permitting both the qualitative and quantitative determination of bile acids and related compounds to be made with high sensitivity and specificity (Sjövall, Lawson and Setchell, 1985; Lawson and Setchell, 1988a). While LC-MS has become the more fashionable tool of late, GC-MS remains the workhorse for detailed metabolic profiling of complex mixtures of bile acids (Almé, Bremmelgaard, Sjövall, et al., 1977), and its real value becomes evident when analyzing samples from patients with pathological conditions.

As described above, most bile acids and their conjugates lack sufficient volatility for GC analysis and for this reason require conversion by derivatization (Blau and King, 1979) (Section 10.3.2) to enable passage through the GC column and introduction to the mass spectrometer. Furthermore, derivatization serves to facilitate ionization by directing fragmentation to specific sites or characteristics of the molecule that provide important structural information, such as, number and position of hydroxy or oxo groups, length and type of side-chain, presence or absence of double-bonds, and even stereochemistry of a particular substituent. A good example of the latter is the marked difference in the electron ionization spectra of Me-TMS ether derivatives of the dihydroxy bile acids, chenodeoxycholic acid (3α,7α-dihydroxy-) and ursodeoxycholic acid (3α,7β-dihydroxy-), where major differences are observed in the intensity of the ion at *m/z* 460 due to differences in the ease of loss of the C-7 trimethylsiloxy group (Lawson and Setchell, 1988a). Similarly, more subtle differences in fragmentation are observed as a function of the orientation of the C-5 hydrogen (5α versus 5β). This is evident when comparing the spectra of the Me-TMS ether derivatives of cholic and allocholic acids (Lawson and Setchell, 1988a) or their respective C_{23} analogues, norcholic and allonorcholic acids (Kihira, Shimazu, Kuwabara, et al., 1986). It is this type of information, coupled with retention data, that enables one to make certain assignments of bile acid structures when pure or authentic reference standards are unavailable.

Ionization of the eluting peaks from the GC column is mainly accomplished by two main approaches, and the choice should be based largely upon the type of information and sensitivity required. The two principal ionization modes for GC-MS are electron (impact) ionization (EI) and chemical ionization (CI) and these differ markedly in ionization energy. Where high sensitivity is required for a specific bile acid measurement there may be advantages to using the softer ionization energy imparted by CI (see later) because it tends to yield protonated molecular ions or adducts with little fragmentation thus resulting in a greater signal/noise ratio at higher mass. This approach is helpful in determining the molecular weight of a compound and has advantages for quantification with techniques such as stable-isotope dilution analysis where the selective measurement of a bile acid is performed (a biased analysis). However, if the composition of the biological fluid or extract is largely unknown or is complex, and the analysis is to be 'unbiased', then the higher energy of EI yields greater fragmentation and consequently more comprehensive structural information. For metabolic profiling this is generally the best approach. For this reason, EI has largely been the ionization mode adopted in most GC-MS

applications to bile acid analysis. This section will describe general features of the fragmentation of bile acid derivatives with the main focus on Me-TMS ethers because these have been more favoured for structural elucidation studies as they generally provide more useful information than many other bile acid derivatives. Me-DMES derivatives, which have been preferred in many Japanese studies, give analogous fragmentations to those of Me-TMS ethers. Nevertheless, complementary derivatization methods can be very helpful in elucidating structures of unknown bile acids or bile alcohols and examples of these are mentioned.

Early comprehensive studies of the mass spectrometry of bile acids examined methyl cholanoates only (Sjövall, Eneroth and Ryhage, 1971; Elliott, 1972), and there also exists much information on the fragmentation of acetate and trifluoroacetate derivatives of methylated bile acids (Sjövall, Eneroth and Ryhage, 1971; Galeazzi, Kok and Javitt, 1976; Kuksis, 1976; Szczepanik, Hachey and Klein, 1976; Yousef, Fisher, Myher, et al., 1976; Szczepanik, Hachey and Klein, 1978; Nakashima, Ban, Kuriyama, et al., 1979). Many excellent reviews on this topic have consequently been published (Sjövall, Eneroth and Ryhage, 1971; Elliott, 1980a; Szczepanik, Hachey and Klein, 1976; Elliott, 1980; Lawson and Setchell, 1988a; Sjövall, Lawson and Setchell, 1985) covering most permutations and substitutions of functional groups in the steroid nucleus and side-chain and these should be consulted for more detail. A comprehensive library of the EI (70 eV) mass spectra of Me-TMS ether derivatives of 112 reference bile acids obtained under identical mass spectrometric conditions is published elsewhere (Lawson and Setchell, 1988a,b) accompanied by retention indices. For the purpose of this review, focus is directed to the general and specific mass spectrometric behavior of the bile acids, intermediates and their metabolites that can be expected to be found in biological fluids of humans and some animal species.

Molecular ions are often not observed, or are of low relative intensity in the EI (70 eV) spectra of bile acids; however, lowering the ionization potential (20–30 eV) may enhance the molecular ion due to reduced fragmentation. Often the molecular ion has to be deduced from the [M-15] fragment ion arising from either loss of an angular methyl group, or a methyl from the trimethylsiloxy of the TMS ethers. If this ion is not evident then other fragments, such as loss of a trimethylsiloxy group [M-90] can be used to draw conclusions regarding the mass of the molecular ion. In the case of other silyl ether derivatives, such as alkyldimethylsilyl ethers, characteristic ions are usually formed due to the loss of the alkyl group and this can help in assigning the mass of the molecular ion. For dimethylalkylsilyl ethers (Miyazaki, Ishibashi and Yamashita, 1978; Fukunaga, Hatta, Ishibashi, et al., 1980; Andersson and Sjövall, 1984), the fragment corresponding to the [M-15] loss in the TMS ether is observed at [M-29], [M-43], or [M-57], respectively, for dimethylethylsilyl, dimethylpropylsilyl and dimethylbutylsilyl ethers and overall the fragmentation patterns mimic those observed for TMS ethers. Table 10.3 lists the molecular weights of the parent compound and various derivatives of the main classes of bile acids. Bile acids with unsaturation, or with an oxo group have masses that differ by −2 and +14 Da (Dalton), respectively. For long chain (C_{27}) bile acids, the eight carbon side-chain results in a molecular ion of 42 Da higher mass than the corresponding

Table 10.3 Molecular weights of the major groups of (C_{24}) bile acids and the more commonly used volatile derivatives

Structure[a]	Parent	Me Ester	Me-TMS	Me-DMES
Cholanoic acid (C_{24}) nucleus	360	374	N/A	N/A
Monohydroxy-cholanoic	376	390	462	476
Dihydroxy-cholanoic	392	406	550	578
Trihydroxy-cholanoic	408	422	638	680
Tetrahydroxy-cholanoic	424	436	726	782
Cholestanoic (C_{27}) nucleus	402	416	N/A	N/A
Monohydroxy-cholestanoic	418	432	504	518
Dihydroxy-cholestanoic	434	448	592	620
Trihydroxy-cholestanoic	450	464	680	722
Tetrahydroxy-cholestanoic	466	480	768	824

[a]Where there is unsaturation in the molecule these mass values decrease by 2 Da for each double-bond. Where there is an oxo group the mass values increase by 14 Da.

C_{24} bile acid, while bile alcohols (C_{27} nucleus) that lack the methyl ester are 2 Da lower in mass than the corresponding C_{24} bile acid, and therefore some care is required to avoid confusing neutral sterols with unsaturated bile acids. Where unsaturation is present the chromatographic separation of the bile acid may be minimal relative to the corresponding saturated bile acids under certain conditions, and this is where the specificity of GC-MS is advantageous over GC-FID or ECD for identification.

After establishing the molecular weight, important information can be gleaned regarding the general structure of the side-chain and the number of functional groups in the steroid nucleus. For methyl cholanoates, cleavage of the complete side-chain is a conspicuous fragmentation resulting in a loss of 115 Da somewhere in the mass spectrum, although not usually from the molecular ion, as it is generally accompanied in combination with loss of trimethylsiloxy groups. Differences in the length of the bile acid side-chain, either shorter, or longer lead to a corresponding mass difference in this fragment. Thus, methyl norcholanoates (C_{23} bile acids) that have a four carbon atom side-chain yield a fragment represented by loss of 101 Da, a difference of [-CH_2]. For very short-chain bile acids (referred to as acidic steroids, such as etianic acids up to methyl bisnorcholanoates) with a C_{20}, C_{21}, or C_{22} carbon skeleton the side-chain is not readily cleaved but instead is lost along with the D-ring, carbons C-15 to C-17, and a trimethylsiloxy to produce a fragment at *m/z* 215. By contrast, homo-bile acids (C_{25} bile acids), and cholestanoic acids (C_{27} bile acids) produce fragment losses of 129 and 157 Da, respectively. However, often in cholestanoic acids there may be one or more hydroxyl groups in the side-chain, particularly in clinical cases involving peroxisomal disorders (Hanson, Isenberg, Williams, et al., 1975; Lawson, Madigan, Shortland, et al., 1986; Clayton, Lake, Hall, et al., 1987; Une, Tazawa, Tada, et al., 1987) and in this case one should anticipate facile loss of the side-chain trimethylsiloxy followed by a side-chain fragment loss corresponding to 155 rather than 157 Da somewhere in the fragmentation sequence. Similarly, for neutral sterols and bile alcohols, side-chain fragmentation readily occurs and the

mass of this fragment is highly informative in not only discerning sterols and bile alcohols from plant sterols that have branched side-chain with methyl or ethyl groups, but also in determining the number and position of any hydroxylations in the side-chain. This is exemplified in two clinical scenarios: the characterization of the first case of an oxysterol 7α-hydroxylase (CYP7B1) deficiency in an infant where numerous side-chain hydroxylated bile alcohols lacking a C-7 hydroxy group were characterized (Setchell, Schwarz, O'Connell, et al., 1998), and in the sterol 27-hydroxylase (CYP27A1) deficiency causing cerebrotendinous xanthomatosis (CTX) that is characterized by the synthesis of an array of polyhydroxylated bile alcohols featuring extensive side-chain hydroxylation, except at the C-27 position (Hoshita, Yasuhara, Une, et al., 1980; Karlaganis, Karlaganis and Sjövall, 1984; Une, Shinonaga, Matoba, et al., 1986).

Irrespective of the type of derivative prepared, important general structural information is obtained from the fragment comprising the steroid ABCD rings, after cleavage of side-chain and loss of derivatized nuclear functional groups. Diagnostically significant ions arise at *m/z* 257, 255, 253, or 251, respectively for monohydroxy-, dihydroxy-, trihydroxy, or tetrahydroxy bile acids, or bile alcohols. The presence of unsaturation, or in some cases an oxo group in the nucleus, shifts the mass of this ABCD-ring fragment by −2 Da, so that an unsaturated dihydroxy-cholenoate structure, or an oxo-hydroxy-cholanoate will give rise to the same nuclear fragment (*m/z* 253) to that of a trihydroxy-cholanoate. In most cases a nuclear oxo group shifts the mass of the ABCD-ring ion by +14 Da.

The hydroxy groups in bile acids and alcohols are eliminated under EI conditions as consecutive losses of trimethylsiloxy groups for Me-TMS ether derivatives and a conspicuous pattern of fragments with integers of 90 Da in the spectrum serves to provide reliable information regarding the number of hydroxyls in the parent molecule. In many cases with polyhydroxy compounds loss of the first trimethylsiloxy group may not be obvious and the number of hydroxyl groups in the parent molecule will be underestimated. In such cases, use of ESI- or FAB-MS will provide the correct molecular weight. Alternatively, Me-DMES ether derivatives can produce an intense [M-29] ion which is helpful in assigning molecular weight. Additionally, the preparation of [$^2H_9$]TMS ethers using [$^2H_9$]trimethylchlorosilane/pyridine can be helpful in determining the molecular weight and the number and/or position of hydroxy groups by a shift of 9 Da for each [$^2H_9$]trimethylsiloxy formed (Axelson, Mörk and Sjövall, 1988). In the Me-TMS ethers of tetrahydroxy-bile acids ions at *m/z* 636 ([M-90]), 546 ([M-2 × 90]), 456 ([M-3 × 90]), and 366 ([M-4 × 90]) are seen, consistent with consecutive losses of four trimethylsiloxy groups from the molecular ion, *m/z* 726. Similarly, ions at *m/z* 548 ([M-90]), 458 ([M-2 × 90]) and 368 ([M-3 × 90]) establish a trihydroxy-bile acid structure, and likewise for dihydroxy- and monohydroxy-bile acids or bile alcohols analogous differences are observed. In the case of vicinal substitutions, such as hydroxy groups in positions C-2 and C-3, C-3 and C-4, or C-6 and C-7, a difference of 89 rather than 90 Da occurs during sequential loss of the trimethylsiloxy groups and this is highly informative in providing structural information. For acetates and trifluoroacetates the analogous loss of acetic acid or trifluoroacetic acid, respectively, is observed for

each hydroxyl function. For other alkylsilyl ethers, the same fragmentations occur and the masses of the fragments will reflect the particular alkyl function.

The position and stereochemistry of the hydroxy groups influence the abundance of the fragments, as does the ionization energy. The relative intensity of these fragments can yield important structural information and this is particularly the case when no reference standards are available for comparison. Therefore, on a practical note, where quadrupole analysers are used some care needs to be taken in calibration and set-up to avoid mass discrimination that can alter the relative ratio of low/high mass ions. Definitive identification of a bile acid or bile alcohol requires comparison of both the retention index and the mass spectrum with those of a reference compound. However, in many cases references are unavailable, but the wealth of information that has been amassed in the literature regarding fragmentation of steroids and bile acids (Sjövall, Eneroth and Ryhage, 1971; Elliott, 1972; Szczepanik, Hachey and Klein, 1976; Elliott, 1980a; Sjövall, Lawson and Setchell, 1985; Lawson and Setchell, 1988a) can permit reasonable confidence in assigning structures even in the absence of pure standards.

In humans, and many animal species the majority of bile acids possess hydroxy groups at positions C-3, C-7, and C-12 (Russell and Setchell, 1992). Differences in the stability or lability of the TMS ethers at these positions are reflected by comparisons of the mass spectra of the 3-, 7-, and 12-monohydroxy methyl cholanoates (Lawson and Setchell, 1988a). Marked differences in the relative intensities of the molecular ions (*m/z* 462), and the fragments representing losses of trimethylsilanol ([M-90]), the side-chain ([M-(90−115)]), and the D-ring cleavage ion yielding the ABC ring fragment ([*m/z* 215]) are observed. The stereoisomerism of the C-5 hydrogen also influences the relative intensities of the molecular ion, which when present can be informative in differentiating structures. Comprehensive descriptions of the features of the mass spectra of Me-TMS ethers of bile acids have been published previously (Sjövall, Lawson and Setchell, 1985; Lawson and Setchell, 1988a) and for this reason only key features of the mass spectra that permit elucidation of structures are discussed below, and these are arranged in sequence according to the position and type of substitution on the steroid nucleus and side-chain.

C-1 hydroxylation is a pathway that is prominent in early life (Lester, St. Pyrek, Little, et al., 1983; Setchell, Dumaswala, Colombo, et al., 1988; Shoda, Osuga, Mahara, et al., 1989; Nakagawa and Setchell, 1990; Shoda, Tanaka, Osuga, et al., 1990; Kimura, Yamakawa, Ushijima, et al., 1994; Kimura, Suzuki, Murai, et al., 1997) and is also induced in advanced cholestatic liver disease (Bremmelgaard and Sjövall, 1979; Bremmelgaard and Sjövall, 1980; Shoda, Mahara, Osuga, et al., 1988) as a mechanism to reduce cytotoxicity and facilitate renal excretion of bile acids. Most C-1 hydroxy bile acids are formed of 3,7-dihydroxy-, 3,12-dihydroxy-, or 3,7,12-trihydroxy-cholanoates and, consequently, 16 or 32 stereoisomers can potentially occur, respectively, in biological samples. Pure standards do not exist for all of these isomers; nevertheless, evidence for a C-1 hydroxyl, in the presence of a C-3 hydroxy group, is discernible from an intense *m/z* 217 ion that arises from cleavage of the A-ring analogous to the formation of *m/*z 129 from a 3-trimethylsilyloxy-Δ^5-structure. It should not be confused with the *m/z* 217 of similar structure

that is a base peak with the Me-TMS ether derivatives of intact bile acid glucuronides (and other glycosides), which has an accompanying ion at *m/z* 204 (Almé and Sjövall, 1980). The analogous fragment in the Me-DMES ether derivative is at *m/z* 245 (217 + [2 × CH_2]) (Shoda, Osuga, Mahara, et al., 1989). It is the base peak in the spectra of the derivative of 1β,3α,7α,12α-tetrahydroxy-5β-cholanoic and many other 1β-hydroxy isomers (Almé, Bremmelgaard, Sjövall, et al., 1977; Carlström, Kirk and Sjövall, 1981; Tohma, Mahara, Takeshita, et al., 1985; Lawson and Setchell, 1988a) and consequently when fragment ion recordings of *m/z* 217 (Almé, Bremmelgaard, Sjövall, et al., 1977; Setchell, Dumaswala, Colombo, et al., 1988; Kimura, Yamakawa, Ushijima, et al., 1994), or *m/z* 245 (Tohma, Mahara, Takeshita, et al., 1986; Kimura, Suzuki, Murai, et al., 1997) are plotted after repetitive scan recording, 1β-hydroxy bile acid isomers are easily recognized (Fig. 10.12). Less intense diagnostic ions at *m/z* 182 and 195 are also observed for the Me-TMS ether derivatives of both C-1 and also C-2 hydroxyl structures, these being formed from cleavage of carbons C-9,10 with either C-5,6 or C-6,7, while the presence of an ion at *m/z* 314 confirms two trimethylsiloxy groups in the BCD-rings.

Hydroxylation at C-2 also occurs early in life and 2β,3α,7α,12α-tetrahydroxy-5β-cholanoic acid is a normal constituent of human infant urine (Clayton, Muller and Lawson, 1982; Strandvik and Wikström, 1982; Kimura, Suzuki, Murai, et al., 1997), and was identified in gastric contents of neonates with intestinal obstruction (Clayton, Muller and Lawson, 1982). The chemical synthesis of several 2β-hydroxylated bile acids and characteristics of the mass spectra of the Me-TMS ether derivatives have been reported (Iida, Komatsubara, Chang, et al., 1991a). Like 1β-hydroxy bile acids, ions at *m/z* 182 and 195 are observed, but *m/z* 243 is the most diagnostically important ion and base peak in the 2β,3α,12α-, and 2β,3α,7α,12α-structures, being formed by cleavage across the AB-rings to yield a fragment comprising carbons C-3 to C-7 and the two trimethylsiloxy groups. This fragmentation is also evident in 3,7- and 3,6-dihydroxy bile acids, but is more abundant in the C-2 hydroxy structures and should not be confused with the *m/z* 243 fragment ion arising from loss of side-chain plus C-15 to C-17 and the trimethylsiloxy group in C-15 hydroxylated bile acids. Ions at *m/z* 181 and 271 due to cleavage across C-9,10 and C-5,6 are also of diagnostic help.

Most naturally occurring bile acids, unless they have undergone oxidoreduction, have a hydroxy group at C-3. All four possible isomers of the C-3 monohydroxy bile acid, lithocholic acid, are found in biological fluids of humans and many animal species, and particularly in faeces because of extensive intestinal bacterial degradation of primary bile acids (Setchell, Lawson, Tanida, et al., 1983). The molecular ion of the C-3 monohydroxy-cholanoate Me-TMS ether is more evident with the 5α-H (allo-) than the 5β-H isomers. This rule however does not apply to other monohydroxy bile acids, such as 7α-, 7β-, and 12α-hydroxy-cholanoates. The intensity of the ions at *m/z* 215 and 372 ([M-90]) formed from loss of the trimethylsiloxy group is relatively high when the C-5 hydrogen is orientated 5β, while the ABCD-ring ion at *m/z* 257 is of much lower abundance. Confident identification of the individual isomers in a GC-MS profile is obtained by combining knowledge of these subtle

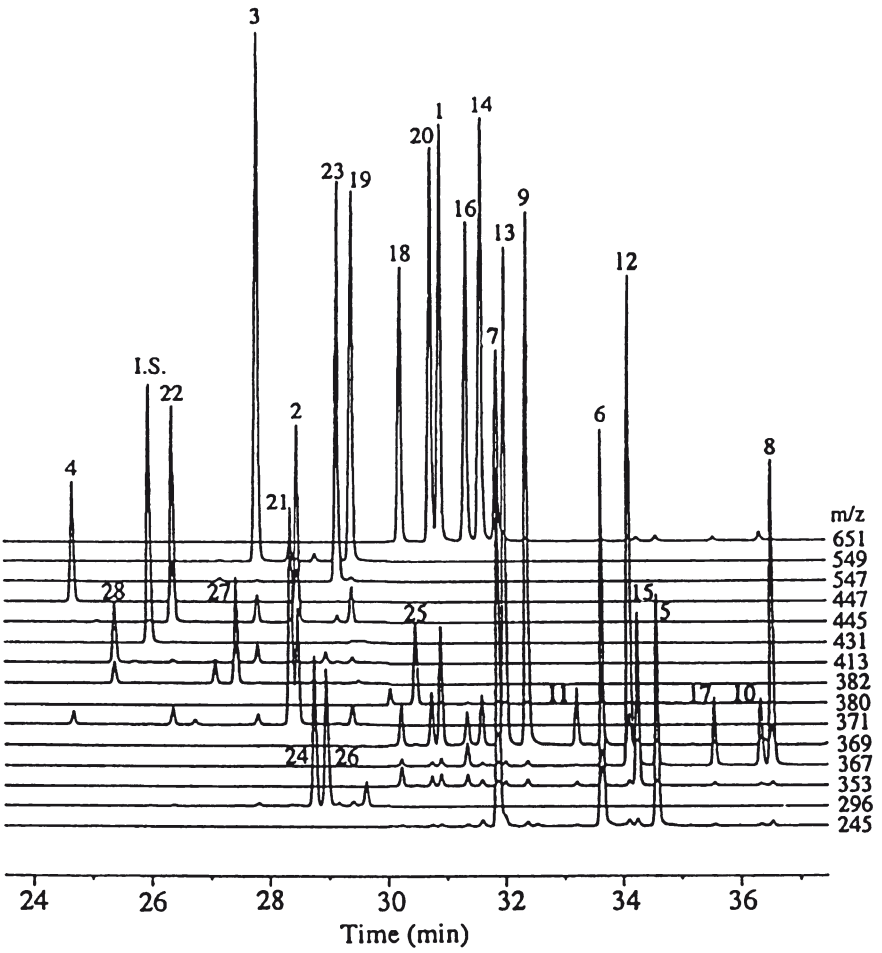


Fig. 10.12 Selection of GC-MS fragment ion profiles of methyl ester-dimethylethylsilyl ether-methyloxime derivatives of bile acids. The following bile acids are indicated by the numbers: 1. cholic; 2. chenodeoxycholic; 3. deoxycholic; 4. lithocholic; 5. 1β,3α,7α,12α-tetrahydroxy-5β-cholanoic; 6. 1β,3α,7α-trihydroxy-5β-cholanoic; 7. 1β,3α,12α-trihydroxy-5β-cholanoic; 8. 2β,3α,7α,12α-tetrahydroxy-5β-cholanoic; 9. 2β,3α,7α-trihydroxy-5β-cholanoic; 10. 3α,4β,7α,12α-tetrahydroxy-5β-cholanoic; 11. 3α,4β,7α-trihydroxy-5β-cholanoic; 12. 3α,6α,7α,12α-tetrahydroxy-5β-cholanoic; 13. hyocholic; 14. 3α,6α,12α-trihydroxy-5β-cholanoic; 15. 3α,7α,12α,19-tetrahydroxy-5β-cholanoic; 16. 3β,7α,12α-trihydroxy-5β-cholanoic; 17. 3β,4β,7α,12α-tetrahydroxy-5β-cholanoic; 18. 3α,7β,12α-trihydroxy-5β-cholanoic; 19. ursodeoxycholic; 20. allo-cholic; 21. allo-chenodeoxycholic; 22. 3β-hydroxy-5-cholenoic;23.3β,12α-dihydroxy-5-cholenoic;24.7α,12α-dihydroxy-3-oxo-5β-chol-1-enoic; 25. 7α,12α-dihydroxy-3-oxo-4-cholenoic; 26. 12α-hydroxy-3-oxo-4,6-choladienoic; 27. 7α-hydroxy-3-oxo-4-cholenoic; 28. 3-oxo-4,6-choladienoic; I.S. 3α,7α-dihydroxy-24-nor-5β-cholan-23-oic (from Kimura, Suzuki, Murai, et al., 1997, with permission)

differences in fragmentation with information on the retention indices that provide a guide to the order of elution (e.g. in the monohydroxy bile acids the elution order is 3α-hydroxy-5α < 3β-hydroxy-5β < 3α-hydroxy-5β < 3β-hydroxy-5α.

Since bile acids are synthesized from cholesterol, which has a 3β-hydroxy-Δ^5 structure, multiple pathways for synthesis lead to structures retaining this unsaturation in the nucleus (Axelson, Mörk and Sjövall, 1988; Axelson and Sjövall, 1990). In early life, the monohydroxy bile acid, 3β-hydroxy-5-cholenoic acid is a normal constituent of biological samples (Makino, Sjövall, Norman, et al., 1971; Back and Ross, 1973) and is easily recognized by a distinct intense ion at *m/z* 129, comprising carbons C-1 to C-3 as well as the trimethylsilanol, and a concomitant loss of 129 Da (*m/z* 331) from the molecular ion. This is also the case for 3β-hydroxy-5-cholestenoic acid, an unconjugated bile acid normally present in human plasma (Axelson, Mörk and Sjövall, 1988). This fragmentation pattern is observed in TMS ethers of sterols as well, but is not a prominent feature when there is substitution at C-7. Because of the Δ^5 double bond, the ABCD-ring fragment is seen at *m/z* 255, and not *m/z* 257 as would be anticipated for a monohydroxy bile acid, and in common with dihydroxy bile acids a fragment ion at *m/z* 249 (CD-rings plus side-chain) is prominent.

Bile acids (C_{24} and C_{27}) and sterols with a 3-oxo group are commonly found either as products of intestinal bacterial biotransformation (Setchell, Lawson, Tanida, et al., 1983; Setchell, Street and Sjövall, 1988), or as intermediates in the pathways for primary bile acid synthesis (Axelson, Mörk and Sjövall, 1988; Axelson, Shoda, Sjövall, et al., 1992). Oxo-bile acids make up a significant proportion of urinary bile acids of healthy infants (Wahlén, Egestad, Strandvik, et al., 1989), infants with advanced liver disease (Clayton, Patel, Lawson, et al., 1988; Kimura, Suzuki, Murai, et al., 1998; Kimura, Mahara, Inoue, et al., 1999) and are the major and diagnostically significant bile acids of patients with liver disease due to a Δ^4-3-oxosteroid 5β-reductase deficiency (Setchell, Suchy, Welsh, et al., 1988). These structures are usually not measured by immuno- or enzyme assays and rarely considered in LC methods. The mass spectrometric behavior of many methyl-oxocholanoates has been extensively reviewed in older literature (Sjövall, Eneroth and Ryhage, 1971; Elliott, 1972; Elliott, 1980a; Lawson and Setchell, 1988; Sjövall, Lawson and Setchell, 1985). In the case of a suspected oxo-group the use of several different and combined derivatives (e.g. methyloxime with Me-TMS or Me-DMES) can be very helpful in structural elucidation (Kimura, Suzuki, Murai, et al., 1997; Almé and Sjövall, 1980; Axelson, Mörk and Sjövall, 1988), while oxidation of any unknown hydroxy to its oxo group can be very informative in assigning the position of the substituent (Carlström, Kirk and Sjövall, 1981). In contrast to the EI spectra of oxo bile acids where the oxo group directs fragmentation (Sjövall, Eneroth and Ryhage, 1971), the -OTMS groups in Me-TMS ether derivatives of oxo-hydroxy bile acids dominate the initial fragmentation sequence. The mass spectra of oxo-hydroxy bile acids are easily distinguishable from bile acids lacking an oxo group by the difference of 14 Da for each substituent. The molecular ion is evident in all but the methyl 3-oxo-12-hydroxy-cholanoates (Lawson and Setchell, 1988a) which otherwise have a clearly discernible [M-15] fragment. The position of hydroxyl groups in 3-oxo structures can be deduced from the difference in relative intensities of the fragments formed from loss of the -OTMS groups and side-chain. This is well illustrated in a compilation of mass spectra of Me-TMS ethers of a series of different positional oxo-bile acids published previously (Lawson and Setchell, 1988a).

In most instances the base peak or dominant ion in the spectrum of 3-oxo bile acids is the loss of -OTMS, or loss of -OTMS + side-chain. The 3-oxo group is readily eliminated under EI as water from several of the fragment ions, while rearrangement loss (retro-Diels-Alder elimination) of the A-ring with the 3-oxo group takes place with a clearly visible loss of 70 Da. This accounts for the ions at *m/z* 201 (*m/z* 271 → 201) in all isomers and *m/z* 316 (*m/z* 386 → 316) in 7-hydroxy-3-oxo structures. In monohydroxy 3-oxo bile acids, an ion at *m/z* 208, described above, characterizes a 12-hydroxy substituent, while *m/z* 229 (ABC-rings + C-18, −90), is observed for 3-oxo-7α-hydroxy- and 3-oxo-6α-hydroxy- structures.

In the pathway for bile acid synthesis, C_{27} sterol intermediates possessing a Δ^4-3-oxo group are formed and these undergo side-chain oxidation to form both cholestenoic (C_{27}) and cholenoic (C_{24}) acids that retain this structure. While 7α-hydroxy-3-oxo-4-cholestenoic acid has been identified in low but significant concentrations in human plasma (Axelson, Mörk and Sjövall, 1988), in patients with liver disease due to a Δ^4-3-oxosteroid 5β-reductase deficiency, 7α-hydroxy-3-oxo-4-cholenoic and 7α,12α-dihydroxy-3-oxo-4-cholenoic acids are the major bile acids in urine (Setchell, Suchy, Welsh, et al., 1988). The Me-TMS ethers of Δ^4-3-oxo C_{24} and C_{27} bile acids generally have a recognizable molecular ion, an intense [M-15], and a dominant ion which is often the base peak representing loss of the -OTMS with or without the side-chain (Axelson, Mörk and Sjövall, 1988; Setchell, Suchy, Welsh, et al., 1988). A prominent ion at *m/z* 269 representing the steroid nucleus after loss of side-chain and trimethylsilanol is visible in the EI spectrum of both the C_{24} and C_{27} acidic and neutral structures. Ions at *m/z* 224 and 209 are indicative of a 7-hydroxy-3-oxo-4-ene structure being formed from cleavage of the C-7,8 and C-9,10 bonds to give a fragment consisting of the AB-rings with and without the angular methyl group. A pair of ions at [M-121] and [M-122] reflecting loss of -OTMS and methoxy group of the methyl ester is diagnostically significant. In the low mass region, ions at *m/z* 174 and 161/162 probably arise from C-ring cleavage after elimination of the C-7 trimethylsiloxy group. These ions serve to distinguish 3-oxo from 7-oxo bile acids which otherwise have similar mass spectra (Lawson and Setchell, 1988a). Conversion of the oxo-group to a methyloxime permits confirmation of the presence of an oxo group by a shift in mass of 29 Da for the molecular ion and a corresponding loss of the methoxy group (−31 Da) from the molecular ion, or in the fragmentation sequence (Almé and Sjövall, 1980; Axelson, Mörk and Sjövall, 1988). In common with neutral steroids (Thenot and Horning, 1972a, b; Bournot and Ramirez, 1989), the formation of a methyloxime of a 3-oxo-4-ene structure yields two peaks due to derivative formation of *syn* and a*nti* isomers (Axelson and Sjövall et al. 1978) but this can be diagnostically helpful in establishing this structural feature in an unknown bile acid. Additionally, the formation of an oxime yields a single or two chromatographic peak, depending on the bile acid structure once this is converted to the TMS ether and an increase in mass of 87 Da. Distinctive fragment ions at [M-89/90] formed from loss of the trimethylsiloxy groups, and lower mass fragment ions become evident for the steroid rings after loss of the trimethylsiloxy group from the oxime. Figure 10.13 is a good example of differences in mass spectra of these different derivatives for a C_{27}, and C_{24} bile acid, and a C_{27} sterol possessing a 3-oxo-4-ene structure (Axelson, Mörk and Sjövall, 1988).

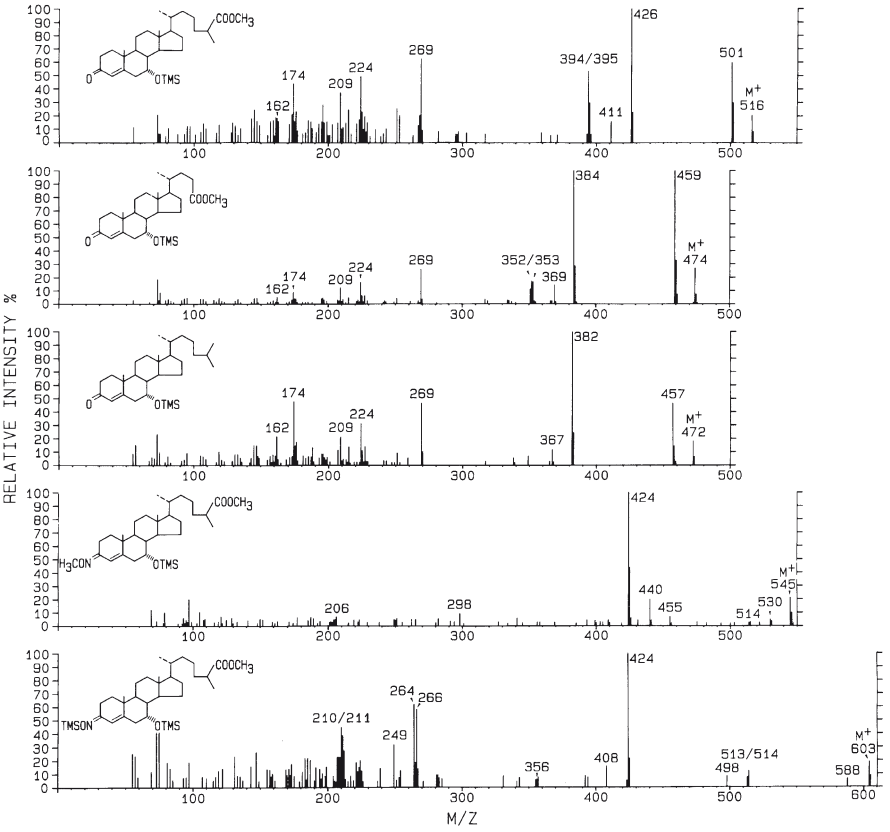


Fig. 10.13 Mass spectra of derivatives of a bile acid in plasma identified as 7α-hydroxy-3-oxo-4-cholestenoic acid (*upper* and the two *bottom* traces). Mass spectra of analogous compounds differing by the side-chain structure are shown for comparison (from Axelson, Mörk and Sjövall, 1988, with permission)

The mass spectrum of the Me-TMS ether derivative of a bile acid with a $\Delta^{4,6}$ -3-oxo bile acid structure (Axelson, Mörk and Sjövall, 1988) reveals fragment ions similar to those with a 7-hydroxy-3-oxo-4-ene structure and some caution is needed in interpreting the significance of their presence because such structures can be formed as artifacts during preparation and work-up of samples. 7α,12α-Dihydroxy-3-oxo-5β-chol-1-enoic acid is present in infant urine (Wahlén, Egestad, Strandvik, et al., 1989; Kimura, Suzuki, Murai, et al., 1997; Kimura, Mahara, Inoue, et al., 1999). The Δ^1 bile acids give interesting spectra. The 3-oxo-Δ^1 structure gives ions at *m/z* 261 and 267, while the 3,7,12-triOTMS-Δ^1 structure gives very intense ions at *m/z* 182 and 195, also seen in C-1,3 trimethylsiloxy structures. The combined presence of a 3-oxo group and unsaturation elsewhere in the bile acid nucleus is rare and more likely to be found as a product of organic synthesis or as an artifact generated during sample preparation. More information on the characteristics of mass spectra of unsaturated bile acids is available elsewhere (Sjövall, Eneroth and Ryhage, 1971; Child, Kuksis and Marai, 1979; Kuksis and Child, 1980; Lawson and Setchell, 1988a).

C-4 hydroxylated bile acids are synthesized in early life and account for 5–15% of the biliary bile acids in early gestation (Setchell, Dumaswala, Colombo, et al., 1988; Dumaswala, Setchell, Zimmer-Nechemias, et al., 1989). These developmentally expressed bile acids are also found in amniotic fluid (Nakagawa and Setchell, 1990), neonatal faeces (Dumaswala, Setchell, Zimmer-Nechemias, et al., 1989) and newborn urine (Kimura, Suzuki, Murai, et al., 1997), and are readily characterized by a unique ion at *m/z* 181 that varies markedly depending on the stereochemistry of the hydroxyls. However, it is the base peak in the spectra of the Me-TMS ether derivatives of 3α,4β,7α-trihydroxy-5β-cholanoate, and 3α,4β,7α,12α-tetrahydroxy-5β-cholanoate. Loss of 129 Da is seen as a feature in the sequential loss of hydroxyl groups throughout all mass spectra of the C-4 hydroxy isomers, because this fragment is formed by a mechanism analogous to that observed for 3β-hydroxy-5-ene structure after elimination of the C-4 trimethylsiloxy group. Ion plots of *m/z* 181 after repetitive scans of GC-MS profiles permit the identification of those bile acids with a C-4 hydroxyl.

C-5 hydroxylated bile acids were found as novel metabolites of exogenously administered 24-norursodeoxycholic and 24-norchenodeoxycholic acids in rodents (Schteingart, Hagey, Setchell, et al., 1993). The mass spectra of the Me-TMS and Me-DMES ethers have been described and predictably the fragmentation patterns were similar for the two derivatives differing by 14 Da due to difference of the $–CH_2$ in the alkyl group. The assignment of a C-5 hydroxy group was based on an 89 Da loss in the cleavage sequence of trimethylsiloxy groups, and from the ion at *m/z* 259 (shifted to *m/z* 287 for the DMES ether). This ion (*m/z* 259) is accounted for by *m/z* 243 + 16 i.e. C-3 to C-7 with the two trimethylsiloxy groups and the underivatized hydroxyl at C-5. This can be confirmed by using stronger conditions for silylation, resulting in the *m/z* 259 ion shifting to *m/z* 331 (259 + 72) as the free hydroxyl group becomes derivatized. This was the approach used to demonstrate 5-hydroxylation of ursodeoxycholic acid in women with intrahepatic cholestasis of pregnancy given ursodeoxycholic acid (Meng, Reyes, Palma, et al., 1997). Ions at *m/z* 243 and 143 presumably are fragments C-3 to C-7 including the 3-trimethylsiloxy-Δ^4 structure with and without loss of the C-5 hydroxy group, and these ions are also observed in cholestane-3,5-diol isomers (Brooks, Henderson and Steel, 1973; Aringer and Nordström, 1981).

C-6 hydroxylation is a major metabolic pathway for bile acid synthesis in several animal species, particularly rats, mice and pigs (Haslewood, 1967; Haslewood, 1971; Haslewood, 1978; Kuramoto, Miyamoto, Konishi, et al., 2000). As with C-1, C-2 and C-4 hydroxylation, the formation of 6-hydroxylated bile acids is prominent in early human life (Setchell, Dumaswala, Colombo, et al., 1988; Kurosawa, Mahara, Nittono, et al., 1989; Shoda, Tanaka, Osuga, et al., 1990; Kimura, Suzuki, Murai, et al., 1997) and in cholestatic diseases (Summerfield, Billing and Shackleton, 1976). Hyocholic acid (3α,6α,7α-trihydroxy-5β-cholanoic) (Almé, Bremmelgaard, Sjövall, et al., 1977) and 3α,6α,12α-trihydroxy-5β-cholanoic (Almé and Sjövall, 1980) are found in the urine of healthy adults. Methods for the synthesis of 6-hydroxylated bile acids have been described (Kurosawa, Mahara, Nittono, et al., 1989). Hydroxylation at C-6 yields distinct and well-recognizable mass spectra (Summerfield, Billing and Shackleton, 1976; Lawson and Setchell, 1988a; Kurosawa, Mahara, Nittono, et al., 1989). The spectra of Me-TMS ethers of

3,6-dihydroxy-cholanoates are discernible from 3,7- and 3,12-dihydroxy bile acids. Spectra and fragmentation of TMS derivatives of 6-hydroxylated steroids and sterols have been studied in great detail (Harvey and Vouros, 1979) and analogous fragmentation pathways are seen with Me-TMS derivatives of 6-hydroxylated bile acids. The molecular ion, in common with most other dihydroxy-cholanoates, is usually absent or very small, but molecular weight can be determined from the [M-15] fragment and from the sequential losses of substituents. Cleavage of the side-chain tends to occur after loss of the trimethylsiloxy groups, and spectra show the expected ABCD-ring fragment at *m/z* 255. The 3,6-dihydroxy-cholanoates, however, are best distinguished from other positional dihydroxy-cholanoates by the diagnostically significant ion at *m/z* 405, which is more intense for the 6β- than the 6α-isomers. This ion is derived from fragmentation across C-1 and C-4 with an intact 3-OTMS [M-145], and the proximity of the C-6 trimethylsiloxy group facilitates this fragmentation. Additionally, a cleavage across the B- and C-rings between C-5,6, C-8,9 and C-12,13 yields a relatively dominant fragment at *m/z* 323. Retention indices become important in assigning exact stereoisomerisms.

Tri- and tetra-hydroxy bile acids with C-6 hydroxyls generally have distinct spectra when there is an accompanying hydroxyl at C-7. While this vicinal, or allylic, structure provides diagnostically important fragmentations of the Me-TMS ethers, the configurations of the 3,6,7-hydroxyls dramatically influence the fragmentation pattern. The most striking feature is a B-ring fission across C-9,10 and C-6,7 bonds to yield an A-ring fragment with the C-6 trimethylsiloxy group intact that is at *m/z* 285. This ion is very intense in the spectra of 3,6α/β,7β isomers and only of much lower intensity in the corresponding 7α isomers. Loss of the trimethylsiloxy group from this fragment generates an ion at *m/z* 195 that is the base peak in the 3,6,7β-stereoisomers, but of minor importance in 7α-isomers such as hyocholic acid (3α,6α,7α-trihydroxy-5β-cholanoate). These general observations apply also to tetrahydroxy-bile acids containing a C-6 hydroxy group, and for polyhydroxylated bile alcohols (Kuroki, Schteingart, Hagey, et al., 1988). If the hydroxyl is moved from C-7 to C-12 (i.e. as in 3,6,12-trihydroxy), then these two diagnostically important fragments are not formed. During sequential cleavage of the trimethylsiloxy groups a loss of 89 Da is observed due to the vicinal arrangement of the 6,7 trimethylsiloxy groups, as in C-2,3 and C-3,4 hydroxylated structures (Sjövall, Eneroth and Ryhage, 1971; Elliott, 1980a). The vicinal hydroxy groups may be further established by alternative derivatization methods, as these structures will form cyclic boronate esters (Brooks, Cole, McIntyre, et al., 1980), or in the case of a *cis* configuration, acetonides (Bremmelgaard and Sjövall, 1980) that generate a distinctive [M-75] loss of 2,2-propanediol, or a [M-58] loss of acetone, respectively. Conversion of vicinal hydroxyl groups to cyclic diethylsilylene derivatives was discussed in Section 10.3.2. Preparation of acetonides has also permitted both nuclear and side-chain stereochemistry of vicinal hydroxyls to be deduced for several bile alcohols with a 3,6,7,25,26-pentol structure (Kuroki, Schteingart, Hagey, et al., 1988).

The presence of a 6-oxo rather than a 6-hydroxy group results in quite different fragmentation. Bile acids with a 6-oxo group are readily enolized with isomerization at C-5. The Me-TMS ether derivative of 6-oxo-lithocholic acid, a major bile acid

of pigs, is quite distinctive from other monohydroxy-oxo in having a base peak at *m/z* 461 [M-15] and very few other ions of abundance. Bile acids with a C-6,7 double bond can be formed during intestinal bacterial 7α-dehydroxylation, and 3α-hydroxy-6-cholenoic acid has been shown to be an intermediate in the formation of lithocholic acid from ursodeoxycholic and chenodeoxycholic acids (Malavolti, Fromm, Nsien, et al., 1993). The presence of a C-6,7 double-bond induces cleavage through the B-ring and formation of an ion at *m/z* 249 (shifted to *m/z* 247 in the presence of a C-12 hydroxyl group) and the ABCD-ring fragment is 2 Da lower than expected for the number of hydroxyls (Kelsey, Mui and Elliott, 1971; Sjövall, Eneroth and Ryhage, 1971; Child, Kuksis and Marai, 1979; Lawson and Setchell, 1988a).

With the exception of secondary bile acids that are formed by intestinal bacterial 7α-dehydroxylation (Setchell, Lawson, Tanida, et al., 1983; Setchell, Street and Sjövall, 1988), almost all bile acids and bile alcohols possess a substituent at C-7, and it is usually accompanied by a C-3 hydroxyl, or oxo group. C-7 Monohydroxy- or 7, 12-dihydroxy- bile acids are to our knowledge not normally found in biological fluids but can be products of chemistry. In Me-TMS ethers the fragmentation of the C-7 trimethylsiloxy group is unremarkable but also influenced by its stereochemistry. In 3,7-dihydroxy-cholanoates, the side-chain is not readily lost and consequently *m/z* 370 ([M-2 × 90]) becomes a prominent feature of the spectrum. Loss of the side-chain then yields the ABCD-ring fragment at *m/z* 255. The corresponding fragment is at *m/z* 253 in 3,7,12-trihydroxy isomers. The M-90 fragment (*m/z* 460) is considerably more intense when the configuration is 7β and this permits clear recognition of UDCA while being of help in assigning stereochemistry of other 7β-hydroxy isomers. A distinctive quartet of ions at *m/z* 243 (AB-ring rupture with 3,7-OTMS), *m/z* 249 (CD-rings plus side-chain), *m/z* 255 (ABCD-rings) and *m/z* 262 (CD-rings and C-7 plus side-chain) permit the C-3,7 dihydroxy bile acids to be differentiated from 3,6- and 3,12-dihydroxy structures. Major differences in the fragmentation of Me-TMS ethers of 3,7-dihydroxy bile acids and sterols are seen with the introduction of a double-bond at C-5,6. Bile acids with a 3β,7α-dihydroxy-Δ^5 structure are normal constituents of human blood (Axelson, Mörk and Sjövall, 1988), and are the major bile acids found as sulphate and glyco-sulphate conjugates in the urine of patients with cholestatic liver disease due to a deficiency in 3β-hydroxy-Δ^5-C_{27}-steroid oxidoreductase (Clayton, Leonard, Lawson, et al., 1987). Unless mild conditions for hydrolysis/solvolysis and sample work-up are used, degradation of the allylic hydroxyl readily occurs and artifacts arise (Clayton, Leonard, Lawson, et al., 1987; Ichimiya, Egestad, Nazer, et al., 1991). Methyl ethers are readily formed if samples are stored for lengthy periods in methanol. The facile loss of the C-7 trimethylsiloxy group yields an intense and dominant fragment that is the base peak in Me-TMS ether derivatives of 7α-hydroxycholesterol (*m/z* 456, [M-90]), 3β,7α-dihydroxy-5-cholenoic acid (*m/z* 458, [M-90]), and 3β,7α-dihydroxy-5-cholestenoic acid (*m/z* 500, [M-90]) derivatives, few other fragments being apparent (Axelson, Mörk and Sjövall, 1988).

An oxo group at position C-7 is readily lost as water under EI conditions leading to prominent ions at [M-18] and [M-18 + 90] for Me-TMS ether derivatives of

7-oxo bile acids (Lawson and Setchell, 1988). This water loss is also apparent throughout the fragmentation sequence. Furthermore the 7-oxo group promotes B-ring fragmentation with cleavage across bonds C-5,6 and C-9,10 to produce distinctive ions at *m/z* 292/293. These ions help to differentiate a 7-oxo-3-hydroxy- from a 3-oxo-7-hydroxy bile acid. As discussed above, conversion to methyloxime or oxime derivatives can also aid in structural elucidation.

A bile acid with a C-7,8 double-bond was identified in the bile of a fish (Kallner, 1968). The methyl ester acetate and trifluoroacetate have been studied (Child, Kuksis and Marai, 1979) and no diagnostically significant ions are evident for a Δ^7 structure but the lack of an ion at *m/z* 247 distinguishes it from a Δ^6 bile acid where this fragment is present. It should be noted that the Δ^7 bond can readily isomerize to a $\Delta^{8(14)}$ bond, and the mass spectrum of this structure shows a pronounced loss of 142 Da due to the cleavage of the side-chain with C-16 and C-17, facilitated by an allylic cleavage of the C-15,16 bond. Bile acids with a double-bond at the C-7,8 position may be synthesized by patients with the Smith–Lemli–Opitz syndrome (SLO) (Smith, Lemli and Opitz, 1964), a condition caused by a deficiency in 3β-hydroxysterol Δ^7 reductase (Honda, Tint, Salen, et al., 1995; Shefer, Salen, Batta, et al., 1995). The accumulated Δ^7 sterol intermediates that characterize this defect become substrates for 27-hydroxylation (Honda, Salen, Shefer, et al., 1999) and subsequent side-chain oxidation leads to formation of 3β-hydroxy-cholest-5,7-dienoic acid and 3β-hydroxy-cholest-5,8-dienoic acid. Both bile acids were identified as metabolites in liver homogenates from rats treated with MB 15.766, an inhibitor of the Δ^7 reductase. The main features of the spectra of the Me-DMES ethers were tabulated (Honda, Tint, Salen, et al., 1995). The base peak in the spectra of both compounds was at *m/z* 409 [M-119] and an intense ion at [M-104] from loss of dimethylethylsilanol is observed. The presence of these unsaturated bile acids in the urine of SLO patients was proposed from FAB-MS (Natowicz and Evans, 1994); however, this is an unreliable means of structural identification and to our knowledge structural confirmation of these bile acids has yet to be published. By contrast, the mass spectrometric characteristics of Δ^7 and Δ^8 sterols are well documented (Axelson, 1991; Wolthers, Walrecht, van der Molen, et al., 1991; Tint, Irons, Elias, et al., 1994). These dehydrocholesterols have been identified in plasma from healthy adults (Axelson, 1991), patients with SLO syndrome (Tint, Irons, Elias, et al., 1994) and patients with CTX disease (Wolthers, Walrecht, van der Molen, et al., 1991). The mass spectra of TMS ether derivatives of Δ^7 and Δ^8 sterols are remarkably similar, as are the $\Delta^{5,7}$ and $\Delta^{6,8}$ sterols. In the latter two structures the base peak in the TMS ethers is at *m/z* 351[M-131]. An important comprehensive study of mass spectra of 26 unsaturated C_{27} sterols as their acetate and TMS ether derivatives has been published, from which the influence of double bonds in different positions on the fragmentation can be seen (Gerst, Ruan, Pang, et al., 1997). As with bile acids, the ABCD-ring fragment after loss of the trimethylsiloxy group and side-chain is 2 Da lower for each double-bond than would be expected for a saturated monohydroxy structure and care is needed in interpretation to avoid confusion with the possible presence of additional trimethylsiloxy groups in the steroid rings.

Hepatic 12-hydroxylation serves to direct bile acid intermediates into the cholic acid pathway, a major pathway for bile acid synthesis in most animal species

(Russell and Setchell, 1992). C-12 Monohydroxy bile acids do not naturally occur, while 3α,12α-dihydroxy-5β-cholanoate (deoxycholic) is the major secondary bile acid in humans and the predominant bile acid in faeces (Setchell, Lawson, Tanida, et al., 1983; Setchell, Street and Sjövall, 1988). In rats and mice, deoxycholic acid is found only in traces, because unlike in humans, it is efficiently 7α-hydroxylated to form cholic acid (Bergström, Rottenberg and Sjövall, 1953). There are eight possible isomers of 3,12-dihydroxy-cholanoates and the mass spectrum of each is remarkably similar (Lawson and Setchell, 1988). This makes distinguishing isomers in a complex GC-MS profile difficult and places an emphasis of utilizing retention indices to complement subtle differences in the mass spectrometric data. The base peak in Me-TMS ethers of all eight isomers is at *m/z* 255 and it is formed by the double elimination of the trimethylsiloxy groups and facile loss of the side-chain. Some differences are observed in the relative abundances of the ions. The C-12 trimethylsiloxy group is lost before the C-3, and in 5α-(allo) isomers, because the latter is less readily eliminated, the *m/z* 345 ion is higher in abundance relative to the *m/z* 370 ion and this can be of help in distinguishing 5α-isomers. As mentioned earlier, this also holds true for 3,7,12- trihydroxy structures where the analogous fragment at *m/z* 343 is of higher abundance than the *m/z* 368 ion in the allo-series (Kihira, Shimazu, Kuwabara, et al., 1986). An exception to this general rule is the 3β,12β-dihydroxy-5β isomer where the intensity of *m/z* 345 > 370 (Lawson and Setchell, 1988a). C-ring cleavage results in the formation of a fragment at *m/z* 208 comprising the D-ring, C-12 and the side-chain and this is a prominent ion that is diagnostically significant for all 12-hydroxylated bile acids, although it tends to be of lower intensity in trihydroxy-cholanoates containing a trimethylsiloxy group at C-7.

Bile acids having a 12-oxo group are normal metabolites found in faeces from humans and animals (Setchell, Lawson, Tanida, et al., 1983; Setchell, Street and Sjövall, 1988; Tandon, Axelson and Sjövall, 1984). These are easily recognized from several characteristics, most notably a D-ring cleavage with a McLafferty rearrangement resulting in loss of 155 Da and production of the ion *m/z* 321 in oxo-monohydroxy bile acids and the subsequent loss of a trimethylsiloxy to give an ion at *m/z* 231, that is the base peak in methyl 3α-hydroxy-12-oxo-5β-cholanoate.

Hydroxylation at C-15 is not common to humans but has been shown to occur in other species (Lund, Boberg, Byström, et al., 1991; Mikami, Ohshima, Mosbach, et al., 1996; Kakiyama, Iida, Goto, et al., 2006; Kakiyama, Tamegai, Iida, et al., 2007). The Me-TMS ether derivative of C-15 hydroxy bile acids shows an ion at *m/z* 243, containing C-15 with its -OTMS group, C-16, C-17 and the side-chain. An accompanying loss of 245 Da from the molecular ion yields a fragment at *m/z* 393 that confirms the C-ring substitution. This is observed in the spectrum of the Me-TMS derivative of 3α,7α,15β-trihydroxy-5β-cholanoic acid (Carlström, Kirk and Sjövall, 1981). Recently, the taurine conjugate of 3α,7α,15α-trihydroxy-5β-cholanoic acid was described as a normal constituent of the bile of swans, tree ducks and geese. Its identification was based upon LC-MS and NMR (Kakiyama, Iida, Goto, et al., 2006).

Pythocholic acid, so named by Haslewood and Wootton because it was first isolated from snakes (Haslewood, 1951; Haslewood and Wootton, 1951), is a C-16 hydroxylated deoxycholic acid derivative. Almost 50 years later, the 16-hydroxy

analogue of chenodeoxycholic acid was found in the bile of some birds (Hagey, Schteingart, Ton-Nu, et al., 2002) and the chemical synthesis and chemical properties of a series of 16-hydroxy bile acids were described (Iida, Hikosaka, Kakiyama, et al., 2002). The mass spectrum of the Me-TMS ether derivative of 3α,7α,16-trihydroxy-5β-cholanoic acid is similar to that of 3,7,12-trihydroxy bile acids, save a significant ion at *m/z* 129 and a corresponding loss of 129 Da to yield an ion at *m/z* 329 in the fragmentation sequence. This ion was suggested to be derived from cleavage of the side-chain with C-17. It seems more likely that C-18 was lost together with the side-chain. Confirmation of the 16-hydroxyl was obtained by the formation of a lactone (Hagey, Schteingart, Ton-Nu, et al., 2002) that cyclized the 16-hydroxyl with the carboxylic acid, an approach originally adopted in the identification of pythocholic acid in 1951 (Haslewood, 1951).

Hydroxylation at C-19, was first shown to take place *in vitro* in human foetal liver homogenates following the identification of 3α,19-dihydroxy-5β-cholanoic acid as a metabolic product of lithocholic acid (Gustafsson, Anderson and Sjövall, 1987). A characteristic fragment in the spectrum of the Me-TMS ether of this bile acid was a base peak at *m/z* 357 [M-(90 + 103)], corresponding to the consecutive losses of two derivatized hydroxyls, one of which must be a primary hydroxyl and therefore located at either of the C-18, or C-19 angular methyl groups. A loss of 103 Da is common to TMS ethers of steroid structures possessing a primary hydroxyl (Setchell, Almé, Axelson, et al., 1976). The identification of 19-hydroxy-lithocholate was assumed from the ion at *m/z* 249 which established a lack of hydroxylation in the CD-rings or side-chain. Many years later a series of C-19 hydroxylated C_{24} bile acids were chemically synthesized and features of the mass spectra of both Me-TMS and Me-DMES ethers described (Kurosawa, Nomura, Mahara, et al., 1995). The identity of 19-hydroxy-lithocholic acid, originally isolated from foetal liver homogenates (Gustafsson, Anderson and Sjövall, 1987), was corroborated from comparison of the Me-TMS of the pure reference standard with the published data. The fragmentation patterns of the Me-DMES ethers are essentially identical to those of Me-TMS ethers, save a difference of 14 Da reflecting the mass difference in alkylsilyl groups. Consequently, loss of the derivatized C-19 hydroxy is revealed from a loss of 117 Da in the DMES ether rather than 103 Da in TMS ethers. In this study, 3α,7α,12α,19-tetrahydroxy-5β-cholanoic was identified in human neonatal urine from comparison of the Me-DMES derivative with that of the reference compound, and this bile acid accounted for 1.5–7% of the total bile acids excreted (Kurosawa, Nomura, Mahara, et al., 1995).

Modifications to the normal five-carbon atom bile acid side-chain can be distinguished by mass spectrometry. Such modifications, which include hydroxylations, unsaturation, and differences in chain length, arise during the normal pathway for bile acid synthesis and also as a result of defects in cholesterol and bile acid biosynthesis and metabolism. For the Me-TMS ether derivative, the length of the side-chain can be deduced from the molecular ion, or when absent, the [M-15], or [M-90] fragments, and the mass difference in the fragment ions that eliminate the side-chain. A loss of 115 Da in the fragmentation sequence signifies a normal unsubstituted cholanoic acid (C_5) side-chain methyl ester, while analogous losses

of 101, 129 and 157 Da establish nor-bile acid (C_{23}) (Shalon and Elliott, 1976; Almé, Bremmelgaard, Sjövall, et al., 1977), homo-bile acid (C_{25}) (Cohen, Tint, Kuramoto, et al., 1975), and cholestanoic acid (C_{27}) (Hanson and Williams, 1971) structures, respectively.

The C_{27} bile acids, 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA, referred to as coprostanic acid in older literature (Eyssen, Parmentier, Compernolle, et al., 1972)) and 3α,7α-dihydroxy-5β-cholestanoic acid (DHCA) are key intermediates in the normal synthesis of bile acids in humans and most animals (Russell and Setchell, 1992), but accumulate in high concentrations in urine, serum and bile of patients with disorders of peroxisomal β-oxidation. Features of the mass spectra of the free and derivatized compounds have been well described (Hanson and Williams, 1971; Eyssen, Parmentier, Compernolle, et al., 1972; Kamat and Elliott, 1972; Kuramoto, Kikuchi, Sanemori, et al., 1973; Mui, Kamat and Elliott, 1974; Hanson, Isenberg, Williams, et al., 1975). The fragmentation patterns of the Me-TMS ethers resemble those of the corresponding C_{24} bile acids. The molecular ions are either absent, or of low intensity and consecutive losses of the trimethylsiloxy groups result in prominent and diagnostically significant ions at *m/z* 410 ([M-3 × 90]) and 412 ([M-2 × 90]) for THCA and DHCA, respectively. A loss of 157 Da due to the C_8-side-chain leads to the expected ABCD-ring ions typical of trihydroxy- and dihydroxy-structures (*m/z* 253 or 255, respectively). There are potentially numerous isomers of cholestanoic acids and in the absence of pure reference compounds for comparison it is suggested that the general rules that apply to the fragmentation of C_{24} bile acids discussed earlier, be used to help in assignment of stereochemistry of functional groups in the steroid nucleus. Furthermore, due to asymmetric carbons in the side-chain, diastereoisomers exist for cholestanoic acids and some care is required in sample work up, particularly avoidance of strong alkaline hydrolysis, to prevent racemization of chiral centres. The synthesis and detailed characterization of a number of diastereoisomers of cholestanoic acids have been reported (Kihira, Morioka and Hoshita, 1981; Kurosawa, Sato, Nakano, et al., 1996). Analysis of Me-TMS ethers and related derivatives is not particularly helpful in assigning configuration because the diastereoisomers are difficult to chromatographically resolve. Other complementary approaches are preferable, including LC-MS, NMR and chemistry. For THCA and DHCA both (25*R*)- and (25*S*)-diastereoisomers are formed in humans; however it is only the (25*S*)-diastereoisomer that is the substrate for the branched chain acyl-CoA oxidase that initiates side-chain oxidation and formation of primary bile acids (Pedersen, Veggan and Björkhem, 1996; Van Veldhoven, Croes, Asselberghs, et al., 1996). Mutations in the gene encoding the 2-methylacyl racemase responsible for racemization of (25*R*)-3α,7α-dihydroxy- and (25*R*)-3α,7α,12α-trihydroxy-5β-cholestanoic acids have been shown to cause liver disease in early life (Setchell, Heubi, Bove, et al., 2003) and neurologic disease in adults (Ferdinandusse, Denis, Clayton, et al., 2000). Therefore, the ability to define the absolute configuration of C-25 of the cholestanoic acids is important in the diagnosis of this genetic defect in bile acid synthesis, where only the (25*R*) enantiomer is found in serum and bile (Setchell, Heubi, Bove, et al., 2003). This is particularly important because the urinary and serum bile acid

profile of these patients resemble those of patients with Zellweger syndrome, however the latter synthesize both (25*R*)- and (25*S*)-diastereoisomers in an approximate ratio of 2:1. Therefore, differential diagnosis requires measurement of both diastereoisomers and this is best accomplished by use of LC-MS where chromatographic resolution is possible (Une, Tazawa, Tada, et al., 1987; Ferdinandusse, Overmars, Denis, et al., 2001; Setchell, Heubi, Bove, et al., 2003).

A unique C_{29} dicarboxylic acid is consistently found in the serum of infants with Zellweger syndrome (Parmentier, Janssen, Eggermont, et al., 1979) and the mass spectra of its methyl ester acetate and trimethylsilyl ether derivatives were originally reported by Parmentier, Janssen, Eggermont, et al. (1979). In the Me-TMS ether, a molecular ion was evident at *m/z* 752, and ions at *m/z* 737 and 721 signified losses of methyl and methoxy groups, respectively. Three consecutive losses of trimethylsiloxy groups led to a diagnostically important fragment at *m/z* 482 (also observed in the acetate derivative) and the difference in mass of this fragment from the ABCD-ring ion at *m/z* 253 established a side-chain of mass 229 Da. A C_{29} dicarboxylic acid was suspected and verified after formation of ethyl esters, which led to shift in the molecular ion of 28 Da, and not 14 Da as would be expected with only one terminal carboxylic acid. This is a good example of how the use of complementary derivatives can be of value in structural elucidation. NMR spectrometry confirmed the mass spectrometric interpretation of 3α,7α,12α-trihydroxy-27a,27b-dihomo-5β-cholestane-26,27b-dioic acid (Parmentier, Janssen, Eggermont, et al., 1979; Janssen and Parmentier, 1981; Janssen, Toppet and Parmentier, 1982). Later, the [^{14}C] labelled analogue was synthesized and characterized by mass spectrometry and NMR (Parmentier, Busson, Janssen, et al., 1993).

Hydroxylation of the side-chain is not that common for cholanoic acids or bile acids of shorter side-chain length, but for cholestanoic acids and particularly bile alcohols, extensive side-chain hydroxylation can occur. There is a wealth of literature on the mass spectrometric features of side-chain hydroxylated bile alcohols (Tint, Dayal, Batta, et al., 1978; Karlaganis, Karlaganis and Sjövall, 1984; Kuwabara, Ushiroguchi, Kihira, et al., 1984; Kuroki, Shimazu, Kuwabara, et al., 1985; Shimazu, Kuwabara, Yoshii, et al., 1986; Hiraoka, Kihira, Kajiyama, et al., 1987; Une and Hoshita, 1994) because these are found in high concentrations as glucuronides and sulphates in the urine, bile and plasma of patients with the rare lipid storage disease of CTX. This genetic defect in bile acid synthesis, caused by mutations in the sterol 27-hydroxylase gene (*CYP27A1*), results in markedly impaired primary bile acid synthesis and a compensatory production of an array of bile alcohols with side-chain hydroxylations at most carbon positions, except C-27 (Karlaganis and Sjövall, 1984). The typical side-chain peaks for TMS ethers of C_{27}-sterols are *m/z* 173 for the 22-hydroxy-, 159 for the 23-hydroxy-, 159 and 145 for the 24-hydroxy-, 131 for the 25-hydroxy- and no significant diagnostic peak for the 27-hydroxysterols. When several hydroxyl groups are in the side-chain, the fragment ions are either increased by 88 Da or decreased by 2 Da due to addition or loss of the additional -OTMS group. Vicinal -OTMS substituents are indicated by a pronounced fragmentation between the two groups (e.g. a very intense *m/z* 131 in spectra of sterols with a 24,25-diOTMS substitution). Similarly, side-chain hydroxylation of

cholestanoic acids is a characterisitic feature of patients with peroxisomopathies (Hanson and Williams, 1971; Parmentier, Janssen, Eggermont, et al., 1979; Clayton, Lake, Hall, et al., 1987). Structural elucidation is facilitated by conversion to the methyl ester trimethylsilyl ether derivative that generally directs side-chain cleavage *alpha* to the trimethylsiloxy group under EI conditions, or it may lead to its migration to the nucleus.

The Me-TMS ether derivative of a proposed C-21 hydroxylated metabolite of orally administered ursodeoxycholic acid showed a mass spectrum consistent with a trihydroxy-cholanoic acid but with a prominent fragment ion at *m/z* [M-103] assumed to represent the loss of the primary trimethylsiloxy group at the C-21 position. The base peak in the spectrum was at *m/z* 329 (Koopman, Wolthers, van der Molen, et al., 1987).

In C_{27} bile acids, hydroxylation at C-22 results in an intense fragment ion at *m/z* 217 containing the distal part of the side-chain and the -OTMS group after cleavage across the C-20,22 bond. Further fragmentation of this side-chain ion with loss of 90 and 32 Da results in low intensity ions at *m/z* 127 and 185, respectively. Fragmentation patterns denoting functional groups in the steroid rings follow the general principles described above for other bile acids. Thus, 3α,7α,12α,22-tetrahydroxy-5β-cholestanoic acid, identified in some reptiles (Haslewood, Ikawa, Tökés, et al., 1978), shows a typical ABCD-ring ion at *m/z* 253 after loss of the three nuclear trimethylsiloxy groups and side-chain. The 22-hydroxyl will also form a lactone (Haslewood, Ikawa, Tökés, et al., 1978), which after cleavage of the C-20,22 bond yields an ion at *m/z* 113 for the lactone structure. The formation of lactones of two unknown urinary bile acids was used as a means of confirming 22-hydroxylation. The mass spectrum (mass range 200–600 Da only) of 3α,7α,12α,22-tetrahydroxy-5β-cholestano-26,22-lactone formed during alkaline hydrolysis of a urine extract from a Zellwegers patient served to establish the identity of 3α,7α,12α,22-tetrahydroxy-5β-cholestanoic acid as one of the urinary bile acids, the other was proven to be 23-hydroxy analogue (Une, Tsujimura, Kihira, et al., 1989). Interestingly, reduction of the lactones with lithium aluminium hydride yielded the 5β-cholestane-3α,7α,12α,22,26-pentol and 5β-cholestane-3α,7α,12α,23,26-pentol structures, respectively, and the TMS ether derivatives then gave highly informative side-chain cleavage ions at *m/z* 171 and 157, respectively. These fragments are typical for bile alcohols with a 22,26- and 23,26-*bis*-trimethylsiloxy groups and definitively establish the position of hydroxylation while illustrating the value of performing additional chemical reactions on unknowns to facilitate identification.

In the spectrum of the Me-TMS ether of 22-hydroxy-chenodeoxycholic acid ((22*S*)-3α,7α,22-trihydroxy-5β-cholanoic acid) an intense ion is observed at *m/z* 284 corresponding to loss of the trimethylsiloxy groups and carbons C-22 to C-24 with transfer of a hydrogen to the ABCD-ring nucleus (Kihira, Morioka and Hoshita, 1981). A triplet of ions at *m/z* 253, 254 and 255 represent the ABCD-ring structure after migration of one or two hydrogens from the nucleus to the side-chain fragment and this is distinctive for this structure. An anticipated intense fragment at *m/z* 175 (likely to be the base peak) generated by cleavage *alpha* to the C-22 trimethylsiloxy group was not apparent because the reported spectrum showed only

ions above *m/z* 200 (Kihira, Morioka and Hoshita, 1981). However, this fragment is the base peak in 22-hydroxy-ursodeoxycholic acid (3α,7β,22-trihydroxy-5β-cholanoic acid), a metabolite of orally administered ursodeoxycholic acid in patients with CTX (Koopman, Wolthers, van der Molen, et al., 1987).

Bile acids with an unsaturated side-chain have been found in many GC-MS studies of bile (Kern, Eriksson, Curstedt, et al., 1977; Kuriyama, Ban, Nakashima, et al., 1979; Nakashima, Ban, Kuriyama, et al., 1979; Parmentier, Janssen, Eggermont, et al., 1979; Noma, Une, Kihira, et al., 1980; Ali, Stephenson and Elliott, 1982), but one should always be cautious in interpreting their significance because depending upon the methods used for extraction, solvolysis, hydrolysis and purification, these can be artifacts. The formation of bile acids with a C-22,23 double-bond is a quantitatively important pathway for bile acid synthesis in rats (Thompson, Davis and Morris, 1993; Setchell, Yamashita, Rodrigues, et al., 1995; Rodrigues, Kren, Steer, et al., 1996), but one that is seemingly rarely considered by researchers studying rodent bile acids. Most LC methods used for biliary bile acids are unable to separate the Δ^{22} metabolites from saturated analogues. Synthesis of Δ^{22} bile acids is specific for bile acids possessing a 7β-hydroxyl group and consequently tauro-Δ^{22}-β-muricholic and tauro-Δ^{22}-ω-muricholic acids are normal constituents of rat bile and plasma (Thompson, Davis and Morris, 1993; Setchell, Yamashita, Rodrigues, et al., 1995; Rodrigues, Kren, Steer, et al., 1996). Furthermore, Δ^{22}-ursodeoxycholic acid is a major metabolite in the bile, plasma, intestinal contents and liver of Sprague-Dawley rats administered UDCA (Setchell, Yamashita, Rodrigues, et al., 1995; Rodrigues, Kren, Steer, et al., 1996). The mass spectra of the Me-TMS ether of Δ^{22} bile acids show similar fragmentation patterns to the corresponding saturated analogues except for a shift of 2 Da due to the side-chain unsaturation. In the published spectrum of Δ^{22}-ursodeoxycholic, an ABCD-ring ion at *m/z* 255, and a 113 Da side-chain loss establish two ring hydroxyls and an unsaturated C_5 side-chain (Setchell, Yamashita, Rodrigues, et al., 1995). The presence of a 3α,7β-*bis*-trimethylsiloxy group gives a base peak at [M-90] analogous to that seen in the spectra of other dihydroxy bile acids with this stereochemistry. The mass spectrum could easily be confused with that of a trihydroxy-cholanoic with one hydroxyl in the side-chain. A C-22,23 double bond does not give any diagnostically significant fragment ions or losses to indicate its position. This was also the case for a Δ^{22} C_{28} bile acid (Noma, Une, Kihira, et al., 1980). A dihydroxy bile acid structure was however confirmed after methylation and chromatographic separation of dihydroxy- from trihydroxy-bile acids on Lipidex 5000 and confirmation of the Δ^{22} structure was established by comparison against the authentic bile acid (Setchell, Yamashita, Rodrigues, et al., 1995). Methods for chemical synthesis and mass spectral characteristics, including retention indices, have been reported (Kihira and Hoshita, 1985).

23-Hydroxylation occurs in some snakes and in seals, Hammarsten's α- and β-phocaecholic acids (Haslewood, 1978). It may be mentioned that perhaps the first application of mass spectrometry to the analysis of an unknown naturally occurring bile acid was in the determination of the structure of a bile acid from one of Hammarsten's original preparations. Mass spectra were recorded of the methyl ester acetate derivatives of the original acid and a glycol cleavage product, norcholic acid,

which showed the structure of α-phocaecholic acid to be 3α,7α,12α,23-tetrahydroxy-5β-cholanoic acid (Bergström, Krabisch and Lindeberg, 1959).

The EI mass spectra of the Me-TMS ethers of 23-hydroxy bile acids are unremarkable, yielding few ions of diagnostic significance. Almé et al. published the mass spectrum of the Me-TMS ether of 23-hydroxycholic acid (3α,7α,12α,23-tetrahydroxy-5β-cholanoic acid) and it resembles that of many tetrahydroxylated bile acids in showing a lack of molecular ion and consecutive losses of the trimethylsiloxy groups. However, the ABCD-ring fragment was at *m/z* 253 and not 251 signifying that three of the hydroxyls were in the nucleus and one in the side-chain. Significant ions at *m/z* 159 and 143 were evident, but the origins unknown. Whether these ions come from cleavage across the C-22,23 bond to release the terminal carbons with the trimethylsiloxy group and the methyl ester is uncertain, but the mass spectrum was found to be identical to that of the Me-TMS ether of an authentic standard of 23-hydroxy-cholic acid (Almé, Bremmelgaard, Sjövall, et al., 1977). Interestingly, the *m/z* 143 ion is also seen as a dominant ion, accompanied with a base peak at *m/z* 131 in the bile alcohol (23*S*)-5α-cholestane-3α,7α,12α,23,25-pentol (Kihira, Okamoto, Ikawa, et al., 1991). The identification of the C_{27} bile acid, 3α,7α,12α,23-tetrahydroxy-5β-cholestanoic acid was established following analysis of its lactone and this is discussed previously (Une, Tsujimura, Kihira, et al., 1989). The presence of a C-23,24 double-bond in dihydroxy- or trihydroxy-cholestenoic acids results in an allylic cleavage of the C-20,22 bond and base peaks at *m/z* 283 ([M – (2 × 90 + 127)]) and 281 ([M – (3 × 90 + 127)]), respectively in the Me-TMS ether derivative (Noma, Une, Kihira, et al., 1980; Ali, Stephenson and Elliott, 1982). Since these ions represent an ABCD-ring with retention of C-20 and C-21 they are present, albeit at low intensity, in the spectra of saturated C_{27} bile acids (Lawson and Setchell, 1988a).

Hydroxylation at C-24 is a prominent feature of bile acid synthesis in patients with peroxisomal disorders. An impairment in side-chain β-oxidation due to lack of peroxisomes, or because of the deficiency of a single enzyme involved in the reaction cascade, leads to the accumulation of cholestanoic acids that become hydroxylated at the C-24 position. Varanic acid (3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid) (Kuramoto, Kikuchi, Sanemori, et al., 1973; Hanson, Isenberg, Williams, et al., 1975) is found in high concentrations in the urine of these patients. It is excreted mainly as a taurine conjugate and the presence of a negative ion at *m/z* 572 is of diagnostic importance in screening for peroxisomal disorders by FAB-MS (Lawson, Madigan, Shortland, et al., 1986; Setchell and Heubi, 2006). Varanic acid is a major bile acid of several animal species (Kuramoto, Kikuchi, Sanemori, et al., 1973; Gustafsson, 1975; Ali, Stephenson and Elliott, 1982; Une, Kuramoto and Hoshita, 1983). The corresponding 12-deoxy analogue (3α,7α,24-trihydroxy-5β-cholestanoic acid) is also found in the bile of the *Varanus monitor* (Ali, Stephenson and Elliott, 1982). The chemical synthesis of varanic acid diastereoisomers has been described (Kurosawa, Sato, Nakano, et al., 1996). The EI (Kuramoto, Kikuchi, Sanemori, et al., 1973; Ali, Stephenson and Elliott, 1982) and CI (Hanson, Isenberg, Williams, et al., 1975) mass spectral characteristics of methyl ester and trimethylsilyl ethers of these C_{27} bile acids have been extensively studied and in general,

fragmentation patterns are similar to those of C_{24} bile acids with regard to the ABCD-rings and their substituents. The molecular ion is usually absent or of very low intensity in both the trihydroxy and tetrahydroxy 24-hydroxy-C_{27} bile acids and the molecular weight has to be deduced from the [M-15] fragment. Consecutive losses of 90 Da confirm the number of trimethylsiloxy groups while the ABCD-ring fragment at *m/z* 255, or 253 in the mass spectra of trihydroxy and tetrahydroxy derivatives, respectively, establishes in each case that one of the hydroxyls is in the side-chain. A loss of 245 Da in the fragmentation sequence occurs due to cleavage and loss of the complete side-chain with its trimethylsiloxy group and results in ions at *m/z* 345 or 343, respectively for the trihydroxy and tetrahydroxy C_{27} bile acids. A concomitant loss of 155 Da is also seen for the side-chain minus the C-24 trimethylsiloxy group. As mentioned previously, cleavage *alpha* to the C-24 trimethylsiloxy group occurs (Une, Kuramoto and Hoshita, 1983) and an ion at *m/z* 321 (323 in the trihydroxy derivative) arises from fragmentation across the C-24,25 bond and loss of the trimethylsiloxy group. Recognition of the C-24 hydroxyl is possible from ions resulting after loss of the side-chain trimethylsiloxy group. This gives a 24,25-double bond that activates the allylic C-20, 22 bond to result in cleavage with the formation of a fragment consisting of the ABCD-ring with carbons 20 and 21 and an ion at *m/z* 283, or 281, respectively for the trihydroxy and tetrahydroxy structures (Ali, Stephenson and Elliott, 1982). The same fragment is observed for a Δ^{23} structure (Noma, Une, Kihira, et al., 1980; Ali, Stephenson and Elliott, 1982) as mentioned above.

The presence of a Δ^{24} structure in trihydroxy-cholestenoic acid not surprisingly leads to the same fragment but additionally, due to the migration of nuclear hydrogens, results in a distinctive cluster of ions at *m/z* 279–282 (Ali, Stephenson and Elliott, 1982). This same cluster is also seen in 5β-cholest-24-ene-3α,7α,12α-triol and 5β-cholest-25-ene-3α,7α,12α-triol (Tint, Dayal, Batta, et al., 1978) and the relative proportion of the ions in the cluster helps to differentiate the Δ^{24} from the Δ^{25} structures. The synthesis and mass spectra of the diastereoisomers of methylated 3α-, 3α,7α-, 3α,12α-, and 3α,7α,12α-hydroxylated Δ^{24} cholestenoic acids have been reported (Iqbal, Patrick and Elliott, 1991) and sufficient differences were observed in the relative intensity of the fragment resulting from loss of side-chain and all nuclear hydroxyls to afford assignment of diastereoisomerism (Iqbal, Patrick and Elliott, 1991).

Since side-chain oxidation proceeds through a cascade of reactions with the formation of a 24-oxo intermediate, the presence of 24-oxo-cholestanoic acids could be anticipated in patients with peroxisomal disorders. The tentative identification of 3α,7α,12α-trihydroxy-24-oxo-5β-cholestanoic acid in duodenal juice from two of three infants with peroxisomal 3-oxoacyl-Coenzyme A thiolase deficiency was reported. Although the mass spectrum was not shown, significant ions of the Me-TMS ether derivative were mentioned; a molecular ion at *m/z* 694 and ions at *m/z* 604, 514, 424, 281 and 253 were observed (Clayton, Lake, Hjelm, et al., 1988; Clayton, Patel, Lawson, et al., 1990).

Hydroxylation at C-25 can be recognized by loss of the carboxymethyl group as 59 Da from the molecular ion. In the Me-TMS ether of 3α,7α,12α,25-tetrahydroxy-5-β-cholestanoic acid this ion is at *m/z* 709 (Clayton, Muller and Lawson, 1982) and

consecutive loss of the trimethylsiloxy groups result in low intensity ions at *m/z* 619, 529, 439 and 349 which distinguish this hydroxylation site from others in the side-chain. Ions of greater abundance are also observed for consecutive losses from the molecular ion of the derivatized hydroxyls, while the typical side-chain loss of 155 Da yields an ion at *m/z* 343 (*m/z* 498 → 343).

The identification of 3α,7α,12α,26-tetrahydroxy-5β-cholestanoic acid in the bile of a frog (*Rana plancyi*) represented the first report of a C-26 hydroxylated bile acid. The reported mass spectrum of the Me-TMS ether did not show a molecular ion but rather the [M-15] fragment at *m/z* 753 as the ion of highest mass. Consecutive losses of trimethylsiloxy groups, together with the ABCD-ring fragments *m/z* 253 and 343, established three hydroxy groups in the nucleus, and one in the side-chain. The mass spectrum was otherwise unremarkable, with nothing of diagnostic significance to indicate the position of the side-chain hydroxyl group. The site of hydroxylation at C-26 was suggested after $LiAlH_4$ reduction of the ester to the 26,27-diol and confirmed after synthesis of the authentic compound (Une, Matsumoto, Kihira, et al., 1980). The corresponding allo-(5α) isomer (3α,7α,12α,26-tetrahydroxy-5α-cholestanoic acid) was also identified in the bile from the bullfrog (*Rana catesbiana*). The fragmentation pattern of the Me-TMS ether of this isomer was essentially the same as that of the 5β-isomer except for differences in the relative intensities of the ions that are similarly observed for 5α-cholanoic acids. Its identity was confirmed after synthesis of the authentic compound (Noma, Une, Kihira, et al., 1980).

Direct analysis of conjugated bile acids in biological samples or extracts by GC-MS is rarely performed because of the difficulty in forming volatile derivatives of all conjugate classes. The use of softer ionization techniques such as direct probe EI (Shaw and Elliott, 1978; Elliott, 1980b), chemical ionization (Back, 1976; Back and Bowen, 1976), thermospray (now largely obsolete) (Setchell and Vestal, 1989), FAB (Lawson, Madigan, Shortland, et al., 1986; Tomer, Jensen, Gross, et al., 1986; Setchell and O'Connell, 1994; Stroobant, de Hoffmann, Libert, et al., 1995), and ES (Roda, Gioacchini, Cerrè, et al., 1995), with or without MS/MS are now the preferred methods for the analysis of intact conjugates and these are discussed in Section 10.4.2.

Nevertheless, GC-MS has been used to characterize the structures of some types of bile acid conjugates and the mass spectrometric characteristics of the fragmentation patterns have been reviewed previously (Elliott, 1980a,b; Sjövall, Lawson and Setchell, 1985; Lawson and Setchell, 1988a). In profiling bile acid metabolism, where detailed information is required on the type of conjugation, the general approach has been to perform a group separation of conjugate classes (Almé, Bremmelgaard, Sjövall, et al., 1977) and then subject these fractions to chemical or enzymic hydrolysis of the conjugates and subsequent analysis of the hydrolysed bile acids by GC-MS. This provides indirect confirmation of the conjugation mode. These approaches are discussed in Section 10.2 and the FAB, ESI, etc. methods in Section 10.4.2. Attempts to prepare volatile derivatives of taurine conjugated bile acids for GC-MS have largely been unsuccessful; however, glycine conjugates and glucuronides are amenable to derivatization and gas chromatography.

Sulphated bile acids are best identified using FAB-MS or ESI-MS techniques where they form strong negative ions, or by the indirect approach after group fractionation and solvolysis mentioned above.

The mass spectra of Me-TMS ether derivative of a bile acid glucuronide is dominated by fragmentation of the derivatized glucuronide group with the result that an ion at *m/z* 217 (not to be confused with a 1β-hydroxylated bile acid structure) becomes the base peak in the spectrum, accompanied by prominent ions at *m/z* 204, and 317 arising from the sugar moiety, and a minor ion at *m/z* 407 for the intact glucuronyl group (Almé and Sjövall, 1980). Ions at *m/z* 369 and 459 (trihydroxy-cholanoates), *m/z* 371 (dihydroxy-cholanoates), or *m/z* 373 (monohydroxy-cholanoates) are formed after loss of the glucuronyl moiety and the oxygen linked to the steroid nucleus (Back and Bowen, 1976) and these are of help in determining the number of functional groups in the nucleus. The position of the glucuronide group however cannot be ascertained from the mass spectrum and the use of chemical reactions is essential when this type of information is required. In this regard, periodate oxidation of the intact bile acid glucuronide to yield the formate ester at the site of conjugation, followed by conversion of the remaining hydroxyls to oxo groups by chromate oxidation, and finally hydrolysis of the formate ester results in an oxo-hydroxy product that can be converted to the methyl ester-methyloxime-TMS ether for analysis by GC-MS. The position of the trimethylsiloxy group can then be distinguished based on the general fragmentation patterns described above, thus permitting positional assignment of the glucuronide moiety. This approach was used effectively to confirm the position of the glucuronide conjugate at C-6 in hyodeoxycholic acid and at C-3 in chenodeoxycholic and cholic acids isolated from human urine (Almé and Sjövall, 1980).

The initial discovery of bile acids conjugated with glucose or *N*-acetylglucosamine was done using GC-MS methodology. Typical ions at *m/z* 204 and 217 are given by the former and at *m/z* 173 and 186 by the latter conjugates (Marschall, Green, Egestad, et al., 1988). Confirmation of the structures and determination of positions of conjugation with FAB-MS are discussed in detail in Section 10.4.2.

Glycine conjugated bile acids can be methylated and give similar mass spectra to the free compounds which have been extensively described in older literature (Elliott, 1980a,b; Sjövall, Lawson and Setchell, 1985; Lawson and Setchell, 1988a). For this reason, and because the newer soft ionization methods discussed in other sections of this review offer a better approach to the analysis of intact glycine and taurine conjugates, only a few points need mentioning. A compilation of the EI mass spectra of Me-TMS ethers of glycine conjugates of LCA, DCA, CDCA, CA, and isocholic acids has been published (Lawson and Setchell, 1988a) and the spectra of all are dominated by an intense ion, usually the base peak, at *m/z* 131 due to rearrangement of the side-chain. Low intensity molecular ions, or an [M-15] fragment are usually evident thereby permitting the molecular weight of the intact conjugate to be established.

A compilation of diagnostically significant fragment ions and fragment losses is given in Table 10.4.

Table 10.4 List of diagnostically significant ions and fragmentations observed for bile acid methyl ester-trimethylsilyl ether derivatives

Observed ion (*m/z*)	Origin	Comments on probable bile acid/alcohol structure
115	Side-chain of C_{24} bile acids	Frequently seen in spectra of C_{24} bile acids
129	A-ring fragment (C-1 to C-3 with -OTMS)	Intense for 3-hydroxy-Δ^5 or -Δ^6 structures
131	Glycine side-chain fragment	Intense for glycine conjugates
131	C-25-C-27 with –OTMS	Intense for 25-hydroxysterols
136	A-ring with C-6 and C-7	Feature of 3-oxo-$\Delta^{4,6}$ structure
143	C-1 to C-4 with –OTMS	Observed for 3,5-dihydroxy structure
142/143	C-1 to C-4 with –OTMS	Observed for 2,3-hydroxy and 3-hydroxy-Δ^4 structures
143/159	Origin unknown	Observed for C-23 hydroxycholanoates
161/162	AB-rings and one oxo group after loss of C-7-OTMS	Diagnostic for 7-hydroxy-3-oxo-Δ^4 or -$\Delta^{4,6}$ structure
174/175	AB-rings + C-11, one oxo group after loss of C-7-OTMS	Diagnostic for 7-hydroxy-3-oxo-Δ^4 or -$\Delta^{4,6}$ structure
173, 186	Fragments of *N*-acetylglucosamine	Feature of *N*-acetylglucosamine conjugated bile acids
175	C-22-C-24 with 22-OTMS	Base peak for 22-hydroxy-UDCA
181	A-ring fragment after loss of C-4-OTMS	Base peak for 3α,4β-hydroxy structures
181	A-ring + C-19 after cleavage of C-9,10 and C-5,6 bonds and loss of TMSOH (*m/z* 271–90)	Observed for C-2,3-dihydroxy structures
182	B-ring fragmentation to form A-ring minus TMSOH	Observed for C-1,3- and C-2,3-hydroxy structures Base peak for 3-hydroxy-Δ^1 structure
195	A-ring + C-19, C-6, and loss of TMSOH (*m/z* 285–90)	Intense for 3,6,7-trihydroxy, seen for C-2,3-dihydroxy structures
208	Side-chain + D-ring, C-18, C-12 and loss of C-12-OTMS	Feature of 12-hydroxy bile acids
211	ABC-rings after loss of C-15 to C-17 and side-chain	Observed for trihydroxycholanoates (low abundance)
213	ABC-rings after loss of C-15 to C-17 and side-chain	Observed for dihydroxycholanoates
215	ABC-rings after loss of C-15 to C-17 and side-chain	Observed for monohydroxycholanoates (prominent)
217	A-ring fragment (1,3-*bis*-OTMS structure, *m/z* 129 + 88)	Usually base peak for 1,3-dihydroxy structures
217	C-22-C-27 with 22-OTMS	Feature of 22-hydroxy-C_{27} bile acids
217, 204, 317	Fragments of a glycosidic moiety	Features of a glycosidic bile acid conjugate
224	A-ring with C-19, C-6 and C-7-OTMS	Indicative of 7-hydroxy-3-oxo-Δ^4 structure
229	ABC-rings + C-18 with an oxo group, loss of TMSOH	Observed for many 3-oxo-6- or 3-oxo-7-hydroxy structures

(continued)

Table 10.4 (continued)

Observed ion (*m/z*)	Origin	Comments on probable bile acid/alcohol structure
231	ABC-rings + C-18 with a 12-oxo group, loss of TMSOH	Typical for 12-oxo-3-hydroxy structures, can be base peak
243	C-3 to C-7 with two -OTMS groups	Feature of 3,7-dihydroxy, intense for 2,3,7-trihydroxy structures
243	C-15 to C-24 with C-15-OTMS	Feature of 15-hydroxylated C_{24} bile acids
247	CD-rings and side-chain (cleavage of C-7,8 and C-9,10)	Analogous to *m/z* 249 with double bond or lost -OTMS, or analogous ion from a C_{27}-sterol
249	CD-rings and side-chain (cleavage of C-7,8 and C-9,10)	Formed from 3,6- or 3,7-dihydroxy, 3-hydroxy-Δ^5 or -Δ^6 or 3-oxo-$\Delta^{4,6}$ structures
251	ABCD-rings after loss of substituents and side-chain	Usually indicative of tetrahydroxy structure
253	ABCD-rings after loss of substituents and side-chain	Trihydroxy, or dihydroxy with double bond or oxo group
255	ABCD-rings after loss of substituents and side-chain	Dihydroxy, or monohydroxy with double bond or oxo group
257	ABCD-rings after loss of -OTMS group and side-chain	Monohydroxy or unsaturated skeleton
261	CD-rings with C-7 and side-chain	Observed for 7,12-dihydroxy structures, e.g. allocholic acid
262	CD-rings with C-7 and side-chain	Distinguishes 3,7- from 3,6- or 3,12-dihydroxy structures
269	ABCD-rings with oxo group after loss of side-chain and TMSOH(s)	Feature of C_{24-27} acids and sterols with nuclear oxo-dihydroxy or oxo-hydroxy-double bond structures
271	ABCD-rings with oxo group after loss of side-chain and TMSOH	Intense for 3-oxo bile acids with one hydroxyl group or a double bond
271	A-ring + C-19 and two -OTMS groups	Feature of 2,3-dihydroxy structures
279–282 cluster	Loss of C-22 to C-27 and hydrogen migration	Observed for Δ^{24} and Δ^{25} trihydroxy-C_{27} acids
281	ABCD-rings and C-20 and C-21 after loss of -OTMS groups (also see *m/z* 253 + CH_2)	Observed for 24-hydroxy-C_{27} tetrahydroxy structures (present in trihydroxy-C_{27} and C_{29}-dicarboxylic structure
283	ABCD-rings and C-20,21 after loss of ring -OTMS groups	Base peak in Δ^{23} C_{27} dihydroxy structures (low intensity and in a cluster with *m/z* 281 and *m/z* 282 in Δ^{24} structures)
284	Loss of C-22 to C-24 with C-22 -OTMS and H-migration	Prominent for 22-hydroxy-CDCA
285	A-ring + C-19 and C-6 and two -OTMS groups	Characteristically intense for 3,6,7β-trihydroxy structures
292/293	C,D-rings + C-6 and C-7 with 7-oxo group	Distinguishes 7-oxo-3-hydroxy from 3-oxo-7-hydroxy
314	BCD-rings + C-19 and side-chain; retro-Diels-Alder loss of A-ring after loss of 3-OTMS	Confirms two -OTMS groups in BCD-rings
316	BCD-rings + C-19 and side-chain	Observed for 3,x-dihydroxy C_{24} bile acids with one hydroxyl in BCD- rings. Low intensity ion for 3-oxo-monohydroxy structures

318	BCD-rings + C-19 and side-chain	Observed for 3-monohydroxy C_{24} bile acids
321	ABC-rings + C-18 with a 12-oxo group ($M^+ - 155$)	Characteristic of 12-oxo-3-hydroxy-C_{24} bile acid with *m/z* 231 ion
323	Entire D-ring + C-6-C-8, cleavage of C-5,6, C-8,9 and C-12,13 bonds	Observed for 3,6-dihydroxy structures
329	ABCD rings after loss of TMSOH, side-chain and CH_4 (see also loss of 129 Da)	Intense in C-16 hydroxy structure, also in C-21 hydroxy (note, characteristic feature of cholesterol [M-129])
331	See loss of 129 Da	Intense for 3-hydroxy-Δ^5-cholenoate
343	ABCD-rings with one -OTMS after loss of two -OTMS	Feature of trihydroxy bile acids, *m/z* 343 > 368 with 5α-H
345	ABCD-rings with one -OTMS and loss of one -OTMS	Feature of dihydroxy bile acids, usually *m/z* 345 > 370 with 5α-H
368	ABCD-rings + side-chain after loss of three -OTMS	Characteristic ion for all trihydroxy-C_{24} bile acids
370	ABCD-rings + side-chain after loss of two -OTMS	Characteristic ion for all dihydroxy-C_{24} bile acids
372	ABCD-rings + side-chain after loss of one -OTMS	Prominent for monohydroxy bile acids, especially if 5β-H
373	See loss of 129 Da	Base peak for 3-hydroxy-Δ^5-cholestenoate
386	ABCD-rings + side-chain, one oxo group, one double bond	Characteristic of oxo-monohydroxy-C_{24} structure
403	See loss of 145 Da	Feature of 3,6,x-trihydroxy C_{24} bile acids (weak)
405	See loss of 145 Da	Feature of 3,6-dihydroxy C_{24} bile acids
407	Intact glucuronyl methyl ester-persilyl moiety	Low intensity for bile acid glucuronides
410	ABCD-rings + side chain after loss of three TMSOH	Prominent ion for cholestanoic acids with three nuclear hydroxyls
412	ABCD-rings + side chain after loss of two TMSOH	Prominent ion for cholestanoic acids with two nuclear hydroxyls
412	See loss of 90 Da	Prominent ion for 3-hydroxy-Δ^5-C_{27} monohydroxy structure
456	See loss of 90 Da	Base peak for 7α-hydroxycholesterol
458	See loss of 90 Da	Base peak for 3,7-dihydroxy-Δ^5-C_{24} acid
460	See loss of 90 Da	Intense ion for 3,7β-dihydroxy-C_{24} acid
461	See loss of 15 Da	Base peak for 6-oxo-lithocholic acid
500	See loss of 90 Da	Base peak for 3,7-dihydroxy-Δ^5-cholestenoate

(continued)

Table 10.4 (continued)

Observed ion (*m/z*)	Origin	Comments on probable bile acid/alcohol structure
Observed losses (Da)		
−15	Loss of methyl from -OTMS (or angular -CH_3)	Usually highest mass in the absence of molecular ion
−18	Loss of H_2O	Seen throughout fragmentation sequence of bile acids with free hydroxyl or oxo groups
−31	Loss of methoxy group	Observed for methyl ethers and for C_{29}-dicarboxylic acid
−59	Loss of a carboxymethyl group	Occurs from M^+ of C-25 hydroxy-cholestanoic acids
−70	Loss of A-ring with 3-oxo group	Characteristic loss for 3-oxo bile acids, minor with additional -OTMS
−89	Loss of -OTMS without hydrogen transfer	Indicates vicinal hydroxyls, e.g. 2,3-, 3,4- or 6,7-hydroxy
−90 or −nx90	Loss of one or n TMSOHs	Indicates presence and number of derivatized hydroxyls
−101	Loss of C_4 side-chain methyl ester	Indicates norcholanoate (C_{23}) structure
−103	Loss of primary -CH_2OTMS	Indicates presence of primary hydroxyl C-18, C-19, C-21, (C-26)
−121/122	Loss of TMSOH and methoxy of methyl ester	Diagnostically significant for 7-hydroxy-3-oxo-Δ^4 structure
−113	Loss of C_5-bile acid side-chain with double bond, or loss of C_{27}-sterol side-chain	Unsaturation or presence of eliminated substituent in side-chain, or spectrum of a sterol
−115	Loss of C_5 side-chain methyl ester	Confirms cholanoic acid (C_{24}) structure
−129	Loss of C_5 side-chain ethyl ester or C_6 side-chain methyl ester	Indicates formation of artifact ethyl esters or a homocholanoic acid (C_{25}) structure
−129	Loss of C-1-C-3 with -OTMS	Characteristic loss for 3-hydroxy-Δ^5 structures
−131	Loss of C-1-C-3 with OTMS	Characteristic for 3-hydroxy-$\Delta^{5,7}$, $\Delta^{5,8}$, $\Delta^{5,8(14)}$ and $\Delta^{6,8}$ sterols
−145	Loss of C-1-C-4 with 3-OTMS	Characteristic for 3,6-dihydroxy bile acids, peak intensities influenced by stereochemistry and additional substituents
−155	Loss of D-ring with C_5 side-chain methyl ester	Characteristic of 12-oxo cholanoates (McLafferty rearrangement)
−157	Loss of D-ring with C_5 side-chain methyl ester	Loss for non-12-oxo bile acids
−155	Loss of C_8 side-chain methyl ester with a double-bond	Indicates cholestanoic side-chain with double-bond, or lost -OTMS
−157	Loss of C_8 side-chain methyl ester	Confirms cholestanoic acid (C_{27}) structure
−173	Loss of C_5 side-chain -OTMS-ester	Artifact formation of -OTMS ester due to incomplete methylation
−229	Loss of C_{10} dicarboxylic acid side-chain	Characteristic of C_{29}-dicarboxylic side-chain structure
−245	Loss of side-chain, C-15 and -OTMS	Establishes D-ring substitution
−245	Loss of C_8 side-chain with -OTMS substituent	Observed for 24-hydroxycholestanoic acids

10.4.1.2 GC-MS – Quantitative Analysis

GC-MS offers high sensitivity, specificity and precision for the quantitative analysis of bile acids in biological samples, especially when the principle of stable-isotope dilution analysis with selected ion monitoring (SIM) is applied (Björkhem, 1979). This technique has also been referred to as selected ion detection, single or multiple ion monitoring, selected ion recording and mass fragmentography. It involves the selection of a single ion, or multiple ions, formed during ionization that are characteristic of either specific structures of the molecule, or generic structures typical of a particular class of compounds, e.g. dihydroxy-cholanoates and trihydroxy-cholanoates. The analogous ion corresponding to that of the added stable-isotope labelled internal standard(s) but shifted in mass according to the number of stable-isotopes incorporated is also selected and these ions are focused and continuously monitored throughout the chromatographic run. The virtual co-elution of the stable-isotope labelled internal standard and the analyte, coupled with retention time adds confidence to the specificity of the measurement. Where magnetic sector instruments are used there are limitations to the dynamic range of ions that can be simultaneously monitored (usually no more than 30% of the lowest mass) when using accelerating voltage switching because of defocusing. This can be overcome by maintaining a constant accelerating voltage and switching the magnetic field, but then scan speed and sensitivity are compromised. Quadrupole instruments that have rapid switching capabilities do not have such limitations. Sensitivity of the assay will be influenced by the ionization characteristics of the bile acid derivative, the choice of ion(s) selected, and the extent of interferences from the biological matrix. Ideally, an ion of highest mass and relative abundance in the spectrum should be chosen. Sensitivity is also inversely proportional to the number of ions monitored, and to the mass spectrometric resolution. In the case of the latter, the most published methods for bile acids have used low resolution. This may result in errors due to presence of contaminating compounds at the same nominal masses as those monitored for the bile acids. The sensitivity of the selected ion monitoring approach is increased by 100–1,000-fold when compared with repetitive scanning GC-MS (Reimendal and Sjövall, 1972), or GC with FID methods. The major limitation of SIM is that the assay shows “bias” because prior selection of the ions of interest is made and additional mass spectral or structural information is not acquired. Therefore, unlike repetitive scanning techniques, the ability to retrospectively examine the data for unexpected or unpredicted metabolites is compromised.

In the early applications of GC-MS-SIM methods (Angelin and Björkhem, 1977; Angelin, Björkhem and Einarsson, 1978; Yanagisawa, Itoh, Ishibashi, et al., 1980; Barnes, Berkowitz, Hirschowitz, et al., 1981; Beppu, Seyama, Kasama, et al., 1981; Angelin, Björkhem, Einarsson, et al., 1982; Bartholomew, Summerfield, Billing, et al., 1982; Beppu, Seyama, Kasama, et al., 1982; Björkhem, Angelin, Einarsson, et al., 1982; Ewerth, 1982; Setchell, Lawson, Blackstock, et al., 1982; Takikawa, Otsuka, Beppu, et al., 1982; Björkhem and Falk, 1983; Setchell and Matsui, 1983; Tohma, Wajima, Mahara, et al., 1984), EI was mostly used and as indicated in the above discussion of bile acid fragmentation, molecular ions are

usually absent or of low intensity, and therefore of limited value for monitoring. Ideally, high mass, high abundance ions are preferred and consequently with EI, the ions formed from the consecutive loss of one or more, or all, of the derivatized hydroxy groups from the bile acid derivative have to be selected. For Me-TMS ether derivatives many of the methods have simultaneously monitored the ions *m/z* 368, 370 and 372 because these are general fragment ions arising from loss of trimethylsiloxy groups produced by all trihydroxy-, dihydroxy- and monohydroxycholanoates, respectively (Setchell and Matsui, 1983). The ion at *m/z* 368 is also prominent in the TMS ether of cholesterol being formed from loss of the trimethylsiloxy group, while a similar loss from saturated C_{27} monohydroxysterols yields an ion at *m/z* 370. Sterols, particularly cholesterol, which can exceed bile acid concentrations by several orders of magnitude in many types of samples, chromatographically elute in the region of bile acid Me-TMS ethers and unless removed can interfere with the analysis. While different derivatives have been used to improve the separation of cholesterol from bile acids (Miyazaki, Ishibashi and Yamashita, 1978; Beppu, Seyama, Kasama, et al., 1981), alternatively separating bile acids from sterols using ion exchange chromatography is an effective and usual approach (Almé, Bremmelgaard, Sjövall, et al., 1977; Setchell and Matsui, 1983; Setchell, Lawson, Tanida, et al., 1983). These general fragment ions can be used with alternative derivatives, such as methyl ester acetate, trifluoroacetates, DMES, pentafluorobutyrates etc. because these same general principles apply. Thus, by monitoring three or four ions it is possible in a single run to quantify all the principal primary and secondary bile acids, and the stable-isotope labelled internal standards based on their chromatographic separation, while other structurally related positional or stereo-isomeric metabolites if present, will be detected.

The basic approach to this type of analysis involves the addition to the biological matrix of stable-isotope labelled analogues of the bile acids as internal standards, and after equilibration, the extraction of bile acids, subjected to either chemical or enzymic hydrolysis, re-extraction, purification (see Section 10.2), and conversion to a volatile derivative for analysis by GC-MS. The advantage of using stable-isotope labelled analogues as internal standards, as opposed to homologs or unrelated compounds is increased precision and accuracy because these more accurately account for any procedural losses in the sample preparation and eliminate effects of response factor variations. Quantification is accomplished by preparing a set of calibration standards of differing concentrations of the bile acids to be measured in the presence of the same concentration of the internal standard(s) that is added to the test samples and then calculating the ratio of the peak areas of the natural to stable-isotope labelled isotope ion responses. The peak area ratio for the test sample is interpolated from the calibration and the accurate concentration determined. Considerations in selection of stable-isotope labelled internal standards include, position and chemical stability of the isotopic atoms (these need to be retained in the ion being monitored), isotopic purity (should be high to minimize interference from naturally occurring isotopes), number of isotopic atoms to molecule (to minimize interference from natural isotopes from the analyte), and type of stable-isotope (to minimize isotopic effects in the assay). While bile acids incorporating ^{13}C or ^{18}O are preferable

(Hachey, Szczepanik, Bergrubes, et al., 1973; Tserng and Klein, 1977; Goto, Hasegawa, Nambara, et al., 1992) these are of limited commercial availability and expensive compared with more commonly used deuterium labelled bile acids (Cowen, Hofmann, Hachey, et al., 1976; Baillie, Karls and Sjövall, 1978). Custom synthesis of the internal standards may therefore be necessary for less common bile acids of interest (Shoda, Axelson and Sjövall, 1993). Deuterium labelled analogues of CA, CDCA, DCA, LCA, and UDCA with anywhere from 2 to 5 isotopic atoms have been commercially available for some time and have been most commonly employed for quantitative analysis of the major primary and secondary bile acids, or for determination of bile acid kinetics in bile (Watkins, Ingall, Szczepanik, et al., 1973; Balistreri, Cowen, Hofmann, et al., 1975; Klein, Haumann and Hachey, 1975; Kern, Everson, DeMark, et al., 1981; Nishida, Miwa, Yamamoto, et al., 1982), or serum (DeMark, Everson, Klein, et al., 1982; Stellaard, Schubert and Paumgartner, 1983; Stellaard, Sackmann, Sauerbruch, et al., 1984). Isotopic effects whereby the internal standard elutes fractionally earlier than the corresponding bile acid are pronounced with increasing number of deuterium atoms in the molecule, and care is required to minimize conditions that might lead to hydrogen-deuterium exchange in work-up. For this reason ^{13}C labelled isotopes are preferred.

Stable-isotope labelled conjugated bile acids have rarely been employed as internal standards (Takikawa, Otsuka, Beppu, et al., 1982; Akashi, Miyazaki and Nakayama, 1983) and therefore the use of the stable-isotope labelled unconjugated bile acids makes the assumption that hydrolysis of the conjugate groups during work-up is complete. In many of the published methods, bile acid sulphates and glucuronides are not included in the measurement unless combined solvolysis and exhaustive enzymic hydrolysis methods are used and consequently total bile acid concentrations are usually underestimated. This may also be true, with samples from patients administered UDCA because *N*-acetylglucosamine conjugates account for a significant proportion of the metabolites (Marschall, Griffiths, Götze, et al., 1994). In one of few methods to use stable-isotope labelled conjugated bile acids, [11,11,12-$^{2}H_{3}$]deoxycholic acid 3-glucuronide and 3-sulphate and [2,2, 4,4-$^{2}H_{4}$] lithocholic acid 3-glucuronide conjugates were employed as internal standards to quantify by GC-MS-SIM, the serum concentrations of bile acid glucuronides and sulphates following their isolation, hydrolysis and conversion to hexafluoroisopropyl-trifluoroacetate derivatives (Takikawa, Otsuka, Beppu, et al., 1982). Bile acid glucuronides and sulphates were found to comprise 8.7% and 11.2% of the total serum bile acids. In another study, five conjugated bile acids labelled with deuterium in the ABCD-rings were used as internal standards to measure and compare bile acid concentrations in liver tissue and bile (Akashi, Miyazaki and Nakayama, 1983), after extraction, solvolysis and hydrolysis, and conversion to ethyl ester-DMES ethers for GC-MS-SIM. Methods employing stable-isotope labelled references as internal standards have been summarized in earlier reviews (Sjövall, Lawson and Setchell, 1985; Lawson and Setchell, 1988a; Setchell and Lawson, 1989).

Oxo-bile acids are generally not included in most of the GC-MS-SIM methods, but depending on the biological sample, they can account for a significant proportion of the total bile acids, e.g. in faeces. One novel approach to quantify oxo-bile

acids in portal venous and systemic blood involves reduction with sodium borodeuteride to yield the monodeuterated hydroxy-cholanoate structure and the subsequent determination of the ratio of these products to the endogenous hydroxy-bile acids (i.e. [M + 1]/[M]) after first quantifying the total hydroxy-bile acid concentrations. However, this approach requires two separate analyses and structural information is lost (Björkhem, Angelin, Einarsson, et al., 1982).

Enhanced sensitivity can often be achieved through alternative ionization techniques. In this regard, CI methods that ionize low-molecular-weight target molecules using mild ion-molecule reactions involving proton transfer can be used to produce intense protonated molecules. Ammonia CI was first used to measure methyl ester acetate derivatives of CDCA and CA in serum after administration of [^{13}C] analogues (DeMark and Klein, 1981). Pool size is determined from the natural abundance of the [^{13}C]/[^{12}C] for each bile acid and calculated by plotting the % atom excess versus time. Common CI conditions for producing positive ions may be complicated by a high level of background noise as is the case in electron ionization. It is well known that negative ions can be produced by capturing low-energy electrons; these ions are generated by passing an electron beam through an inert moderating gas, such as methane, isobutane, or ammonia, or by reaction with an ion, such as hydroxide formed by electron bombardment of a mixture of nitrous oxide and methane. The former method is referred to as electron-capture negative-ion chemical ionization (NICI), whereas the latter is called reactant-ion NICI (Smit and Field, 1978). The effect of the reagent gas on NICI was investigated using different esters of stearic acid as model compounds (Goto, Watanabe, Miura, et al., 1987). When isobutane is used as a reactant gas for CI, alkyl esters, such as methyl ester and ethyl ester, produce protonated molecules due to proton transfer and deprotonated molecules due to hydride abstraction reactions. For active esters, such as *p*-nitrophenyl ester and *N*-hydroxysuccinimidyl ester, elimination of the alcohol group produces a carbonium cation in the positive-ion detection mode. On the other hand, benzyl esters produce a carboxylate anion in the negative-ion detection mode and a benzyl cation in the positive-ion detection mode. According to the base peak intensity ratio of negative ions to positive ions (N/P ratio), benzyl esters enhance the formation of anions in the NICI mode because of the high electron affinity of the benzyl groups, whereas the N/P ratios of alkyl and active esters are less than one. For the pentafluorobenzyl ester of stearic acid, the intensity of the signal for the carboxylate anion in the NICI mode is 1,000-fold higher than that for the pentafluorobenzyl cation. The conversion of bile acids to pentafluorobenzyl ester-dimethylethylsilyl ether derivatives results in the characteristic negative ions, $[M\text{-}181]^-$, as base peaks due to the elimination of the pentafluorobenzyl group in the NICI mode. Approximately 2 fg of each dihydroxylated bile acid can be detected with a signal-to-noise ratio of ten using GC-MS NICI with selected ion monitoring (Fig. 10.14), thereby making it possible to quantify bile acids in biopsy samples of liver tissue (Goto, Miura, Inada, et al., 1988).

This method to derivatize bile acids into pentafluorobenzyl ester-dimethylethylsilyl ethers has also permitted the analysis of bile acids in a dried blood disc from neonates (Murai, Mahara, Suzuki, et al., 1995) and analysis of the 1β- and

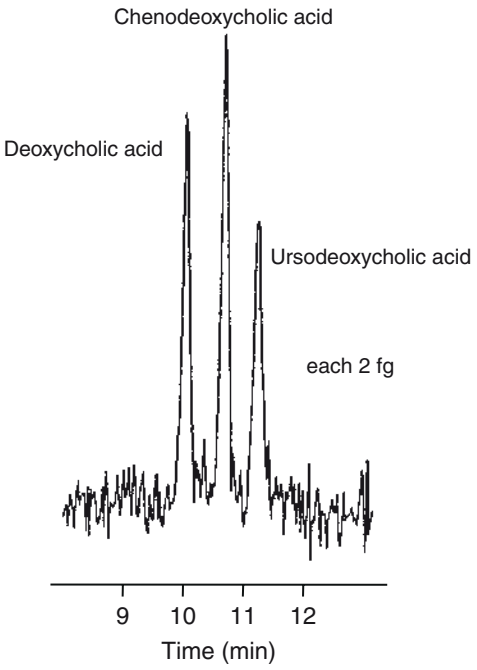


Fig. 10.14 Selected ion recording of pentafluorobenzyl ester-dimethylethylsilyl ether derivatives of three dihydroxylated bile acids using negative-ion chemical-ionization GC-MS. Each peak represents the response from injected derivatives of two femtog underivatized bile acid

6α-hydroxylated bile acids present in human foetuses and neonates (Strandvik and Wikström, 1982; Setchell, Dumaswala, Colombo, et al., 1988; Murai, Mahara, Kurosawa, et al., 1997). Hydroxylation at the 1β- and 6α-positions is important for the urinary excretion of bile acids that accumulate in the liver due to cholestasis associated with congenital biliary atresia. Other 4- or 6-hydroxylated bile acids, with a 1,2-glycol structure, are also found in foetuses and neonates (Setchell, Dumaswala, Colombo, et al., 1988; Dumaswala, Setchell, Zimmer-Nechemias, et al., 1989) (see also Introduction, Section 10.1.3). For analysis of these metabolites, *N*,*O*-bis(diethylhydrogensilyl)trifluoroacetamide is very useful for the derivatization of bile acid pentafluorobenzyl esters (Goto, Teraya, Nambara, et al., 1991; Goto, Hasegawa, Nambara, et al., 1992). As discussed in Section 10.3.2, this silylation converts hydroxyl groups into the corresponding diethylhydrogensilyl ethers and the vicinal glycols (except for the diaxial glycols) form cyclic diethylsilylene derivatives.

The C_{27} bile acid intermediates DHCA and THCA (Parmentier, Janssen, Eggermont, et al., 1979; Eyssen, Eggermont, van Eldere, et al., 1985; Clayton, Lake, Hall, et al., 1987), and the C_{29} dicarboxylic acid (Parmentier, Janssen, Eggermont, et al., 1979), found in the bile, urine, and blood of patients with peroxisomal disorders have been quantified by GC-MS with NICI of the pentafluorobenzyl ester-DMES ethers (Goto, Miura and Nambara, 1989). This approach offers a complementary technique to FAB- or ESI-MS (discussed in Section 10.4.2) for confirmation of the diagnosis.

Pentafluorobenzyl ester-TMS ether derivatives and NICI GC-MS with SIM have also been applied to the analysis of plasma bile acids (Stellaard, Langelaar, Kok, et al., 1989) and because of the high sensitivity have proved useful in the determination of bile acid kinetics after administration of stable-isotope labelled bile acids to humans (Hulzebos, Renfurm, Bandsma, et al., 2001).

Quantitative analysis of metabolic profiles of bile acids in biological samples has been traditionally accomplished by first performing GC analysis and quantifying peaks relative to one, or several internal standards (e.g. coprostanol, nordeoxycholic acid, 5α-cholestane or an *n*-hydrocarbon of suitable chain length are commonly used) and applying response factors to correct for differences in detector responses among different bile acid derivatives; the mass spectrometer being used to merely confirm the identity of the peaks of interest after repetitive scanning of the eluting components. There are a few examples, where quantification has been performed on data acquired from repetitive scanning of the profile (Back, Sjövall and Sjövall, 1974). The most basic approach is to measure the peak area or peak height (Axelson, Cronholm, Curstedt, et al., 1974) from the summed ions or total ion current (TIC) chromatogram and to compare this with either a standard mixture of bile acids analysed under identical conditions (external standards), or to an internal standard (Axelson, Cronholm, Curstedt, et al., 1974). This approach is semi-quantitative because it assumes that equimolar concentrations of bile acids for which standards are unavailable yield similar TIC responses, which may not be the case. Alternatively, if a wide range of stable-isotope labelled bile acid analogues were available for addition to the sample, this would, in principle, greatly improve the accuracy and precision of the measurement. However, there is a limited availability of stable-isotope labelled bile acids. One of the earliest attempts to quantify the principal bile acids in bile compared the ion responses based on peak areas for three different ions derived from EI of the Me-TMS ethers of CDCA, CA and DCA using deuterium labelled internal standards against a mixture of the pure compounds (Miyazaki, Ishibashi, Inoue, et al., 1974). There was no significant difference in the calculated concentrations among the three ions selected and the purity of the eluting peak could be determined from the reciprocal of the relative intensity of each ion. With a similar approach, the permethyl derivatives of a number of bile acids were quantified using deuterium labelled bile acids as internal standards with good linearity, reproducibility (coefficient of variation approximately 5%) and sensitivity (2 ng injected on column) (De Weerdt, Beke, Verdievel, et al., 1980).

With computerized programs, the option to select one, several, or many ions for each compound from a wide mass range scanned (50–800 Da) greatly increases specificity and facilitates quantification but the biggest drawback remains the lack of a sufficient selection of pure standards for use as calibrators. An approach first used by Sjövall's group was to select within a defined retention time window, up to ten different ions characteristic of the bile acid structure, which were summed after background subtraction, and converted to TIC equivalents for comparison with external standards (Axelson, Cronholm, Curstedt, et al., 1974). Coprostanol was added as an internal standard to correct for the proportion of the sample injected on column and to permit concentrations to be calculated. Lists of characteristic ions in

the spectra of 30 bile acid Me-TMS ethers have been published and this general approach to repetitive scanning of bile acid profiles was applied to measure bile acids in plasma and urine (Almé, Bremmelgaard, Sjövall, et al., 1977; Bremmelgaard and Sjövall, 1979; Thomassen, 1979; Bremmelgaard and Almé, 1980). This same approach is applicable to other bile acid derivatives (Elliott, 1980b). For example, metabolic profiling of Me-DMES ethers of bile acids in urine and bile has been described (Fig. 10.12) (Kimura, Suzuki, Murai, et al., 1997).

Overall, irrespective of the method of data handling, quantification by repetitive scanning has limited sensitivity (2–20 ng on column) and precision (5–20% depending on the bile acid measured and its concentration) when compared with selected ion monitoring, but does offer a 'metabolomic' approach. Improvements in sensitivity may be obtained by scanning a restricted mass range but this has mainly to be carried out in stable-isotopic tracer studies (Cronholm, Burlingame and Sjövall, 1974; Vlahcevic, Cronholm, Curstedt, et al., 1980). Information on the rate and extent of incorporation of ^{13}C and ^{2}H into bile acids from [^{13}C], [1,1-$^{2}H_2$] and [2,2,2-$^{2}H_3$] analogues of ethanol administered to rats (Cronholm, Makino and Sjövall, 1972a, b; Cronholm, Burlingame and Sjövall, 1974; Cronholm, Eriksson, Matern, et al., 1975; Vlahcevic, Cronholm, Curstedt, et al., 1980), and of [^{18}O] from inhaled [$^{18}O_2$] (Björkhem and Lewenhaupt, 1979) has been obtained by this repetitive scanning approach and, likewise, information on the turnover of cholesterol and bile acids has been obtained.

Finally, it should be mentioned that mass spectrometers as well as software for quantification and metabolomic analyses have been greatly improved and continue to be developed and made commercially available from several sources.

10.4.2 Mass Spectrometry and Liquid Chromatography

10.4.2.1 FAB-MS and FAB-CID

Following the turn of the century, atmospheric pressure ionization (API), in particular ESI, largely replaced FAB ionization in lipid mass spectrometry (MS), although FAB continues to be favoured by some research groups in the bile acid field (Setchell and Street, 1987; Setchell and O'Connell, 1994; Setchell, Schwarz, O'Connell, et al., 1998; Bove, Heubi, Balistreri, et al., 2004; Setchell and Heubi, 2006; Heubi, Setchell and Bove, 2007). However, as both techniques are used, and lead to the formation of the same ions (i.e. usually $[M\text{-}H]^-$ or $[M + H]^+$), a synopsis of the pioneering work performed using FAB-MS and FAB with collision-induced dissociation (CID) is still relevant.

With the introduction of FAB polar bile acids and bile alcohol conjugates could, for the first time, be directly and routinely analysed by mass spectrometry (Whitney, Lewis, Straub, et al., 1981; Ballatore, Beckner, Caprioli, et al., 1983). Setchell, Lawson and Sjövall, however, first used FAB-MS in 1979 in a clinical setting to determine bile acids in the urine from patients with unexplained liver disease, and developed a

screening strategy that subsequently led to the identification of new inborn errors in bile acid synthesis that cause liver disease and fat-soluble vitamin malabsorption in infants and children (Egestad, Pettersson, Skrede, et al., 1985; Lawson, Madigan, Shortland, et al., 1986; Clayton, Leonard, Lawson, et al., 1987; Setchell, Suchy, Welsh, et al., 1988; Setchell, Heubi, O'Connell, et al., 1997; Setchell, Schwarz, O'Connell, et al., 1998; Setchell, Heubi, Bove, et al., 2003). In healthy infants, children, or adults, bile acids synthesized in the liver are secreted across the canaliculus into bile, where they act as detergents in facilitating the absorption of fats and fat-soluble vitamins from the intestine. Because of an efficient enterohepatic circulation, plasma bile acids are maintained at relatively low concentrations (<4 μmol/L) and there is minimal renal excretion into urine where typically urinary bile acid concentrations are <20 μmol/L, which is below, or at the limit of detection of the FAB-MS technique. However, in cholestatic liver disease when bile-flow is reduced, plasma bile acid concentrations increase markedly, and renal excretion becomes the major route for elimination of bile acids. Under this pathological state, individual bile acid species are readily detected in the urine by negative ion FAB-MS and the intensity of the ions produced is roughly proportional to the severity of the cholestasis. Single ions corresponding to the $[M\text{-}H]^-$ of individual bile acid conjugates present in urine permit the status of hepatic synthesis to be determined. When bile acid synthetic pathways are intact the FAB-MS mass spectrum generated reveals ions corresponding to conjugates of the normal primary bile acids chenodeoxycholic and cholic acids. However if the liver disease is caused by a mutation in a gene encoding one of the more than 15 enzymes involved in the synthesis of primary bile acids from cholesterol, then the FAB-MS spectrum reveals a distinct and highly definitive spectrum of series of ions that characterize and are specific for the particular genetic defect (Setchell and Heubi, 2006). Figure 10.15 highlights the principal negative ions in the spectra of six different bile acid synthetic defects. This approach provides a powerful screening procedure for 'teasing out' specific defects in bile acid synthesis. In the last 20 years an international screening program at Cincinnati Children's Hospital has analysed >8,000 urine samples from infants with unexplained liver disease by this approach and bile acid synthetic defects have been found to account for 2.1% of the cases examined (Setchell and Heubi, 2006).

The first reported studies of the fragmentation of bile acids by FAB-CID were performed by Beynon and colleagues in Swansea (Kingston, Beynon, Newton, et al., 1985; Liehr, Kingston and Beynon, 1985), and Gross and co-workers in Nebraska (Tomer, Jensen, Gross, et al., 1986). Both groups performed high-energy (keV) CID on magnetic sector instruments. In Beynon's studies $[M\text{-}H + 2Na]^+$ ions were fragmented, and the resulting product-ion spectra allowed the differentiation of bile acid isomers which varied in the position of hydroxylation. Gross and colleagues fragmented $[M\text{-}H]^-$ ions and were able to identify the major sites of cleavage in the bile acid skeleton (Scheme 10.1; Table 10.5). Both groups concluded that fragmentation occurred by charge-remote fragmentation (CRF) mechanisms (i.e. fragmentation occurring remote from the site of charge). These initial studies were followed up by a number of other groups who also demonstrated that high-energy CID of bile acid $[M\text{-}H]^-$ ions gives product-ion spectra that can be

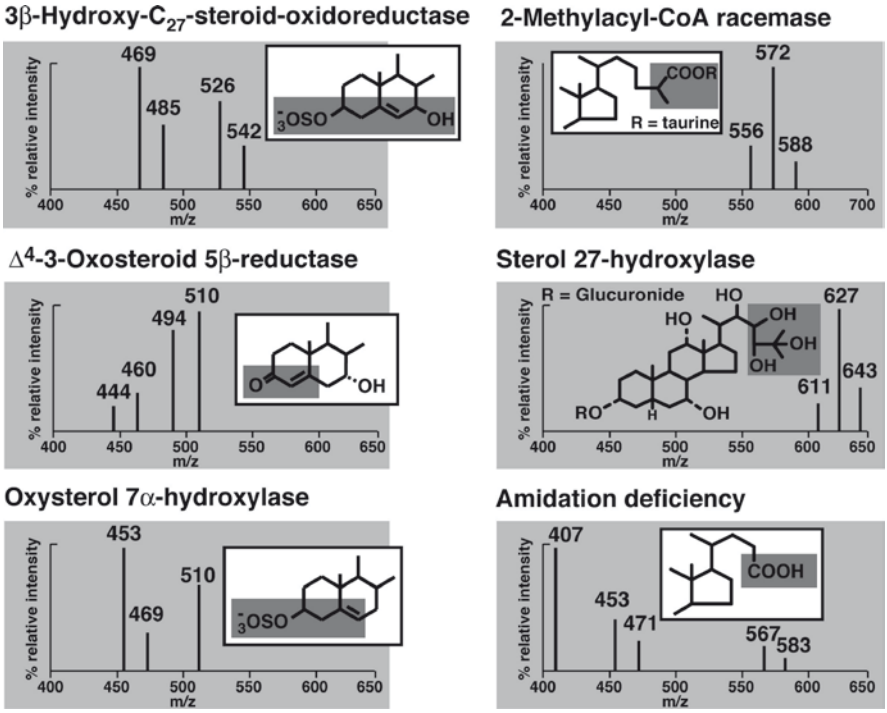
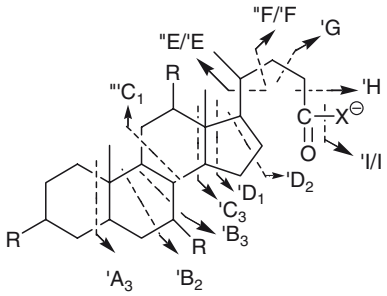


Fig. 10.15 Reconstructed negative ion mass spectra generated from FAB-MS ionization of typical urine extracts from patients with different genetic enzyme defects in bile acid synthesis. Only the key ions are shown for specific metabolites that retain the basic structure of the substrates for the deficient enzyme (Setchell and Heubi, 2006)



Scheme 10.1 Charge-remote fragmentation of bile acids. In taurine conjugates $X^- = NHC_2H_4SO_3^-$, in glycine conjugates $X^- = NHCH_2CO_2^-$ and in free acids $X^- = O^-$. The fragmentation nomenclature employed is described fully in Griffiths, Brown, Reimendal et al. (1996). Briefly, the prime to the left of the fragment describing the letter, e.g. $'A_3$, indicates that the ion is deficient in one hydrogen as compared to a fragment formed by a similar cleavage in the molecular ion (M^- or M^+)

Table 10.5 Characteristic fragment ions observed in CID spectra of bile acid $[M-H]^-$ ions. See Scheme 10.1 for fragment ion nomenclature

Compound	Fragment ion *m/z*															
TLCA[a]	High[b,c] energy	80 (SO_3)	94	107 (“J)	123/124 (‘I)/(I)	151 (‘H)	165 (‘G)	178 (“F)	206 (“E)	234 (‘D_2)	- -	288 (‘C_3)	314 (“C_1’)	342 (‘B_3)	356 (‘B_2)	410 (‘A_3)
TLCA[a]	Low[c,d,f] energy	- -	- -	107 (“J)	124 (I)	151 (‘H)	165 (‘G)	178 (“F)	206 (“E)	234 (‘D_2)	248 (‘D_1)	288 (‘C_3)	- -	342 (‘B_3)	- -	410 (‘A_3)
TLCA[a]	Low[c,e,f] energy	80 (SO_3)	95 (K)	107 (“J)	124 (I)	151 (‘H)	165 (‘G)	178 (“F)	206 (“E)	234 (‘D_2)	248 (‘D_1)	288 (‘C_3)	314 (‘C_1”)	342 (‘B_3)	- -	410 (‘A_3)
GLCA[a]	High[b,c] energy	- -	- -	58 (‘J)	74 (I)	101 (‘H)	115 (‘G)	128 (“F)	157 (‘E)	184 (‘D_2)	- -	238 (‘C_3)	- -	292 (‘B_3)	- -	360 (‘A_3)
GLCA[a]	Low[f] energy	- -	- -	- -	74 (I)	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
LCA[a]	High[b,c] energy	- -	- -	- -	- -	- -	58 (‘G)	71 (“F)	100 (‘E)	127 (‘D_2)	- -	181 (‘C_3)	- -	235 (‘B_3)	- -	303 (‘A_3)
LCA[a]	Low[f] energy	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -

[a]Incorporation of additional functional groups changes the fragment ion mass by the appropriate value, i.e., C–(O)H → +16: C = O → +14: → –2.

[b]High-energy CID: keV (He/N_2) – 400 eV (Xe).

[c]Side-chain cleavage ions may be terminally unsaturated or radical, depending on the collision energy and mode of ionization.

[d]FAB ionization.

[e]ES ionization.

[f]Low-energy CID spectra also contain many additional fragment ions that result from neutral losses (see Table 10.2).

TLCA, taurolithocholic acid; GLCA, glycolithocholic acid; LCA, lithocholic acid.

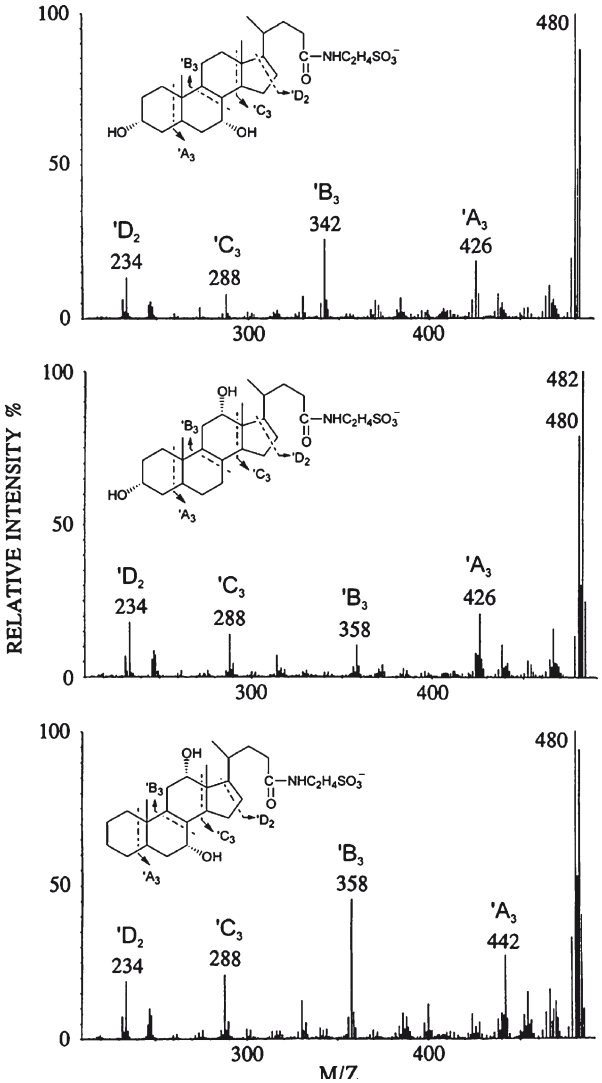


Fig. 10.16 Negative ion FAB-CID spectra of $[M-H]^-$ ions of *m/z* 498. Upper spectrum taurochenodeoxycholic acid, middle spectrum taurodeoxycholic acid, and lower spectrum 7α,12α-dihydroxycholanoyltaurine. The spectra were recorded as B/E linked scans at 6 keV collision energy with He as the collision gas on a double focusing magnetic sector instrument (modified from Zhang, Griffiths, Bergman, et al., 1993; with permission)

used to differentiate between bile acid isomers (Fig. 10.16) (Zhang, Griffiths, Bergman, et al., 1993). It was noted that taurine-conjugated bile acids give more intense $[M-H]^-$ ions, and more structurally informative product-ions than either glycine conjugated or non-aminoacyl amidated bile acids. This observation stimulated

the development of a derivatization method where aminosulphonic acids (e.g. 2-aminoethanesulphonic acid, taurine) were coupled to the carboxylic acid groups of non-aminoacyl amidated and glycine conjugated bile acids via an amide bond (Zhang, Griffiths, Bergman, et al., 1993). Sulphonic acids are more acidic than carboxylic acids (pK_a taurocholic acid 1.5, pK_a glycocholic acid 4.5), and hence are more readily deprotonated, and give more informative high-energy CID spectra than carboxylates. The derivatization procedure gives quantitative conversions and is applicable to labile structures such as, 7α-hydroxy-3-oxo-4-ene. This derivatization procedure has been used to aid in the determination by FAB-CID of the position of conjugation of bile acid glucosides (Glc), *N*-acetylglucosaminides (GlcNAc) and glucuronides (GlcA), and also in a study of the bile acid content of the urine of a child with cholestatic liver disease (Marschall, Griffiths, Götze, et al., 1994; Marschall, Griffiths, Zhang, et al., 1994; Yang, Griffiths, Nazer, et al., 1997).

When high-energy CID spectra of sugar containing bile acids are recorded, they show abundant neutral losses of the sugar moiety, as well as ring and side-chain fragment ions (Fig. 10.17; Scheme 10.2). Table 10.6 lists the characteristic neutral losses associated with sugar conjugation. FAB with high-energy CID was used to show that

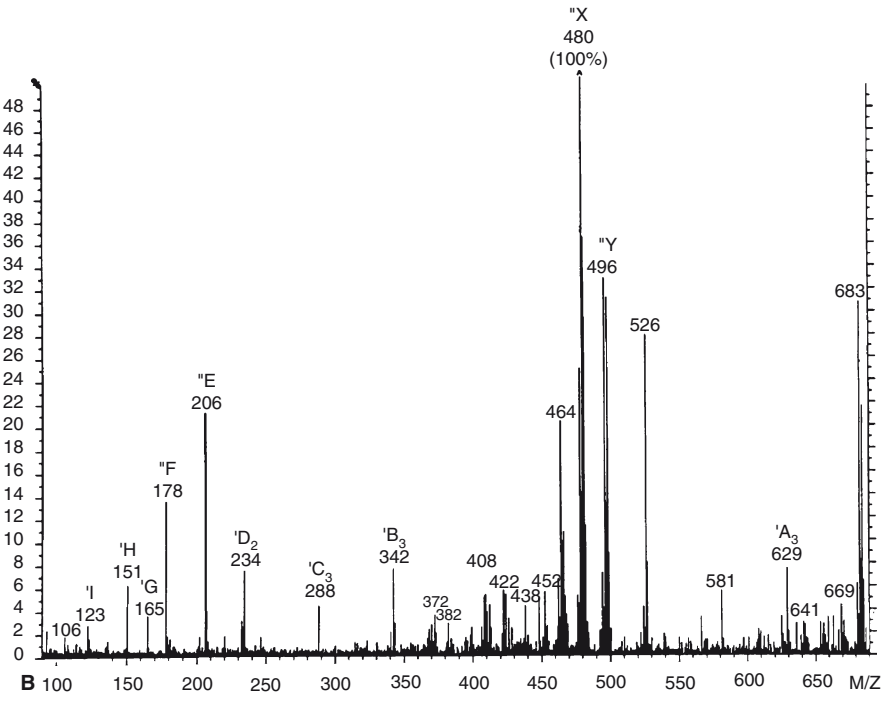


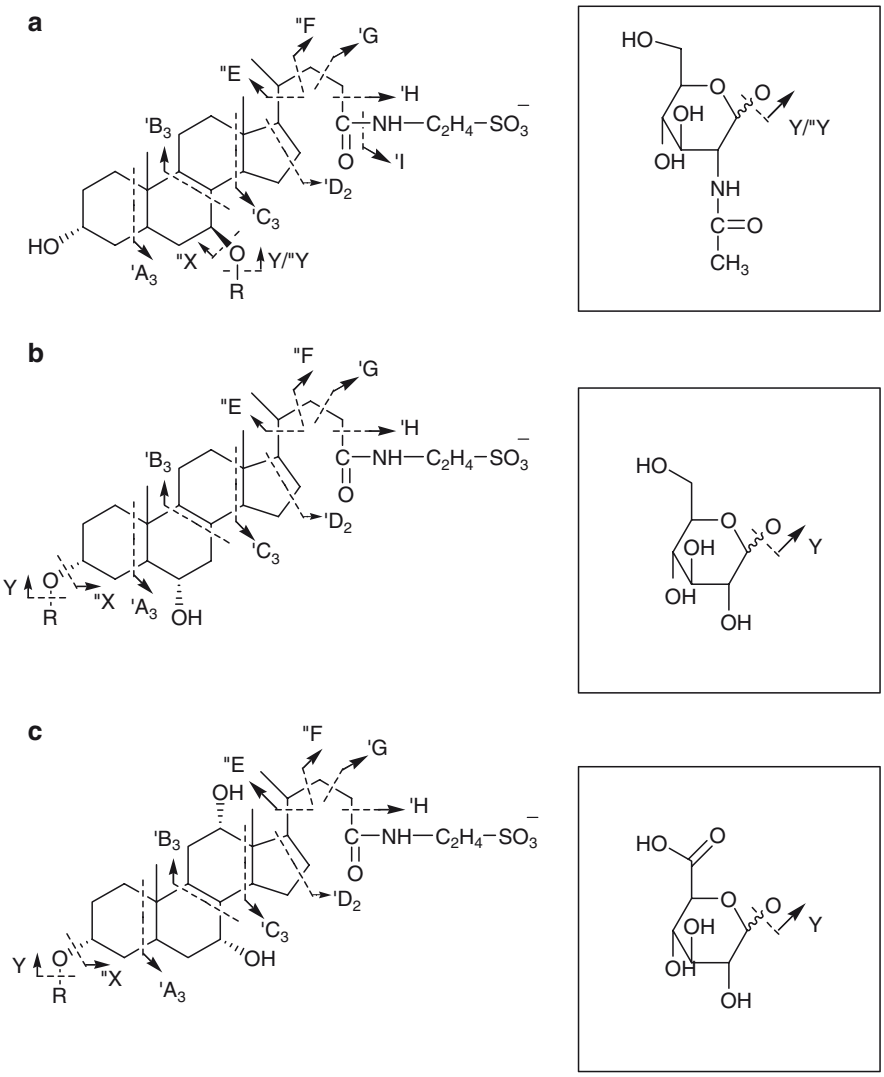
Fig. 10.17 Negative-ion FAB-CID spectrum of $[M\text{-}H]^-$ ions of tauroursodeoxycholic acid *N*-acetylglucosaminide at *m/z* 701. The CID spectrum was recorded as a B/E-linked scan at 8 keV collision-energy with He as the collision gas, on a double focussing magnetic sector instrument (with permission from Marschall, Griffiths, Götze, et al., 1994). See Scheme 10.2(a) for fragmentation and labeling of fragment ions

Table 10.6 Neutral losses characteristic of bile acids and bile alcohols and their conjugating groups (see Scheme 10.2)

Bile acid	Sulphate	GlcA	Glc	GlcNAc	Taurine	Glycine	Free acid
High-energy CID[a]	−80 (SO_3)	−176 ($C_6O_6H_8$)	−162 ($C_6O_5H_{10}$)	−203 ($C_8O_5NH_{13}$)[b]	–	−44 (CO_2)[d]	–
Bile acid [M-H]$^-$		−194 ($C_6O_7H_{10}$)[b]	−180 ($C_6O_6H_{12}$)	−205 ($C_8O_5NH_{15}$)[b]		−62 (H_2CO_3)[d,e]	
				−220 ($C_8O_6NH_{14}$)[c]			
				−221 ($C_8O_6NH_{15}$)			
Low-energy CID	−80 (SO_3)	−176 ($C_6O_6H_8$)	−162 ($C_6O_5H_{10}$)[f]	−203 ($C_8O_5NH_{13}$)	−64 (SO_2)	−44 (CO_2)	−44 (CO_2) or −46 (H_2CO_2)
Bile acid [M-H]$^-$					−66 (H_2SO_2)	−62 (H_2CO_3)	−62 (H_2CO_3)
					−82 (H_2SO_3)		−64 (H_4CO_3)
					−84 (H_4SO_3)		
					−94 (CH_2SO_3)		
					−108 ($C_2H_4SO_3$)		
					−125 ($NH_2C_2H_4SO_3H$)		

Bile alcohol	Sulphate	GlcA	GlcNAc
High-energy CID[a]	−80(SO_3)[d,e]	−176 ($C_6O_6H_8$)[b]	−175 ($C_7O_4NH_{13}$)[b,h]
Bile alcohol [M−H]$^-$		−194 ($C_6O_7H_{10}$)[b]	−203 ($C_8O_5NH_{13}$)[b,h]
			−205 ($C_8O_5NH_{15}$)[b,h]
			−221 ($C_8O_6NH_{15}$)[b,h]
Low-energy CID	−80 (SO_3)[g]	−60 ($C_2H_4O_2$)	–
Bile alcohol [M−H]$^-$		−176 ($C_6O_6H_8$)	
		−178 ($C_6O_6H_{10}$)	
		−194 ($C_6O_7H_{10}$)	
Low-energy CID	−80 (SO_3)	−176 ($C_6O_6H_8$)	–
Bile alcohol [M+H]$^+$	−98 (H_2SO_4)	−194 ($C_6O_7H_{10}$)	
		−212 ($C_6O_8H_{12}$)[i]	

[a]High-energy CID: keV (He/N_2) – 400 eV (Xe).
[b]Observed in FAB keV CID.
[c]The resultant distonic product ion is stabilized by radical delocalization over a double bond.
[d]Observed in ES 800–400 eV CID.
[e]Observed when double conjugated with GlcA.
[f]Glc or Gal.
[g]Observed for di-OSO_3.
[h]Observed for OSO_3/GlcNAc double conjugates.
[i]Observed when sterol contains an additional alcohol or ketone group.



Scheme 10.2 Fragmentation of sugar-containing bile acids. (**a**) GlcNAc is shown conjugated to tauroursodeoxycholic acid, R = $C_8O_5NH_{14}$. (**b**) Glc is shown conjugated to taurohyodeoxycholic acid, R = $C_6O_5H_{11}$. (**c**) GlcA is shown conjugated to taurocholic acid, R = $C_6O_6H_9$. The structures of the sugars are shown in insets

the position of glucose conjugation to bile acids is at the 3(α)-hydroxyl group, and that selective conjugation of GlcNAc occurs at the C-7(β) hydroxyl group (Marschall, Griffiths, Götze, et al., 1994; Marschall, Griffiths, Zhang, et al., 1994).

Not only bile acids, but also bile alcohols conjugated with acidic groups (e.g. sulphate, glucuronide) are readily ionized by negative-ion FAB. For example, Egestad et al. showed that FAB-MS spectra of urine from a patient suffering from

cerebrotendious xanthomatosis (CTX), who lacked the cytochrome P450 enzyme CYP27A1, gave a profile of bile alcohol glucuronide peaks at *m/z* 611, 627, and 643 corresponding to $[M-H]^-$ ions of C_{27} bile alcohol glucuronides with four, five and six hydroxyl groups (Egestad, Pettersson, Skrede, et al., 1985). Similar studies were performed by Dayal, Salen, Tint, et al. (1990), but using FAB-MS in the positive-ion mode (Dayal, Salen, Tint, et al., 1990). As discussed above, FAB is most powerful when combined with high-energy CID, in which case it can be used to identify the location of conjugating groups on the bile alcohol skeleton. This is illustrated in the work of Meng et al. where both negative-ion FAB and ESI with high-energy CID was used to identify the double conjugate (24*S*)-24-hydroxycholesterol-3-sulphate, 24-glucuronide in the urine of a child with severe cholestatic liver disease (Meng, Griffiths, Nazer, et al., 1997). GC-MS was used to characterize the steroid stereochemistry after removal of the conjugating groups, the locations of which were determined by FAB- and ESI-CID (see Fig. 3 in Meng, Griffiths, Nazer, et al., 1997).

As FAB was initially incorporated on magnetic-sector instruments, most of the early CID studies were performed at high collision-energy (keV). However, informative CID spectra of bile acids can also be obtained at low collision-energy (<100 eV) (Table 10.5) (Libert, Hermans, Draye, et al., 1991; Stroobant, de Hoffmann, Libert, et al., 1995; Lemonde, Johnson and Clayton, 1999). Taurine-conjugated Δ^4-unsaturated bile acids give particularly informative low-energy CID spectra (Libert, Hermans, Draye, et al., 1991) (Fig. 10.18). The spectra of Δ^4-unsaturated taurine conjugates give a series of CRF fragment ions from which the position of unsaturation can readily be determined. Libert, Hermans, Draye, et al. (1991) were also able to make use of neutral-loss scans, where the facile 'B_3 fragmentation gives a characteristic neutral loss of 152 Da for 3-oxo-Δ^4 taurine conjugates and 154 Da for 3-hydroxy-Δ^4 taurine conjugates (Libert, Hermans, Draye, et al., 1991) (Fig. 10.18). In a study that involved the analysis of urine from a patient with tyrosinemia type 1 disease, the neutral-loss scan for 152 Da allowed the specific detection of taurine conjugates of 7α-hydroxy-3-oxo-chol-4-en-24-oic acid and 7α, 12α-dihydroxy-3-oxo-chol-4-en-24-oic acid.

10.4.2.2 ESI-MS

Atmospheric pressure ionization has largely replaced FAB ionization in lipid mass spectrometry, although FAB is still favoured by some research groups (Setchell and Street, 1987; Setchell and O'Connell, 1994; Setchell, Schwarz, O'Connell, et al., 1998; Bove, Heubi, Balistreri, et al., 2004; Setchell and Heubi, 2006; Heubi, Setchell and Bove, 2007). In terms of CID, FAB provides definite advantages over the less energetic ionization modes, i.e. ESI and atmospheric pressure chemical ionization (APCI); however, LC is far more compatible with API methods than with FAB (Yang, Griffiths, Nazer, et al., 1997). It should also be noted that LC combined with continuous-flow FAB was used for bile acid analysis during the late 1980s and early 1990s (Ito, Takeuchi, Ishii, et al., 1986; Evans, Ghosh, Evans, et al., 1993; Yang, Griffiths, Nazer, et al., 1997). The first ESI spectra of bile

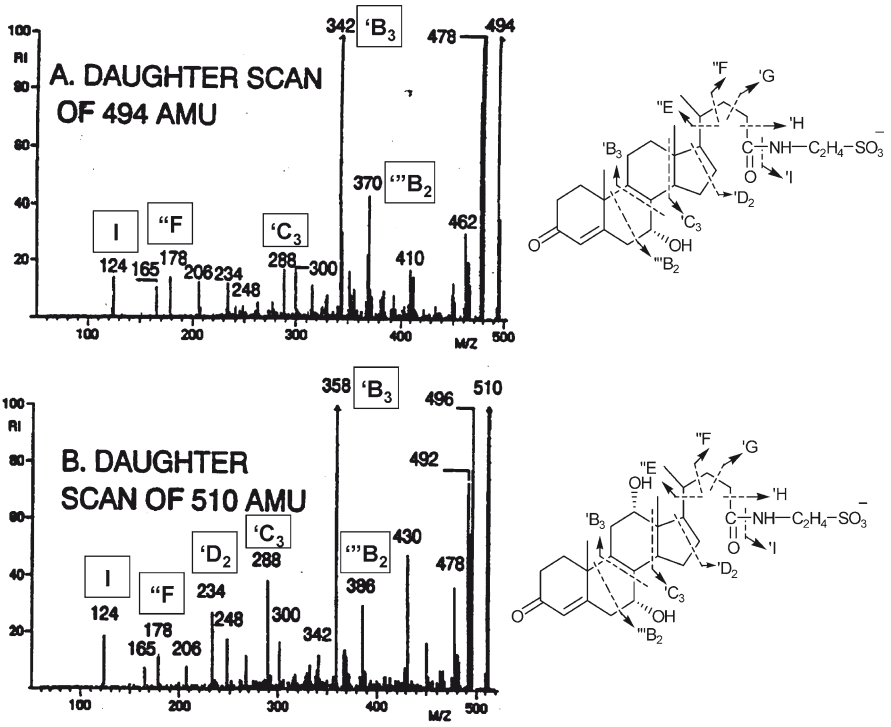


Fig. 10.18 FAB-CID spectra of $[M-H]^-$ of (**a**) 7α-hydroxy-3-oxochol-4-en-24-oyltaurine and (**b**) 7α,12α-dihydroxy-3-oxochol-4-en-24-oyltaurine. Spectra were recorded on a tandem quadrupole using xenon as the collision gas (modified from Libert, Hermans, Draye, et al., 1991)

acids were published by Warrack and DiDonato in 1993 (Warrack and DiDonato, 1993) followed later by Roda et al. in 1995 (Roda, Gioacchini, Cerrè, et al., 1995). Both groups presented data on the LC-MS analysis of bile acids. Warrack and DiDonato used ESI in the positive-ion mode, and by combining LC-MS with SIM achieved detection limits on the order of 50 pg (Warrack and DiDonato, 1993). Roda et al. used ESI in the negative-ion mode and achieved impressive detection limits of the order of 15 pg when monitoring $[M-H]^-$ ions and performing conventional reversed-phase LC on a C_{18} column, which was improved to 5 pg when a capillary column was used (Roda, Gioacchini, Cerrè, et al., 1995). Significantly, Roda et al. observed ESI to be more sensitive for glycine- and taurine-conjugated bile acids than for free bile acids, a finding that was also similarly observed for FAB ionization, and exploited using simple derivatization chemistry (Zhang, Griffiths, Bergman, et al., 1993). LC-ESI-MS has more recently been exploited to identify unconjugated bile acids in the cytoplasmic fraction from rat brain (Mano, Goto, Uchida, et al., 2004), human cecal bile acids (Hamilton, Xie, Raufman, et al., 2007), and sulphated glycine- and taurine-double conjugated bile acids in human urine (Shinka, Inoue, Ohse, et al., 2007). Whether using ESI or FAB as the method of ioniza-

tion, care must be taken in interpreting mass spectra in the absence of extra information, e.g. chromatographic time or MS/MS data. To postulate the presence of a bile acid or its structure based on the mass of an $[M\text{-}H]^-$ ion alone can readily lead to mistaken identifications (Evans, Ghosh, Evans, et al., 1993; Natowicz and Evans, 1994).

LC-MS methodologies have also been used for the analysis of bile acid sulphates and glucuronides. Both conjugating groups are characterized by the presence of $[M\text{-}H]^-$ and $[M\text{-}2H]^{2-}$ ions in their negative-ion ESI mass spectra, and the ratio of the charge-states depends on the ESI interface conditions and the solvent composition. LC-MS methods for analysing bile acid 3-sulphates and 3-glucuronides in human urine have been developed relying on SIM and stable isotope labelled internal standards for quantification (Ikegawa, Murao, Motoyama, et al., 1996; Ikegawa, Yanagihara, Murao, et al., 1997).

Bile acids are conjugated with glucuronic acid via an ether linkage formed between the anomeric hydroxyl group of the sugar and a hydroxyl group on the steroid nucleus (Scheme 10.2c). However, glucuronic acid can also be linked to bile acids through an ester bond to the C-24 carboxylic acid group (Fig. 10.19a) (Goto, Murao, Nakada, et al., 1998; Ikegawa, Okuyama, Oohashi, et al., 1999). The ester bond formed between the C-24 carboxyl group on the bile acid and the hydroxyl group on the anomeric carbon of the sugar is easily hydrolysed under neutral or alkaline conditions and care must be taken to avoid this occurring. Aqueous acetonitrile, buffered with 20 mM ammonium acetate, pH 6, offers a good solvent for ESI analysis and as a mobile phase for chromatographic separation. The solution phase instability of bile acid 24-glucuronides is mirrored in the electrospray interface when operated in the negative-ion mode. Bile acid 24-glucuronides are found to be less stable than 3-glucuronides and undergo in-source fragmentation with the loss of sugar moiety (−176 Da) (Fig. 10.19a, b). This allows the differentiation of bile acid isomers according to the position of glucuronidation. An LC-MS assay for bile acid 24-glucuronides in human urine based on SIM has been developed as bile acid 3-glucuronides are easily separated from 24-glucuronides on a C_{18} column (Fig. 10.19c, d) (Ikegawa, Okuyama, Oohashi, et al., 1999). These experiments were performed on a double-focusing magnetic sector instrument, where it was possible to perform SIM at moderately high resolution (5,000), which lends confidence to the identification and quantification of the bile acids.

Bile acid acyl glucosides have not been identified in either human urine or serum (Goto, Shibata, Iida, et al., 2004; Goto, Shibata, Sasaki, et al., 2005). However, bile acid acyl galactosides (Gal) have been identified in human urine (Goto, Shibata, Sasaki, et al., 2005). Using negative-ion APCI with a post-column addition of octanoic acid and employing LC-MS with SIM for octanoate adducts of bile acid hexosides, deoxycholic acid 24-galactoside and cholic acid 24-galactoside were identified in human urine (Fig. 10.20). Further confirmation was obtained by LC-MS/MS, where DCA 24-Gal and CA 24-Gal were confirmed from retention times and MS/MS data (Fig. 10.21; Table 10.6). Another modern ionization technique, matrix-assisted laser desorption/ionization (MALDI), has recently been exploited by Ikegawa et al. to demonstrate the covalent attachment of lithocholic acid to lysine or arginine residues in a number of proteins from the liver of bile duct-ligated rats (Ikegawa, Yamamoto, Ito, et al., 2008).

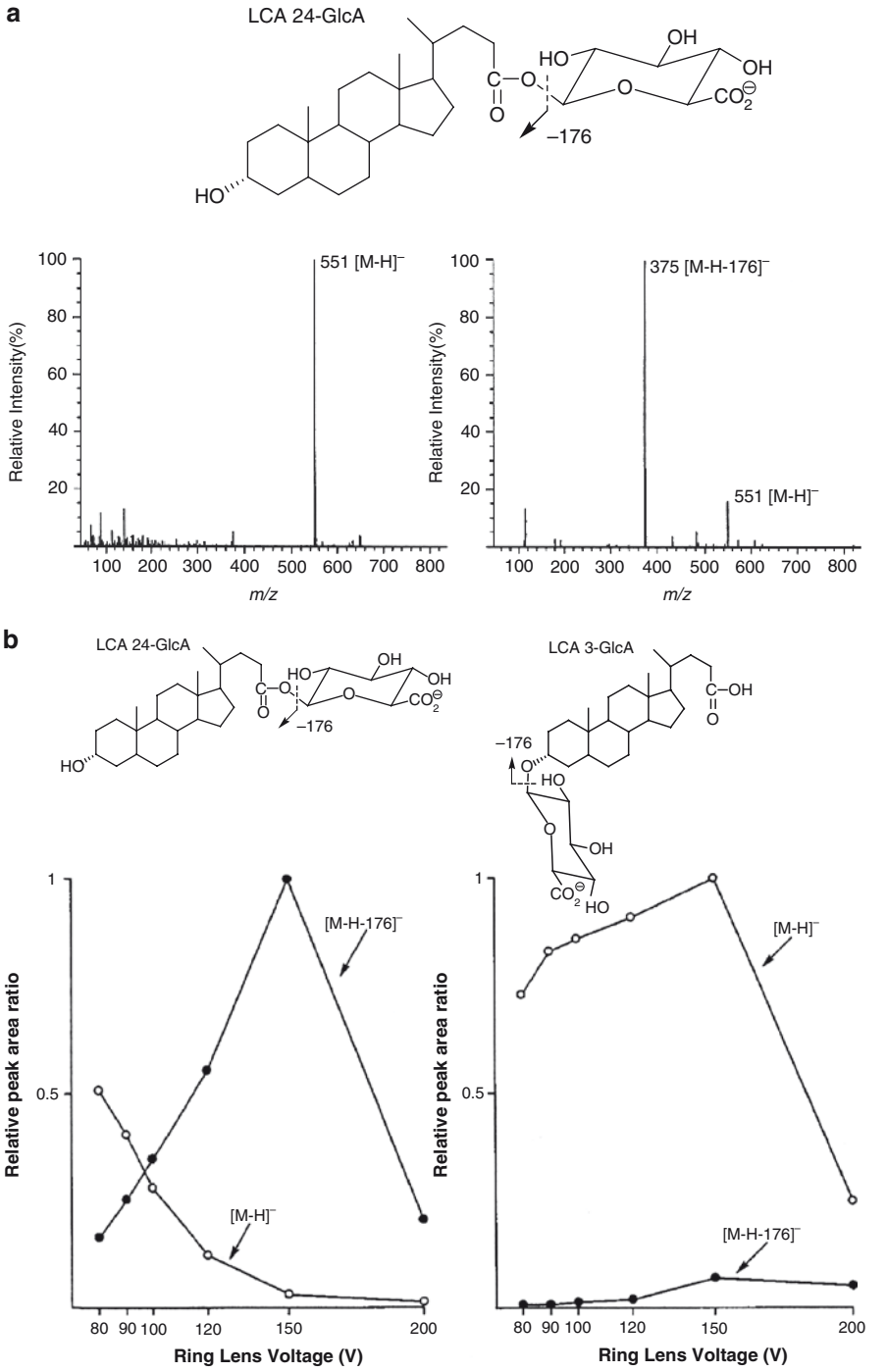


Fig. 10.19 Negative-ion ESI-MS analysis of bile acid glucuronides. (**a**) ESI-MS of lithocholic acid 24-glucuronide recorded under normal (ring voltage 60 V, *left* panel) and energetic (ring voltage 150 V, *right* panel) source conditions. (**b**) The influence of ring voltage on the formation of $[M\text{-}H\text{-}176]^-$ ions; lithocholic acid 24-glucuronide (*left* panel), 3-glucuronide (*right* panel).

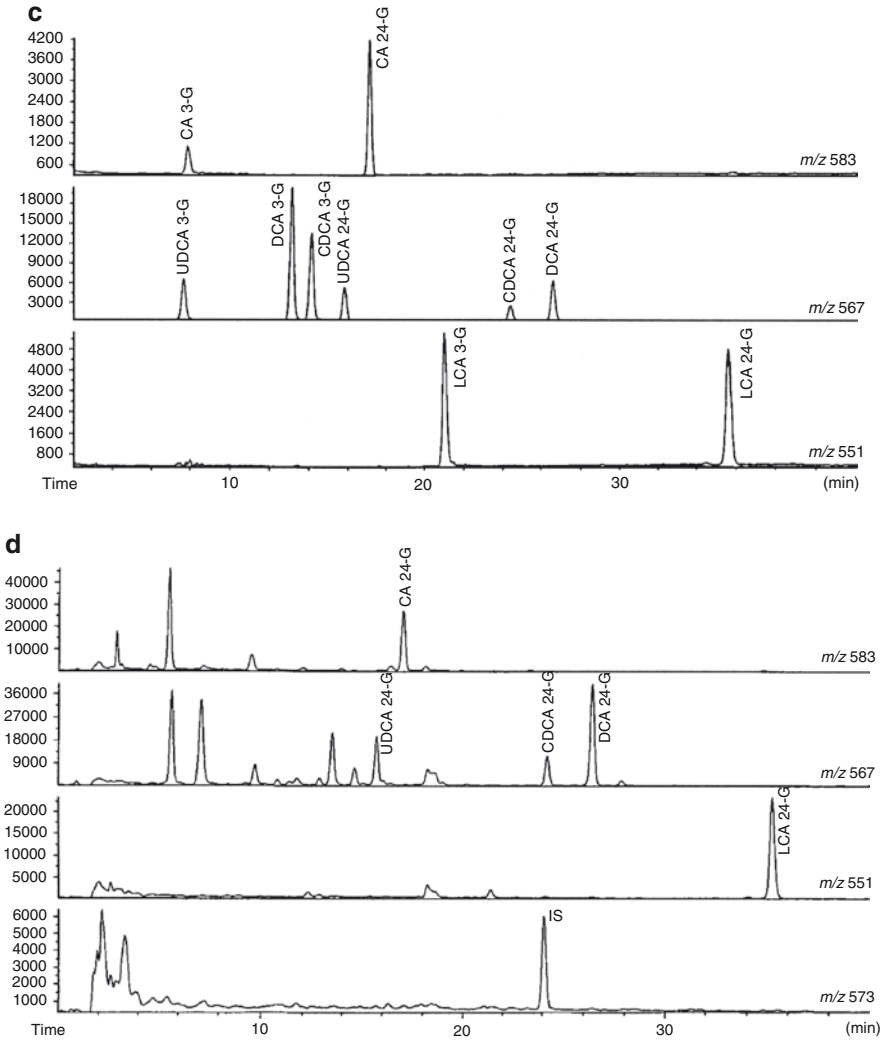


Fig. 10.19 (continued) (**c**) SIM of bile acid 3- and 24-glucuronide [M-H]$^-$ ions. Separation was performed on a C_{18} column using a mobile phase of 20 mM ammonium acetate (pH 6): acetonitrile (3:1 → 3:2, v/v, over 35 min), at a flow rate of 150 μL min^{-1}. (**d**) SIM for bile acid 24-glucuronides in human urine, the internal standard (IS) is [3,7-$^{18}O_2$,$^2H_2$]chenodeoxycholic acid 24-glucuronide. Data collected on a double focussing magnetic sector mass spectrometer (with permission from Ikegawa, Okuyama, Oohashi, et al., 1999). Abbreviations: cholic acid (CA), ursodeoxycholic acid (UDCA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), lithocholic acid (LA)

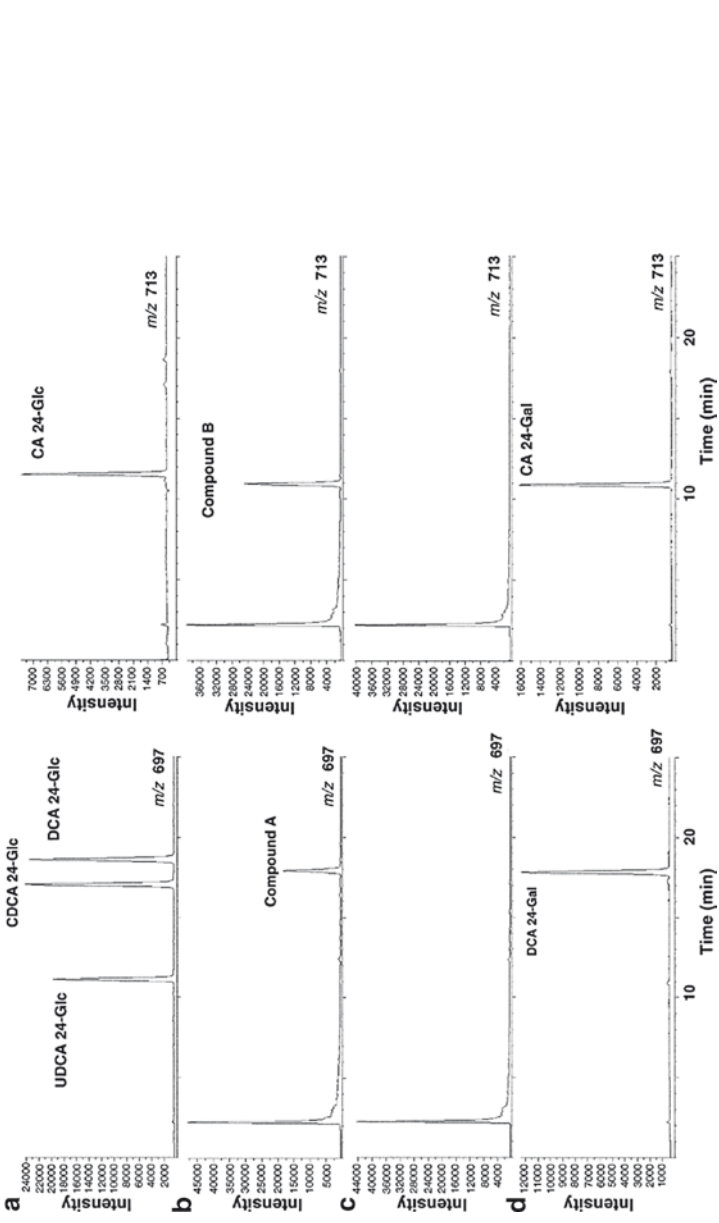


Fig. 10.20 LC-APCI-MS of bile acid glycosides with post column addition of octanoic acid. SIM for (**a**) authentic bile acids: UDCA 24-Glc, CDCA 24-Glc, DCA 24-Glc ($[M\text{-}H + 144]^-$, *m/z* 697) and CA 24-Glc ($[M\text{-}H + 144]^-$, *m/z* 713); (**b**) human urine extract; (**c**) human urine extract following alkaline hydrolysis; (**d**) authentic specimen of DCA 24-Gal ($[M\text{-}H + 144]^-$, *m/z* 697) and CA 24-Gal ($[M\text{-}H + 144]^-$, *m/z* 713). The mass and retention times of compounds **A** and **B** indicate that they correspond to DCA 24-Gal and CA 24-Gal, respectively. The bile acid glycosides eluted from a C_{18} column (150 × 4.6 mm, 5 μm particles) using a mobile phase of 1 mM ammonium acetate, pH 7, and acetonitrile. The flow rate was 0.9 mL/min, and the eluent was supplemented post-column with 200 mM octanoic acid and ammonia at a flow of 0.1 mL/min. Abbreviations are as in Fig. 10.19

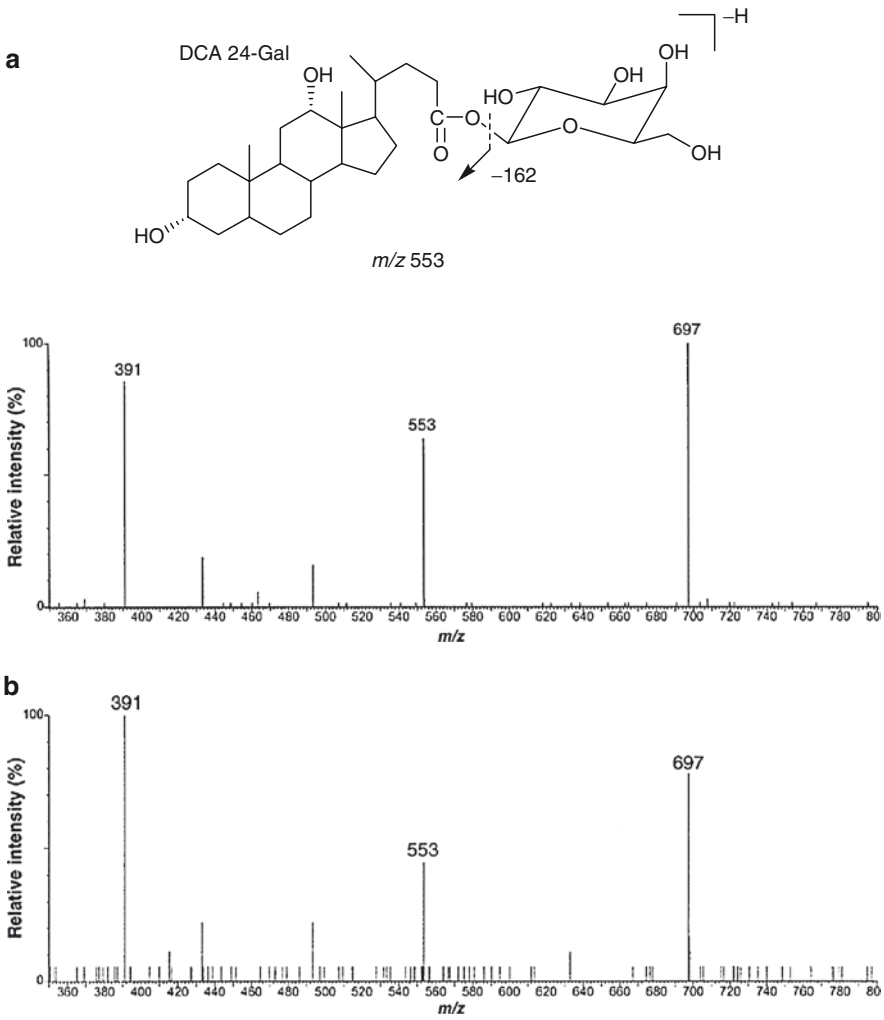


Fig. 10.21 MS/MS spectra of (**a**) DCA 24-Gal octanoate adduct $[M\text{-}H + 144]^-$, *m/z* 697 and (**b**) compound **A** from Fig. 10.20, *m/z* 697. The ions at 553 and 391 correspond to $[M\text{-}H]^-$ and $[M\text{-}H\text{-}162]^-$ ions, respectively. Spectra were recorded on a tandem quadrupole with a collision energy of 15 eV. Ionization was via negative-ion APCI and octanoic acid was a post-column additive

10.4.2.3 ESI-MS/MS

Although very impressive detection limits can be achieved by LC-ESI-MS, and LC-APCI-MS, considerable gains in specificity are possible by incorporating MS/MS. Most, but not all, API-MS/MS instruments now employ low-energy CID (<100 eV), although as discussed below, high-energy CID offers many advantages in terms of structure determination.

Bile acids can be ionized by ESI in either the positive-ion or negative-ion mode. In the positive-ion mode, low-energy MS/MS spectra of bile acid $[M + H]^+$ ions are dominated by $[M + H\text{-}nH_2O]^+$ ions, where *n* is the number of ring hydroxyl groups. Thus, neutral-loss scans for −36 and −54 Da have been used in LC-ESI-MS/MS experiments to detect di- and tri-hydroxy bile acids respectively. Taurine-conjugated bile acids fragment to give prominent ions at *m/z* 126 ($^+H_3NC_2H_4SO_3H$), while glycine-conjugates give ions at *m/z* 76 ($^+H_3NCH_2CO_2H$). Isomer differentiation by low-energy MS/MS of $[M + H]^+$ ions is possible, although ESI-MS/MS spectra of bile acid isomers (e.g. taurochenodeoxycholic acid, TCDCA, and taurodeoxycholic acid, TDCA) are very similar, only differing in fragment-ion intensities. The limited differentiating power of low-energy CID makes an online LC step highly desirable for the differentiation of bile acids from biological fluids.

Negative-ion ESI-MS/MS of taurine conjugated bile acids performed at low collision-energy results in abundant fragment ions at *m/z* 124 ($H_2NC_2H_4SO_3^-$), 107 ($CH_2CHSO_3^-$) and 80 (SO_3^-) (Table 10.5; Fig. 10.22). This can be exploited using the single reaction monitoring (SRM) or multiple reaction monitoring (MRM) transitions $[M\text{-}H]^- \rightarrow$ *m/z* 124, 107 or 80, or in precursor-ion scans for these ions (Mushtaq, Logan, Morris, et al., 1999; Perwaiz, Tuchweber, Mignault, et al., 2001). MRM, SRM and precursor-ion scans are modes of operation that are particularly suited to tandem quadruple instruments. Glycine conjugated bile acids give abundant ions at *m/z* 74 ($H_2NCH_2CO_2^-$) at low collision-energy (Table 10.5; Fig. 10.22) and

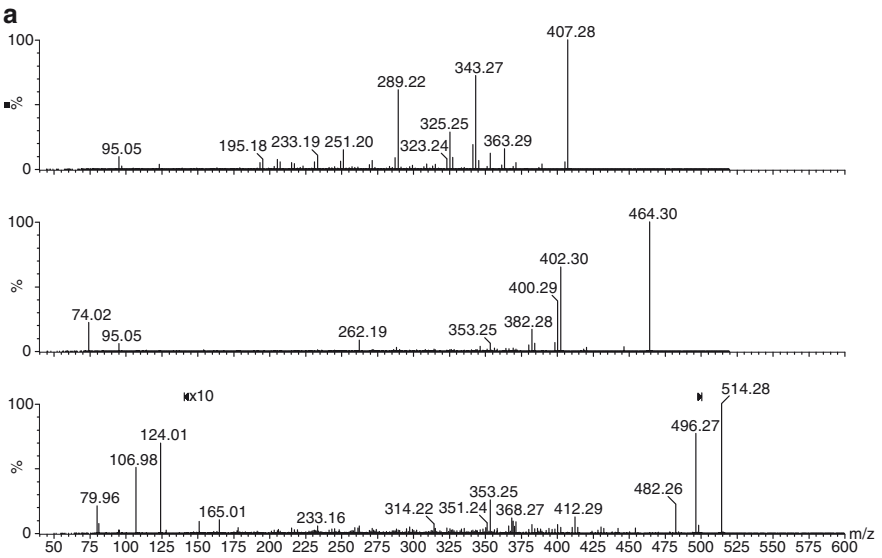


Fig. 10.22 Low-energy CID spectra of ESI-generated $[M\text{-}H]^-$ ions of (**a**) cholic acid *m/z* 407 (1 ng/μL, 40 eV, Ar, *upper* panel), glycocholic acid *m/z* 464 (1 ng/μL, 40 eV, Ar, *centre* panel), taurocholic acid *m/z* 514 (50 pg/μL, 60 eV, Ar, *lower* panel):

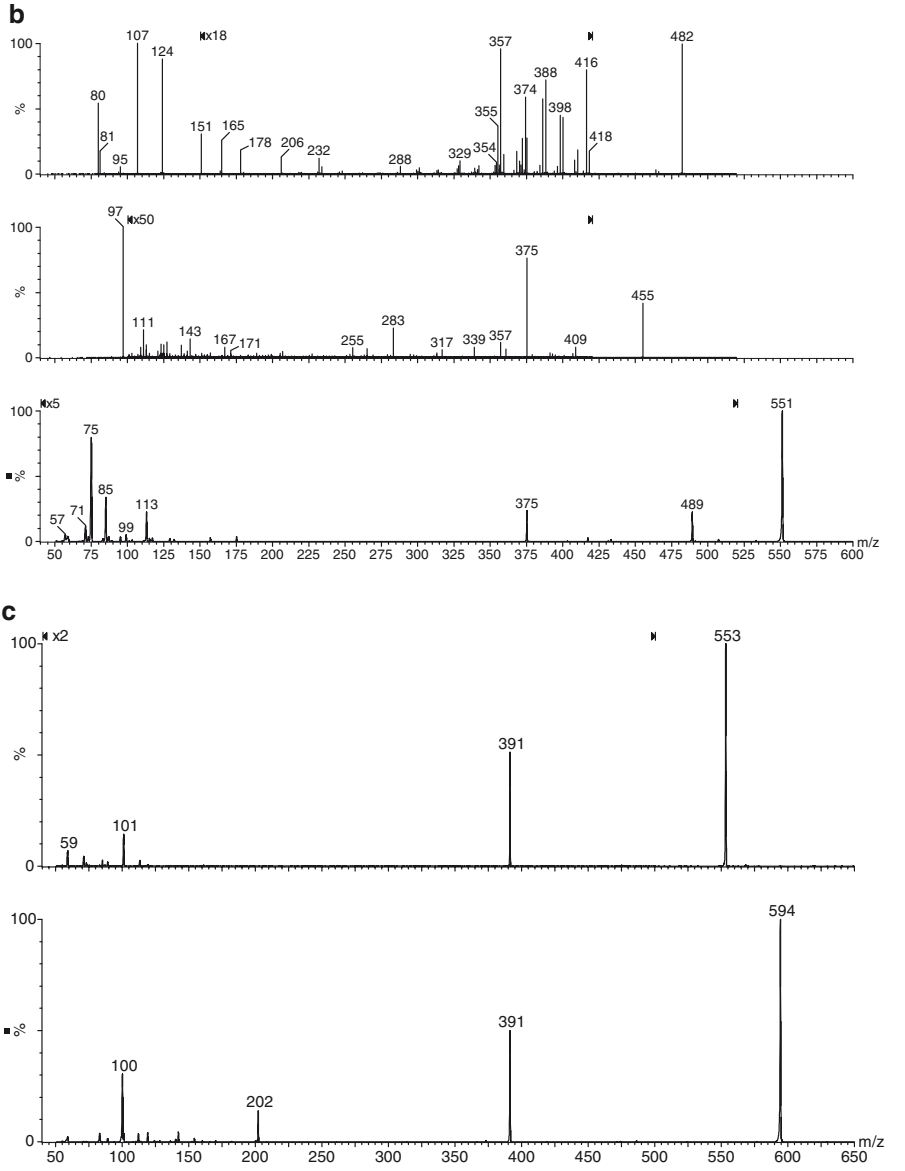


Fig. 10.22 (continued) (**b**) taurolithocholic acid *m/z* 482 (500 pg/μL, 60 eV, Ar, *upper* panel), lithocholic acid 3-sulphate *m/z* 455 (100 pg/μL, 40 eV, Ar, *central* panel), lithocholic acid 3-glucuronide *m/z* 551 (10 ng/μL, 30 eV, Ar, *lower* panel): (**c**) hyodeoxycholic acid 3-glucoside *m/z* 553 (30 eV, Ar, *upper* panel), ursodeoxycholic acid 7β-*N*-acetylglucosaminide *m/z* 594 (30 eV, Ar, *lower* panel). The MS/MS spectra in (**a**)–(**b**, *upper* and *central* panel) were recorded on a quadrupole time-of-flight instrument, while those in (**b**, *lower* panel) and (**c**) were recorded on a tandem quadrupole instrument. Nano-ESI was used in each case and the sample concentration, collision energy and collision gas are given in parenthesis

again this transition can be exploited in MRM and precursor-ion scans (Mushtaq, Logan, Morris, et al., 1999; Perwaiz, Tuchweber, Mignault, et al., 2001). Non-aminoacyl amidated bile acids with a free carboxylic acid group fragment under low collision-energy CID conditions to give weak but reproducible neutral losses of 44 Da (CO_2) or 46 Da (HCO_2H) and of 46 + *n*18 Da (where *n* is the number of hydroxyl groups), which can be exploited in neutral loss-scans. Sulphated bile acids give a characteristic fragment at *m/z* 97 (HSO_4^-), and a neutral loss of 80 Da (SO_3). Table 10.6 lists characteristic neutral losses observed in the low-energy CID of conjugated bile acids and bile alcohols.

LC-ESI-MS/MS using low-energy CID has been used by many workers for the quantification of bile acids in biological fluids (e.g. human bile (Perwaiz, Tuchweber, Mignault, et al., 2001)), human serum (Bootsma, Overmars, van Rooij, et al., 1999; Tagliacozzi, Mozzi, Casetta, et al., 2003; Burkard, von Eckardstein and Rentsch, 2005), human urine (Goto, Myint, Sato, et al., 2007), and rat serum (Ando, Kaneko, Watanabe, et al., 2006). For taurine conjugates the MRM transitions $[M\text{-}H]^- \rightarrow$ *m/z* 124 or 80 are favoured (Perwaiz, Tuchweber, Mignault, et al., 2001; Burkard, von Eckardstein and Rentsch, 2005), while for glycine conjugates the MRM transition $[M\text{-}H]^- \rightarrow$ *m/z* 74 is usually used (Perwaiz, Tuchweber, Mignault, et al., 2001). The residual precursor-ion after MS/MS is often used for the quantification of free bile acids. This practice is not to be recommended because the specificity provided by SRM is lost. An alternative and preferable strategy is to employ $[M\text{-}H]^- \rightarrow [M\text{-}H\text{-}44]^-/[M\text{-}H\text{-}46]^-$, or $[M\text{-}H\text{-}44\text{-}n18]^-/[M\text{-}H\text{-}46\text{-}n18]^-$ transitions which maintain the specificity provided by SRM.

In an effort to improve the speed and sensitivity of analyses of unconjugated bile acids, Johnson and colleagues have taken an alternative strategy based on derivatization to dimethylaminoethyl (DMAE) esters and direct ESI-MS/MS analysis (Johnson, ten Brink, Schuit, et al., 2001). The first step of their analytical protocol was to protect alcohol groups by reaction with acetyl chloride. The product was then treated with oxalyl chloride and then dimethylaminoethanol to give a DMAE ester. Derivatized bile acids give intense $[M + H]^+$ peaks in ESI mass spectra, and the product-ion spectra are dominated by $[M + H\text{-}CH_3CO_2H]^+$ fragments. The $[M + H]^+ \rightarrow [M + H\text{-}60]^+$ transition is particularly useful for MRM studies, and in this way C_{27} bile acids in plasma samples of only 5 μL have been quantified. Patients with peroxisomal disorders show impaired β-oxidation of C_{27} bile acid intermediates and consequently have levels of C_{27} bile acids, notably 3α,7α-dihydroxy-5β-cholest-27-oic acid and 3α,7α,12α-trihydroxy-5β-cholest-27-oic acid in plasma (Clayton, Lake, Hall, et al., 1987; Setchell, Bragetti, Zimmer-Nechemias, et al., 1992). This is illustrated in the ESI-MS/MS neutral loss spectra presented in Fig. 10.23, which show the mass of precursor-ions that lose 60 Da (CH_3CO_2H) in the CID process. Johnson et al. were also able to use this method to quantify dihydroxy- and trihydroxy C_{27} bile acids in blood spots from Guthrie cards (Johnson, ten Brink, Schuit, et al., 2001). The advantage of this method is that it takes less than 1 h for sample preparation and ESI-MS/MS analysis, requires no purification or preparation steps, and uses a simple chemical derivatization step. The disadvantages are the possible interference of other hydroxyacids with similar molecular

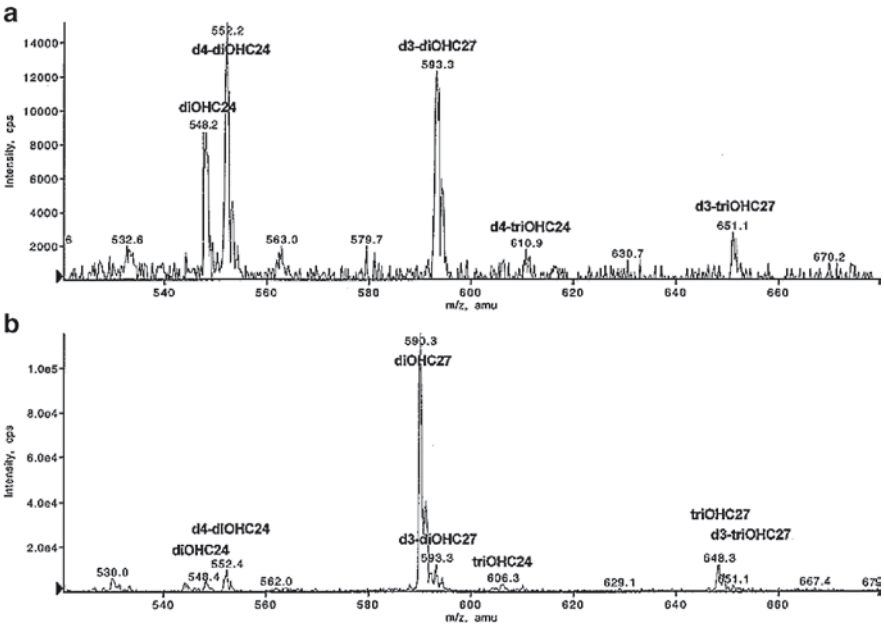


Fig. 10.23 ESI-MS/MS analysis of acetyl dimethylaminoethyl esters of bile acids extracted from 5 μL of plasma. Neutral-loss scans for 60 Da (CH_3CO_2H) are shown. Stable isotope-labelled standards are indicated with the prefix d3 or d4. (**a**) A control plasma sample from a healthy infant. (**b**) A plasma sample from an infant with peroxisome biogenesis defect. Spectra were recorded on a tandem quadrupole instrument. (with permission from Johnson, ten Brink, Schuit, et al., 2001)

weights to those of the bile acids under investigation, and the inability of the method to distinguish between isomers. It should be noted that Peter Clayton's group in London had previously demonstrated the potential for bile acid analysis by ESI-MS/MS of blood spots from Guthrie cards (Mills, Mushtaq, Johnson, et al., 1998; Mushtaq, Logan, Morris, et al., 1999).

Another derivatization method, this time appropriate for the bile acid precursor 7α-hydroxycholest-4-en-3-one has recently been described by Honda, Yamashita, Numazawa et al. (2007). This involves derivatization to the picolinoyl ester and LC-ESI-MS/MS exploiting MRM. The sensitivity of the method is such that 7α-hydroxycholest-4-en-3-one can be quantified in 2–50 μL of plasma. This method has similarly been exploited by the same group for the analysis of oxysterol precursors of bile acids in human serum and rat liver microsomes (Honda, Yamashita, Hara, et al., 2009; Honda, Yamashita, Miyazaki, et al., 2008).

During bile acid biosynthesis, bile acid intermediates become activated as CoA thioesters (Russell, 2003). LC-ESI-MS/MS methods have been developed to permit the identification of synthetic trihydroxycholestanoic acid CoA thioesters (Gan-Schreier, Okun, Kohlmueller, et al., 2005), *in vitro* formed trihydroxycholanoic acid thioesters and adenylate acid anhydrides (Ikegawa, Ishikawa, Oiwa,

et al., 1999), and also glutathione thioesters formed *in vitro* from the latter two substrates (Mitamura, Sogabe, Sakanashi, et al., 2007).

Although most studies performed today are on instruments designed for LC-API-MS, or for API-MS/MS at low collision-energy (<100 eV), bile acids and bile alcohols have also been analysed by ESI-MS/MS under high collision-energy conditions (Meng, Griffiths, Nazer, et al., 1997; Yang, Griffiths, Nazer, et al., 1997). ESI-CID spectra recorded at keV collision-energy are essentially similar to those recorded by FAB-CID (Table 10.5), and provide detailed structural information. Further, CID spectra recorded at intermediate collision energy (800–400 eV) with Xe as the collision gas give fragment-ion patterns similar to those generated at keV energy, and can also be regarded as high-energy CID spectra (Table 10.5). For structure determination, high-energy CID has the advantage over low-energy CID, in that the high-energy CID spectra display pronounced fragment-ion patterns resulting from cleavage of carbon–carbon bonds within the steroid skeleton and side-chain, while low-energy CID spectra are dominated by fragment ions generated by the neutral loss of small molecules, i.e. water, conjugating group (cf. Figs. 10.16 and 10.22). This makes high-energy CID the technique of choice for the identification of novel conjugated bile acids, although the constraints imposed by the requirement of high source potential has limited the development of instruments suitable for ESI-keV-CID.

The advantage provided by high-energy CID is illustrated in a study performed by Alvelius and colleagues who characterized an unusual 7-oxygenated bile acid sulphate from a patient suffering from Niemann-Pick disease type C (Alvelius, Hjalmarson, Griffiths, et al., 2001). The negative-ion ESI mass spectrum of the urine from this patient was unusual in that it contained numerous pairs of $[M\text{-}2H]^{2-}$ and $[M\text{-}H]^{-}$ ions, e.g. 335.6^{2-} and 672.4^{-} (Fig. 10.24a). ESI-high-energy-CID analysis of the $[M\text{-}H]^{-}$ ion at *m/z* 672 indicated the presence of the sulphate group (*m/z* 80) (SO_3^-), 97 (HSO_4^-), and GlcNAc (*m/z* 452, loss of 220 Da) groups (Fig. 10.24b). The sulphate group was located to position C-3 by the fragment ion at *m/z* 123, while location of the GlcNAc group to the ABC ring system by the fragment ion at *m/z* 530, and specifically to C-7 adjacent to the double bond between C-5 and C-6 by the radical fragment-ion at *m/z* 452 (Table 10.26). With the additional aid of GC-MS analysis the new bile acid was identified as 3β,7β-dihydroxychol-5-enoic acid 3-sulphate, 7-*N*-acetylglucosaminide. Iida et al. have subsequently synthesized 3β,7β-dihydroxychol-5-enoic acid 3-sulphate, 7-*N*-acetylglucosaminide and its corresponding glycine and taurine conjugates and recorded the low-energy MS/MS spectra of their $[M\text{-}H]^{-}$, $[M + H]^{+}$, and $[M + Na]^{+}$ ions (Iida, Kakiyama, Hibiya, et al., 2006).

10.4.2.4 Conventional Versus Capillary LC-MS for Qualitative and Quantitative Analysis

The decision to use either conventional or capillary LC depends on the desired application, and it may in fact be preferable to negate an LC separation step all

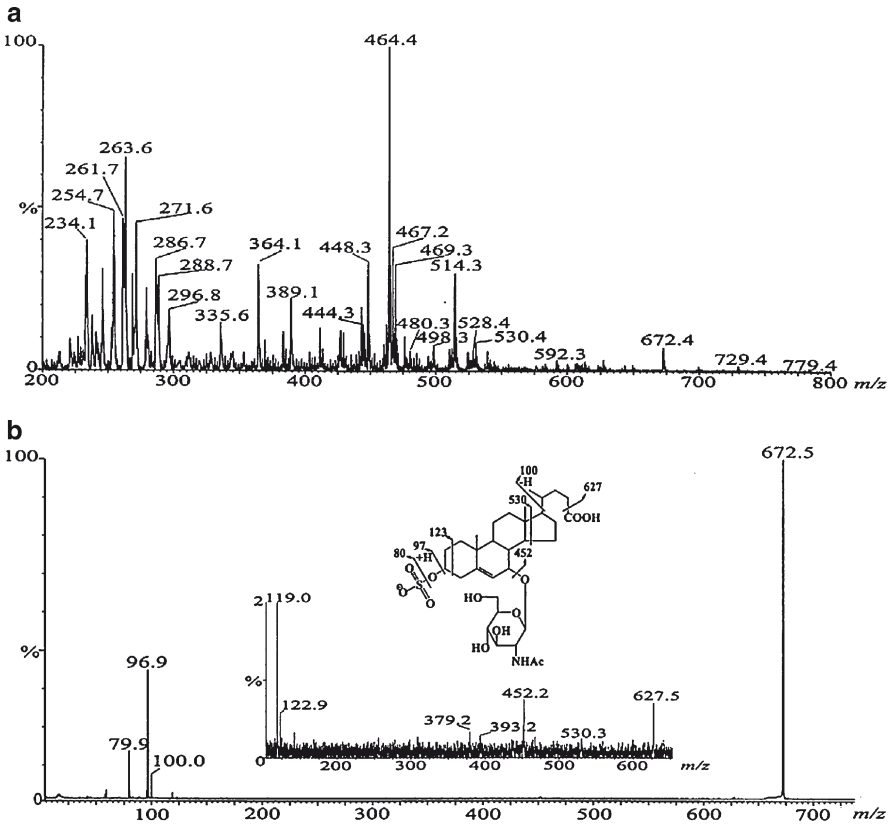


Fig. 10.24 (**a**) ESI-MS analysis of a urine extract from an infant suffering from a Niemann-Pick type C disease and (**b**) ESI-MS/MS spectrum recorded at 400 eV collision energy with xenon as the collision gas of the ion at *m/z* 672 corresponding to the $[M\text{-}H]^-$ ion from 3β,7β-dihydroxychol-5-enoic acid 3-sulphate, 7-*N*-acetylglucosaminide present in the urine extract. The spectrum was recorded on a double focussing magnetic sector-orthogonal acceleration (OA) time-of-flight (TOF) instrument

together when, for example, screening clinical samples for metabolic errors of cholesterol metabolism (Egestad, Pettersson, Skrede, et al., 1985; Lawson, Madigan, Shortland, et al., 1986; Clayton, Leonard, Lawson, et al., 1987; Setchell, Suchy, Welsh, et al., 1988; Setchell, Heubi, O'Connell, et al., 1997; Setchell, Schwarz, O'Connell, et al., 1998; Johnson, ten Brink, Schuit, et al., 2001; Setchell, Heubi, Bove, et al., 2003). Conventional LC encompasses both normal-bore (3–4.6 mm i.d., 0.5–3 mL/min) and narrow-bore (1–2 mm i.d., 20–300 μL/min) columns, while capillary LC includes micro-bore (150–800 μm i.d., 2–20 μL/min) and nano-bore columns (20–100 μm i.d., 100–1,000 nL/min). The earliest generation of ESI interfaces (which are still in use today) is well-matched with flow-rates of the order of 5–100 μL/min, with maximum sensitivity being achieved at the lower end of this flow-rate range, and are most compatible with narrow-bore or micro-bore columns

(Warrack and DiDonato, 1993; Roda, Gioacchini, Cerrè, et al., 1995). It is possible to interface normal-bore LC columns to such ESI interfaces, but a post-column split is required (Warrack and DiDonato, 1993; Roda, Gioacchini, Cerrè, et al., 1995). Pneumatically assisted ESI, sometimes called ion-spray, has been developed to allow the direct coupling of normal-bore columns with the ESI interface modified to receive flow-rates of up to 1 mL/min. APCI interfaces are also capable of operating at this flow-rate and receiving eluate from normal-bore columns (Goto, Shibata, Iida, et al., 2004; Goto, Shibata, Sasaki, et al., 2005).

Theoretically, a reduction in column diameter produces a higher concentration of sample in an eluting peak (Abian, Oosterkamp and Gelpi, 1999). As ESI is also a concentration dependent process, this dictates that maximum sensitivity can be achieved by using miniaturized LC, and has led to the increasing popularity of capillary LC-MS. A new generation of nano-ESI or micro-ESI interfaces have been developed which perform optimally at low-flow-rates (<1 μL/min) and thereby provide maximum sensitivity when coupled with capillary column LC. Despite providing maximum sensitivity, capillary LC performed at low-flow-rate has its limitations. Although the concentration of sample in an eluting peak is dependent on the reciprocal square of column i.d., the column loading capacity and optimum injection volume also follow a similar relation (with respect to column i.d). This creates problems in terms of sample injection and column overloading, particularly for columns of i.d. <300 μm where optimum injection volumes are < μL (Tomer, Moseley, Deterding, et al., 1994). The problem of low injection-volume can be simply overcome by on-line sample pre-concentration on a trap-column arranged in series with the analytical column. Sample pre-concentration is performed at μL/min flow rate on the trap column, which is then flushed, and the sample separated on the analytical column at low-flow-rate (<μL/min). By applying such methodology 5–10 μL of sample can be quickly (<5 min) loaded on to an analytical capillary column. However, the problem of column loading capacity still exists. The best solution to column overloading is to include a group separation step prior to capillary LC. This is illustrated in work performed by Yang et al. in which a urine extract from a child with cholestatic liver disease was separated into four fractions according to acidity on an anion exchange column (Yang, Griffiths, Nazer, et al., 1997). Each fraction was analysed by capillary-LC-ESI-MS and MS/MS in a 1 h run, allowing the partial characterization of over 150 bile acids and conjugated bile alcohols. The remaining drawback with capillary column LC is one of analysis time. For example, in the above study LC runs took 60 min precluding the possibility of high throughput analysis (Yang, Griffiths, Nazer, et al., 1997). However, with the development of ultra-performance liquid chromatography (UPLC, 15,000 psi) using smaller particles (<2 μm), or alternatively monolithic columns at lower pressure, it is likely that the time constraint associated with capillary chromatography will be overcome. Alternatively, UPLC can be employed with conventional column dimensions and flow-rate so as to minimize chromatographic run time. Impressive work in this regard has been performed by Nicholson's group in London (Martin, Dumas, Wang, et al., 2007), Wilson and colleagues in Manchester (Plumb, Johnson, Rainville, et al., 2006) and Hagio, Matsumoto, Fukushima, et al. (2009) in Japan. Capillary electrochromatography

(CEC) has also been combined with ESI-MS/MS for bile acid analysis, providing fast, high resolution (610,000 plates/m) separations with high sensitivity (40 fmol) analysis (Que, Konse, Baker, et al., 2000).

While capillary column chromatography combined with nano-ESI or micro-ESI will provide the maximum sensitivity for bile acid analysis and is preferable for the profiling of the conjugated bile acid and alcohol content of biological samples, many screening and quantitative studies require high-throughput as their main priority in which case narrow-bore chromatography or UPLC combined with negative-ion ESI or APCI is the method of choice.

10.5 Enzymatic Assays

The conversion of 3α-hydroxylated bile acids to the corresponding 3-oxo forms under slightly alkaline conditions by 3α-HSD is accompanied by the reduction of NAD^+ to NADH, and the amount of NADH produced corresponds to the quantity of total 3α-hydroxylated bile acids in the samples (Iwata and Yamasaki, 1964) (Fig. 10.25). Although this simple enzymatic assay is often used for analysis of total bile acids in bile, fluorometric detection of the produced NADH is needed to measure serum bile acids (Murphy, Billing and Baron, 1970) and the method requires 1–3 mL of serum to allow the bile acids to be detected. Mashige et al. described a diaphorase coupling method, which causes the enzymatic conversion of resazurin to resorufin with the conversion of NADH to NAD^+ (Mashige, Imai and Osuga, 1976) (Fig. 10.26). Resorufin can be detected with excitation at 560 nm and monitoring 580 nm as the emission wavelength. Thus, this method allows for simple and sensitive analysis of total 3α-hydroxysteroids with a detection limit of 0.1 nmol. Moreover, when 1-methoxyphenazinemethosulphate, instead of diaphorase, is combined with the 3α-HSD system the method could be used to analyse bile acids in 50 μL of human serum free from the problems associated with the inactivation of the enzyme (Ikawa, Mura and Kawasaki, 1981). Replacing resazurin with nitrotetrazolium blue for an enzymatic colorimetric method has also been reported

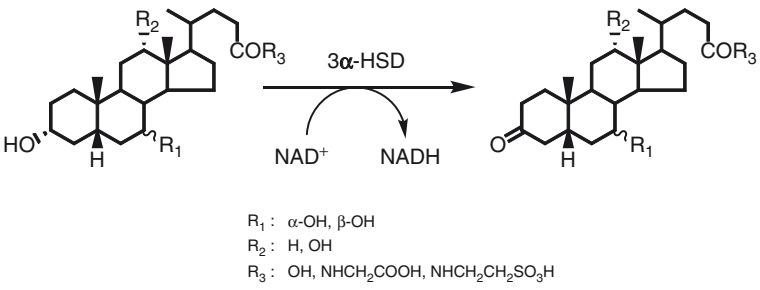


Fig. 10.25 Enzymatic conversion of bile acids into 3-oxo bile acids by 3α-hydroxysteroid dehydrogenase

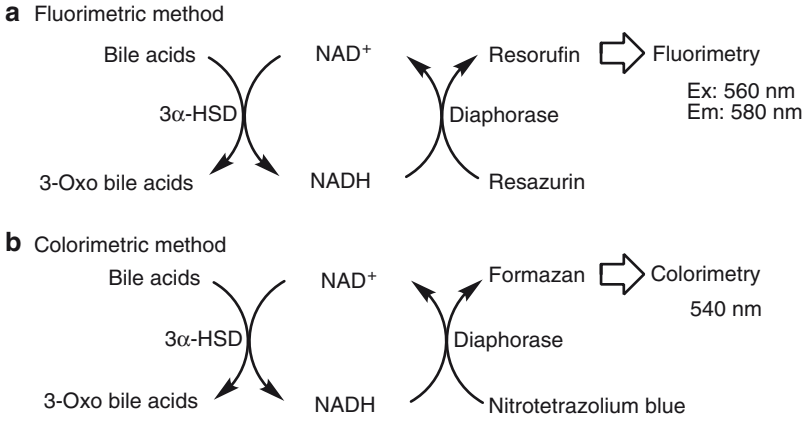


Fig. 10.26 Diaphorase-coupling enzymatic methods using fluorometric (**a**) and colorimetric (**b**) detection

(Fig. 10.26) (Mashige, Tanaka, Makai, et al., 1981). Although this method was useful for simple analysis of biological fluids containing high levels of bile acids, endogenous contaminants, such as a bilirubin, often influence the assay results. This problem was solved by a preceding fractionation of conjugated bile acids using PHP-LH-20 (Kimura, Suzuki, Sato, et al., 1979) (see Section 10.2.2.1).

In 1980, Nicholas et al. reported an enzymatic microassay for serum bile acids with an enzyme amplification technique (Nicholas, Chaintreuil, Descomps, et al., 1980) that was later improved (Komiyama, Adachi, Ito, et al., 1982). In this method, after residual NAD^+ is destroyed by alkalinization and heat, diaphorase catalyses the conversion of the produced NADH into NAD^+ coupled with the conversion of iodonitrotetrazolium salt to formazan. Aldehyde dehydrogenase activity then converts the produced NAD^+ into NADH coupled with the conversion of ethanol into acetaldehyde.

For these enzymatic methods, the substrate specificity and the purity of the enzymes are very important. The presence of other dehydrogenase activities in the enzyme preparation obviously decreases the specificity and results in overestimation of bile acid levels. 3-Oxo and 3β-hydroxy bile acids are not determined, so the method is not suitable for analysis of total bile acids in faeces or large intestine (Eneroth, Hellström and Sjövall, 1968; Setchell, Lawson, Tanida, et al., 1983;Porter, Fordtran, Santa Ana, et al., 2003; Hamilton, Xie, Raufman, et al., 2007). 3α-HSD shows no activity for 3-sulphates or 3-glucuronides of 3α-hydroxysteroids nor does it act on sterols or 7-sulphates of bile acids that have a 3α-hydroxyl group.

3β-HSD, 7α-HSD, 7β-HSD, and 12α-HSD have been isolated and used to assay bile acids (Haslewood, Murphy and Richardson, 1973). 7α-HSD was isolated from a strain of *E. coli*, and a direct enzymatic assay for 7α-hydroxylated bile acids and their conjugates was developed (Haslewood, Murphy and Richardson, 1973). 7α-

HSD can be used to assay the primary bile acids cholic acid and chenodeoxycholic acid (MacDonald, Williams and Mahony, 1974; Fausa and Skålhegg, 1977). Continuous-flow determination of primary bile acids using 7α-HSD and bioluminescence detection has been reported (Roda, Kricka, DeLuca, et al., 1982). This assay uses three enzymes, 7α-HSD, NAD(P)H/FMN oxidoreductase, and bacterial luciferase covalently linked to Sepharose, and can be used to measure 7α-hydroxy bile acids in 5–50 μL of serum.

12α-HSD has been employed to analyse 12α-hydroxylated cholic and deoxycholic acids (Macdonald, Williams and Musial, 1980), while ursodeoxycholic acid can be measured using 7β-HSD (MacDonald, Williams, Sutherland, et al., 1983). A highly sensitive bioluminescent assay for 12α-hydroxy bile acids using immobilized 12α-HSD was described that had a detection limit of 4 pmol (Schoelmerich, Hinkley, Macdonald, et al., 1983).

Enzymatic assays are applicable to the analysis of "total" or groups of bile acids, and are routinely used in clinical work and mass-screening due to their simplicity. Enzymatic assays, however, often overestimate the amounts of bile acids in biological fluids due to insufficient specificity and/or purity of the enzyme used. Therefore, reliable analysis requires the use of the most suitable enzyme for the purpose of the study. Although all of these enzymatic methods using UV, fluorescence, colorimetry, bioluminescence detection, or amplification systems can be used to analyse bile acids in bile and intestinal juice, relatively low concentrations of serum bile acids have been mainly measured with enzymatic assays coupled to fluorescence or bioluminescent detection systems. Urinary bile acids commonly exist in conjugated forms, and as such require time-consuming pretreatment, including solvolysis, for analysis by these methods. The variability and abundance of bile acid isomers make enzymatic methods unsuitable for analysis of urinary bile acids. Enzymatic assays do not measure the individual concentrations of bile acids, but instead determine concentration based on the composition of bile acids having a specific type of structure, e.g. 3-hydroxyl, 12-hydroxyl group(s) etc. Although combinations of multiple enzyme assays can provide estimates of the levels of certain individual bile acids, other methods, such as LC, GC, GC-MS, and LC-MS, are required to corroborate the reliability of the data obtained using multiple enzymatic assays.

An enzymatic method has been described for determination of total 3α-sulphated bile acids in urine (Matsui, Kasano, Yamauchi, et al., 1996) and plasma (Kato, Yoneda, Nakamura, et al., 1996). The sulphates are first hydrolysed by a particular bile acid 3α-sulphate sulphohydrolase to yield 3β-hydroxy bile acids, which are then determined using a 3β-hydroxysteroid dehydrogenase. It is likely that this method will also include other 3α-sulphated steroids, such as the 3α-sulphated progesterone metabolites present at high levels in bile, plasma and urine of pregnant women (Meng, Reyes, Palma, et al., 1997).

The dehydrogenase reactions have been applied as detection methods for LC. In 1979, Okuyama and coworkers reported an online LC method coupled with column-immobilized 3α-HSD for analysis of individual bile acids (Okuyama, Kokubun, Higashidate, et al., 1979). 3α-HSD is immobilized onto aminopropyl-CPG

(Controlled Porous Glass) by Schiff base formation using glutaraldehyde. β-NAD (0.3 mM) in 10 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 0.05% 2-mercaptoethanol is mixed with the LC effluent, and the fluorescence of the quantitatively produced NADH is measured. This method can simultaneously analyse the common unconjugated, glycine-conjugated, and taurine-conjugated bile acids in serum, and has been applied to the analysis of serum from patients suffering from hepatobiliary diseases, and rat serum and liver (see Section 10.3). Hirano et al. analysed 3-sulphated and nonsulphated bile acids using LC coupled with 3αHSD before and after chemical solvolysis and found a significantly high percentage of total GCDCA as the 3-sulphate in the serum of patients with obstructive jaundice (Hirano, Miyazaki, Higashidate, et al., 1987). An LC method using immobilized 3α-HSD coupled with electrochemical detection was described where the eluate containing NADH from the 3α-HSD-immobilized column was mixed with phenazine methosulphate (PMS) in 0.1 M phosphate buffer (pH 7.0) and the level of the reduced form of PMS measured with an electrochemical detector (Kamada, Maeda, Tsuji, et al., 1982). This method is very sensitive and selective, and has a detection limit of 20 pmol. The use of a 1-methoxy-5-methylphenazinium methylsulphate (isoluminol)-microperoxidase-based system was used to identify the 1β- and 6α-hydroxylated bile acids in urine from neonates and pregnant women (Ikegawa, Hirabayashi, Yoshimura, et al., 1992). Although the detection limit of this method is almost the same as that of fluorescence detection methods, the electrochemical detector has greater selectivity. Moreover, with the use of a column of immobilized 3β-HSD instead of a 3α-HSD column with this LC system the measurement of 3β-hydroxylated bile acids, including 3β-hydroxy-5-cholenoic acid is possible (Sugiyama, Okuyama, Imoto, et al., 1986).

The 3-HSD enzyme reaction is also used as a precolumn derivatization method for LC. The resulting 3-oxo bile acids are derivatized with dansylhydrazine (Kawasaki, Maeda and Tsuji, 1983). The dansylhydrazones of the bile acids are then separated by LC on an ODS-silica column, and the eluate is monitored by fluorescence detection (excitation: 340 nm; emission: 520 nm) with a detection limit of 0.5 pmol. This enzyme system coupled with 2,4-dinitrophenylhydrazine has also been applied to the analysis of bile alcohols by UV detection (Une, Harada, Mikami, et al., 1996).

10.6 Immunoassays

Immunoassays using antigen-antibody reactions are “specific”, sensitive, simple, and useful for high-throughput analyses. A radioimmunoassay (RIA) for conjugated cholic acid in serum was described early (Simmonds, Korman, Go, et al., 1973). Immunoassay systems can usually only detect the target hapten molecules, and many immunoassays using different antisera have been reported for analysis of the same conjugated form of cholic acid (Murphy, Edkins, Williams, et al., 1974; Demers and Hepner, 1976; Matern, Krieger and Gerok, 1976; Spenney, Johnson,

Hirschowitz, et al., 1977; Minder, Karlaganis, Schmied, et al., 1979). Immunoassays for chenodeoxycholic acid (Demers and Hepner, 1976; Roda, Roda, Aldini, et al., 1977; Schalm, van Berge-Henegouwen, Hofmann, et al., 1977), ursodeoxycholic acid (Makino, Tashiro, Hashimoto, et al., 1978), deoxycholic acid (Demers and Hepner, 1976), and lithocholic acid (Demers and Hepner, 1976; Cowen, Korman, Hofmann, et al., 1977) have been described. Bile acids are nonantigenic low-molecular-weight haptens. Therefore, complexes of bile acids covalently bound to large immunogenic molecules, such as a bovine serum albumin (BSA), are required for the preparation of specific antibodies. The antibodies produced in the immunized animals usually have a wide range of structural specificities for the hapten-conjugated site on the BSA molecule. Moreover, most antisera used in these immunoassay systems were prepared using immunogens in which the side chain carboxyl of the bile acid was coupled to ε-amino groups on the protein surface. Therefore, most of the immunoassay systems cannot discriminate between the different amino-acid conjugations, although they can differentiate between molecules based on the number and configurations of hydroxyl groups on the steroid nucleus.

In early experiments, [^{14}C]- or [^{3}H]-labelled bile acids were used as tracers for analysis of serum bile acids, whereas [^{125}I]-labelled tracers were used in later publications. RIAs can detect serum bile acids at pmol levels and are useful for high-throughput analysis. Enzyme immunoassays (EIAs) use a hapten-enzyme conjugate as a tracer instead of radioisotope-labelled antigens. EIAs can be used to measure levels of serum bile acids with almost the same detection limit as RIAs, because the basic mechanism of the assays is the specific antigen-antibody interaction. The construction of an effective EIA system requires the preparation of an enzyme-labelled antigen that possesses a high specific activity without decreasing the enzyme activity, or the immunoreactivity. The labelling enzymes that have been employed for these assays include β-galactosidase (Maeda, Setoguchi, Katsuki, et al., 1979), alkaline phosphatase (Ozaki, Tashiro, Makino, et al., 1979), and peroxidase (Baqir, Ross and Bouchier, 1979).

The drawback of most RIAs and EIAs remains the limited specificity of the antibody. Usually, antibodies demonstrate high structural specificity for bile acids but often there is a low level of cross-reactivity with bile acids having similar structure to the target bile acid (Table 10.7). In the case of lithocholic acid and ursodeoxycholic acid, which are typically present in low concentrations in serum, even low levels of antibody cross-reactivity will influence the measured values. Therefore, a quality antibody, preferably a monoclonal antibody, is very important for reliable analysis of bile acids in biological fluids. Recently, reports of the development of enzyme-linked immunosorbent assay systems for particular conjugated bile acids, such as ursodeoxycholic acid 7-*N*-acetylglucosaminides (Kobayashi, Oiwa and Goto, 1998), ursodeoxycholic acid 3-sulphate (Kobayashi, Katsumata, Katayama, et al., 2000), and glycolithocholic acid 3-sulphate (Kobayashi, Katsumata, Uto, et al., 2002), were published (Table 10.7). Immunoassays for these types of metabolites using specific monoclonal antibodies may be useful for disease-specific screening tests, e.g. primary biliary cirrhosis and other hepatobiliary diseases.

Table 10.7 Cross-reactivities of monoclonal antibodies that recognize bile acid metabolites

Bile acids	Cross-reactivity (%)
UDCA 7-N-acetylglucosaminide	
UDCA 7-NAG	100
GUDCA 7-NAG	45
TUDCA 7-NAG	50
UDCA	<0.01
GUDCA	<0.01
UDCA 3-S	<0.01
UDCA 3-sulphate	
UDCA 3-S	100
GUDCA 3-S	170
TUDCA 3-S	50
CDCA 3-S	<0.03
UDCA	<0.01
GLCA 3-sulphate	
GLCA 3-S	100
TLCA 3-S	11
LCA 3-S	40
CA 3-S	0.02
CDCA 3-S	0.63
DCA 3-S	2.2
UDCA 3-S	0.52
LCA	<0.08
GLCA	<0.01
TLCA	<0.01
LCA 3-G	<0.03
CA	<0.01
DCA	<0.01
UDCA	0.01
GUDCA	<0.01

10.7 Conclusions and Future Perspectives

The methods for bile acid analysis have improved continuously over the past few decades. Much of the development is due to improved instrumentation, data acquisition and data evaluation. The single most important advance resulted from the development of soft ionization methods in mass spectrometry. This is exemplified by the diagnosis of inherited and acquired defects in bile acid biosynthesis and metabolism achievable by a simple and rapid analysis of extracts of urine or plasma by FAB- or ESI-MS. With ESI-MS, ESI-MS/MS and their online combination with liquid chromatography, it has become possible to analyse most forms of bile acids and conjugated bile alcohols in biological extracts. The sensitivity of ESI methods can be very high, and high specificity may be achieved by high-energy CID and multiple reaction monitoring. The need for prior sample preparation is determined by the absolute

and relative concentrations of the different types and forms of bile acids to be analysed, and the matrix interference and load. As in the case of GC-MS, accurate quantification requires addition of isotopically labelled versions of the analytes. This strategy can be used for analysis of one or a few bile acids (panels) but is not realistic for analyses of bile acid/bile alcohol profiles (metabolomics, cholanoidomics), in which quantifications will be less accurate in exchange for more detailed, less biased and potentially new information about the nature of the bile acid mixture.

LC-MS/MS and GC-MS are complementary methods for analysis of profiles of bile acids and alcohols. While LC-MS/MS provides information about types and sites of conjugation, the fragmentation by electron impact ionization in GC-MS presently provides more information on bile acid structures (e.g. position and orientation of substituents) than ESI-MS/MS. At present, GC also gives faster separations with higher resolution than LC. Therefore, GC-MS is an essential method for detailed recognition and identification in analyses of mixtures of isomeric bile acids and bile alcohols. Since GC-MS requires cleavage of conjugates and derivatization, a preceding analysis by ESI-MS can be used to determine the nature and site of conjugation, and to evaluate the efficiency of the cleaving method and the potential formation of artefacts. When using this strategy in multicomponent analyses, a sample preparation procedure including anion exchange separation will often provide the highest sensitivity, widest dynamic range, and best removal of contaminants.

There is no single simple method that is satisfactory for analysis of bile acids in all types of biological fluids and tissues. All methods have limitations in comprehensiveness, specificity, speed, or simplicity. Some sample types, e.g. urine and faeces, can contain very complex bile acid mixtures with more than 100 components, other samples, e.g. tissues and cells, contain very low levels of bile acids. The choice of method depends on the particular application and the aim of the analysis. However, it should be emphasized that specificity can only be guaranteed by use of a chromatographic method coupled with mass spectrometric detection. Non-mass spectrometric methods rely on assumptions regarding the bile acid composition of the samples to be analysed and it is assumed that peaks in chromatograms, and enzymatic or immunological reactions are caused by these bile acids. The validity of such assumptions is best established by reasonably unbiassed mass spectrometric methods. This is obviously important when bile acid patterns are compared between different conditions, e.g. in health and disease, and bile acid structures may be changed due to disease or drugs. Methods assumed to measure groups of bile acids or total bile acids rather than the sum of individual bile acids should be used critically.

Considering the rapid developments in mass spectrometry one may assume that the next generation of methods for analysis of bile acids in biological materials will be based on this method. Instruments are already available with extraordinary resolution and accuracy in mass determination, providing increased specificity of detection, accurate calculations of elemental composition and opening possibilities to perform multi-labelling heavy-isotope studies of metabolism and its kinetics. Cheaper instruments, for LC-MS/MS and GC-MS, which are easy to operate and

with intelligent software are also becoming available. Judging from results in steroid analysis, LC-MS/MS methods for high-throughput (100–200 samples/day) routine analysis of selected bile acids should soon be a reality. For analysis of bile acid profiles, methods are needed that give more extensive and/or selective fragmentation of ions produced by the soft ionization methods. In this way the amount of structural information provided by LC-MS/MS could approach that given by GC-MS.

It is unlikely that mass spectrometry without chromatography can replace LC-MS/MS in future methods for analysis of bile acid profiles. Thus, there is a need for improvements in column technology to achieve faster and more efficient capillary LC separations. Ongoing improvements are stimulated by the same need in proteomics and metabolomics. Faster and automated sample preparation methods are needed in many applications for high-throughput analyses. A variety of sorbents are commercially available which can form the basis for sample preparation procedures based on charge, acidity, polarity, and structural features of the bile acids to be analysed. Sorbent beds can also be used as filters to remove sample components such as nonpolar lipids from bile acid fractions. Because of the high sensitivity of ESI-MS methods, sample sizes can be drastically reduced, permitting application of microfluidic systems for sample preparation, coupled either online or run off-line with fraction collection to achieve the most time-efficient combination of sample preparation and final analysis by capillary column LC-MS/MS and GC-MS.

Chromatography-mass spectrometry will be the predominant principle for bile acid analysis for many years. It is sometimes regarded as too exclusive, complicated, slow and expensive. It is not more expensive than analysis by methods which do not give correct results due to lack of specificity. Calculated on the number of bile acids included in a profile analysis, the cost is low. No other method permits the discovery of new bile acids or bile alcohols at low levels in biological materials. The ongoing developments in mass spectrometric, chromatographic, and data evaluation techniques will lead to an increasing use of these techniques in all areas of bile acid and bile alcohol research.

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Chapter 11
Analysis of Vitamins D, Their Metabolites and Analogues

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11.1 Introduction

The analysis of vitamins D and their metabolites and analogues has been reviewed by us on two occasions (Makin et al., 1995; Jones and Makin, 2000) over the last 10-15 years. In this chapter, we have drawn heavily on the 2000 review, up-dating it to take account of the developments in methodology that have occurred in the intervening years, but including elements of our 1995 review so that the reader can get a picture of the historical context as well as the modern developments.

While it might be considered inappropriate to raise the issue of nomenclature at this point, it has become apparent to us that there is a degree of confusion, particularly among clinical colleagues, about what the often ambiguous terms used by vitamin D biochemists actually mean. In this chapter, we will try hard to adopt our own recommendations but occasionally will be guilty of what we criticise here. The structures of vitamin D_2 (9,10-seco-ergosta-5,7,10(19),22-tetraen-3β-ol) and vitamin D_3 (9,10-seco-cholesta-5,7,10(19)-trien-3β-ol) are illustrated in Fig. 11.1. Structures of metabolites of vitamin D_3 and some chemical analogues mentioned in the text are given in Table 11.1 (see also Tables 11.2 and 11.3), together with their trivial and shorthand names. Further structures are given in Figs. 11.2, 11.4 and 11.7. When it is not necessary or not required to distinguish between vitamins D_2 and D_3 (including vitamins D_4 and D_5) or dihydrotachysterols (DHT_2 and DHT_3,) the terms vitamins D or DHTs are used – note the use of the plural. However, great care must be exercised in using such terms as their use may give rise to considerable ambiguity. We suggest that the term 'vitamin D' in the

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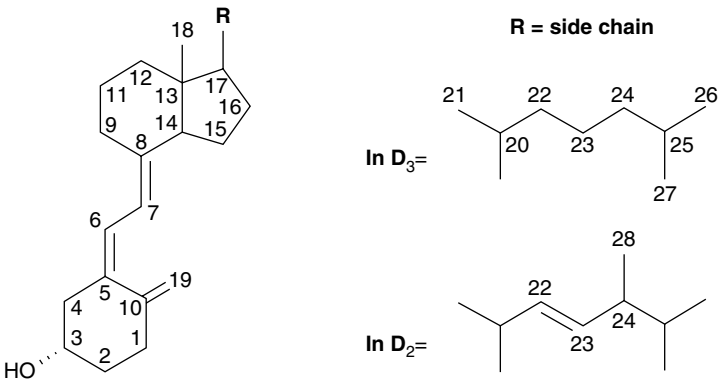


Fig. 11.1 Basic formulae of vitamins D: Each carbon in the nucleus is numbered in the usual way as well as the carbons in the side chains for vitamin D_2 and vitamin D_3.

singular should not be used except as a generic means of describing the particular area of interest. In particular, the term 'vitamin D' should NOT be used, as is often the case (Vieth, 2004), as a generic term to include the hydroxylated metabolites – in particular, the active metabolite 1α,25-dihydroxyvitamin D_3 – e.g. 'vitamin D has an important physiological role in the regulation of calcium homeostasis'. Recognising the nomenclature difficulty, some of the biologically more important

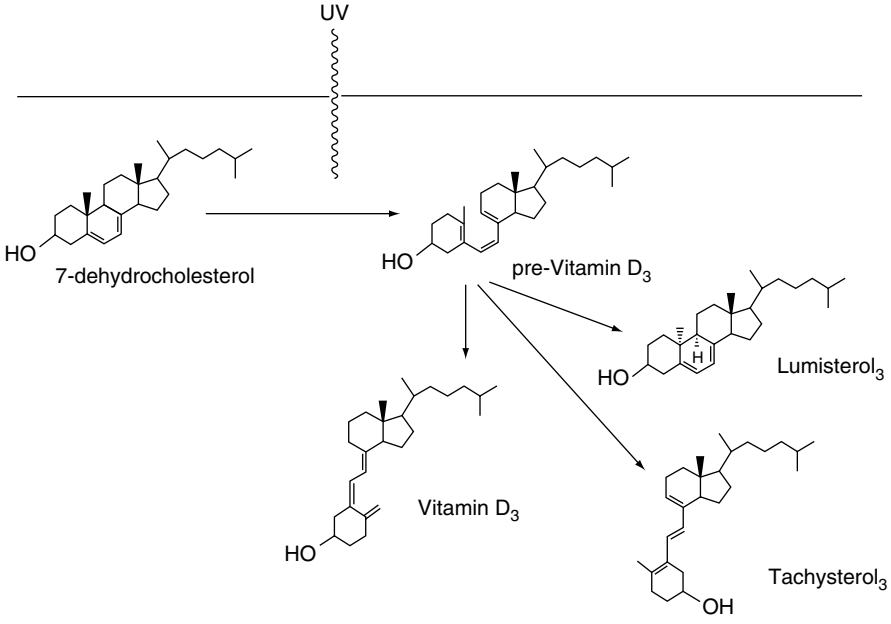


Fig. 11.2 Synthesis of vitamin D_3 in the skin by ultraviolet irradiation of 7-dehydrocholesterol: Only some of the isomers formed are shown. The rate limiting step which controls the amount of vitamin D_3 released into the blood stream is the conversion of pre-vitamin D_3 into vitamin D_3

metabolites of vitamin D_3 have been given shorter names (e.g. 25-OH-D_3 – calcidiol, 25-OH-D_2 – ercalcidiol, 1α,25-$(OH)_2D_3$ – calcitriol – IUPAC-IUB, 1981).

As an example, there is some discussion at the present time (2004–2010) about the desirable concentration of '25-hydroxyvitamin D' (25-OH-D) in human serum, which under our recommendations should be referred to as '25-hydroxyvitamins D' (25-OH-Ds). What is often not made clear during these discussions is precisely what this term means, as oral vitamin D supplements may be either vitamin D_2 or vitamin D_3 and the analytical value for '25-OH-Ds' achieved will depend on the methodology used (DeLuca, 2004) and whether it discriminates between 25-OH-D_2 and 25-OH-D_3. In physiological/therapeutic terms, is a given concentration or dose of 25-OH-D_3 equivalent to the same concentration or dose of 25-OH-D_2 (Trang et al., 1998; Armas et al., 2004)? Does supplemental vitamin D_2 have the same effect as vitamin D_3 (Houghton and Vieth, 2006; Holick et al., 2008), and what is the maximum dose of vitamin D which can be safely used therapeutically (Hathcock et al., 2007)? Concentrations of vitamins D and their metabolites are expressed in molar terms (e.g. nmol/L) or in mass terms (e.g. μg/mL). As the molecular weight of 25-OH-D_3 is 400, concentration in nmol/L × 0.4 = concentration in μg/L. For 25-OH-D_2, the factor is 0.412. A convenient list of molecular weights of vitamins D and their metabolites is given in Norman (1979) – a valuable and recommended source of information on vitamins D.

'Activity' (or potency) is another ambiguous term which is widely used but often not defined or poorly understood. In our view, it should only be used when the end point used to measure the activity is clearly defined, and even then, the term may have little physiological meaning (e.g. Ovesen et al., 2003). The use of 'Vitamin D', a nutritional term coined in the 1920s meaning a substance with full antirachitic activity, should not be used, unless very clearly defined. Is the statement 'the biologic activity of vitamin D_2 and vitamin D_3 is identical' (Lim et al., 2005) meaningful, unless there is some explanation of what particular activity is being considered?

11.2 Chemistry and Biochemistry

11.2.1 Chemistry

Vitamins D, secosteroids derived from steroid precursors by the opening of the steroid B-ring between carbons 9 and 10, exist in a number of different chemical forms (Fig. 11.1). The natural vitamin D (D_3) has the cholestane C-17 side chain, but the ergostane analogue (D_2) has long been used as a nutritional supplement and other steroidal counterparts (such as D_4 and D_5) are currently being investigated for possible therapeutic use. Vitamin D_3 (cholecalciferol) arises by the photolytic action of UVB light (290–320 nm) on the sterol precursor, 7-dehydrocholesterol, which is found in the upper dermis layers of the skin. The concentration of 7-dehydrocholesterol in the skin is relatively constant but declines in the elderly (MacLaughlin and Holick, 1985), thus reducing Vitamin D_3 production for a given UVB exposure.

The chemical reactions involved are shown in Fig. 11.2. It can be seen that apart from the production of the pre-vitamin and subsequent conversion into vitamin D_3, a number of other isomers are also formed. The proportion of these different products from 7-dehydrocholesterol varies depending on the conditions under which they are formed. Vitamin D_2 (ergocalciferol) arises in a similar fashion, in that it is produced by the UV irradiation of the 5,7-diene sterol precursor, ergosterol. Ergosterol is widely distributed in plants and fungi. Vitamin D_2 has an extra methyl (C28) attached to carbon 24 on the vitamin D_3 side chain together with an extra double bond at C22(23). Because the further metabolism of vitamins D involves hydroxylations on their side chains, it is inevitable that the metabolism and/or physiological effects of vitamin D_2 and vitamin D_3 may well be slightly different (Horst et al., 2005). For historical reasons, vitamin D_2 is more readily available commercially and is used in a number of countries throughout the world (though not in Europe) to supplement milk and other foodstuffs; calciferol BP (British Pharmacopeia) is in fact vitamin D_2.

The 5,7-diene structure together with the C10–19 double bond, forming a *cis*-triene system in vitamins D, has a characteristic UV spectrum (molar extinction coefficient for vitamin D_3 = 18,300, λ_{max} 264 nm and λ_{min} 228 nm (Bell, 1978; Norman, 1979) which can be distinguished from the other isomers of vitamins D which are formed during the ultraviolet irradiation of the pro-vitamin. Although we are now in the 21st Century, more than 70y after the isolation of vitamins D_2 and D_3, agreed molar extinction coefficient values are still elusive, values of 18,200/18,300 for both vitamins D_2 and D_3 (Bell, 1978; Norman, 1979) being quoted as well as 19,400 for vitamin D_2 (Penau and Hagemann, 1946 quoted in Norman, 1979). Some of the differences between the UV spectra of these compounds are pronounced but others are simply subtle shifts in the UV absorption peaks. Figure 11.3 illustrates some examples of the UV spectra of the irradiation products of cholecalciferol showing the UV spectra of vitamin D_3, the pro-vitamin, 7-dehydrocholesterol and the two isomers of vitamin D_3, lumisterol$_3$ and tachysterol$_3$. These spectra were obtained using a photodiode array detector monitoring eluent from a liquid chromatography (LC) system. Careful inspection of the retention times of peaks shown in this figure also indicates that the chromatographic resolution of three of these four compounds was achieved in the LC system used. Although the *in vivo* formation of vitamin D_3 occurs in the skin, exactly the same process can be achieved in the test tube, and it can be seen that separation and identification of the different isomers formed in this process is possible, although it may be difficult to resolve completely all the possible isomers which may be formed in the process of this reaction. It may be necessary indeed to utilise different LC systems for complete resolution (Jones et al., 1985).

11.2.2 Biochemistry

Once the pre-vitamin is formed in the upper layers of the skin as a result of the reaction of ultraviolet light, it filters down to the lower layers where it is slowly

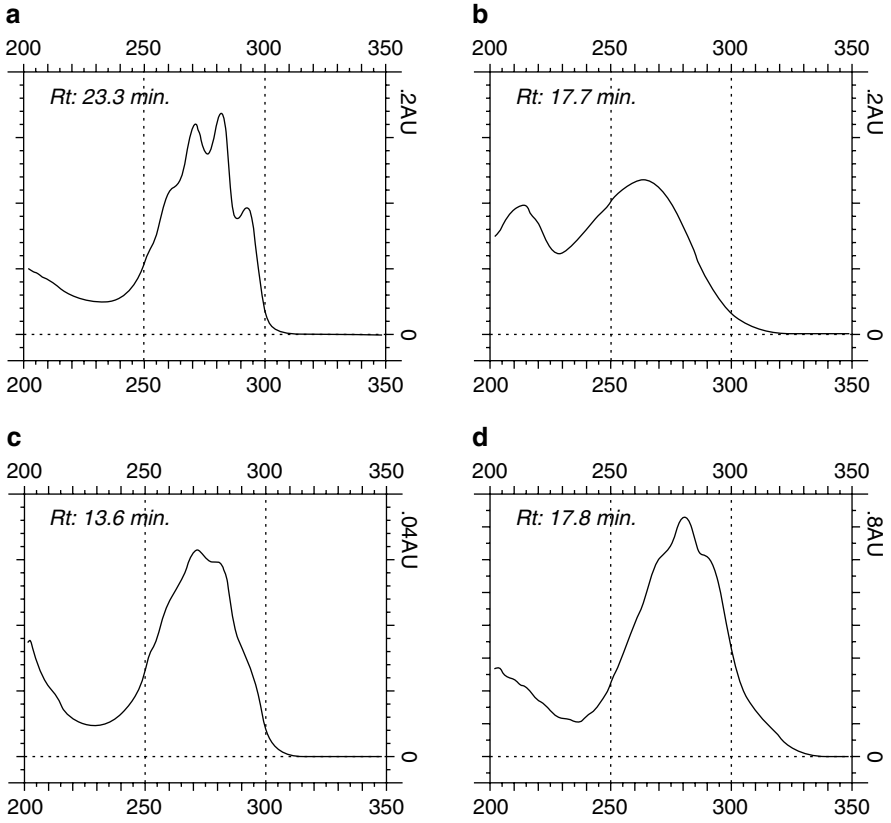
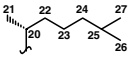
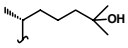
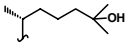
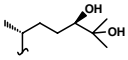
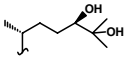
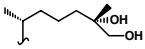
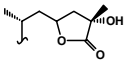


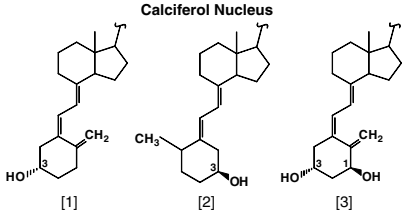
Fig. 11.3 Ultraviolet spectra of some irradiation products of 7-dehydrocholesterol. Retention times in a straight-phase LC system (Zorbax-SIL – 0.8%IPA in Hx) are given in the upper left-hand corner of each panel. (a) 7-dehydrocholesterol, (b) vitamin D_3, (c) lumisterol$_3$, (d) tachysterol$_3$ (from Jones and Makin, 2000)

transformed thermally into Vitamin D_3 (Holick, 2005). Vitamin D_3 and its isomers, like all other neutral steroids, are primarily hydrophobic and thus present a transport problem in the aqueous medium of the body fluids. This transport is achieved with the aid of a specific globulin, vitamin D binding protein (DBP), which binds vitamin D_3 and its metabolites and carries them in the plasma from tissue to tissue. The affinity of the DBP for vitamin D_3 is 1,000 times greater than that for the pre-vitamin. This difference in affinity is advantageous in that it protects against the possibility of hypervitaminosis after prolonged exposure to sunlight since effectively only vitamin D_3 is removed from the skin by combination with DBP, and the slow thermal conversion of the pre-vitamin to vitamin D_3 is the rate-limiting step in this process. Other metabolites of vitamin D_3 also bind to DBP with differing affinities (Table 11.1), the preference being 25-OH-D_3-26,23-lactone > 25-OH-D_3 ≥ 24,25-$(OH)_2D_3$ > vitamin D_3 > 1α,25-$(OH)_2D_3$. The high human blood concentration

Table 11.1 Vitamin D_3 and its natural metabolites

Vitamin D metabolite [ring structure][a]	Side chain structure	Site of synthesis	Relative VDR-binding affinity[b]	Relative DBP-binding affinity[c]	Reference
Vitamin D_3 [1]		Skin	? 0.001	3,180	Mellanby, 1919 McCollum et al., 1922
25-OH-D_3 [1]		Liver	0.1	66,800	Blunt et al., 1968
1α,25-$(OH)_2D_3$ [3]		Kidney	100	100	Fraser and Kodicek, 1970 Holick et al., 1971
24(*R*),25-$(OH)_2D_3$ [1]		Kidney	0.02	33,900	Holick et al., 1972
1α,24(*R*),25-$(OH)_3D_3$ [3]		Target tissues[d]	10	21	Holick et al., 1973
25(*S*),26-$(OH)_2D_3$ [1]		Liver?	0.02	26,800	Suda et al., 1970
25-OH-D_3-26,23-lactone [1]		Kidney	0.01	250,000	Horst et al., 1979

[a] Structure of the calciferol nucleus (secosterol ring structure).
[b] Values reproduced from previously published data (Stern, 1981).
[c] Values reproduced from previously published data (Bishop et al., 1994)
[d] Known target tissues included intestine, bone, kidney, skin, and the parathyroid gland.



[1] [2] [3]

of DBP (~8 μM) ensures that there is a large binding capacity which is rarely more than 2–5% saturated. Vitamins D can also be derived from the diet, as both vitamin D_3 and vitamin D_2. The absorption of vitamins D by the gastrointestinal tract occurs by the usual mechanism of fat absorption and can be compromised by other components of the diet or lack of bile salts, which may inhibit or prevent absorption. Dietary vitamins D are delivered to the liver on chylomicrons and thus can be distinguished from UV-induced *in vivo* vitamin D_3, which, in large part, arrives bound to DBP.

Vitamins D from skin or dietary sources do not circulate for long in the bloodstream but, instead, are immediately taken up by adipose tissue or the liver for storage or activation. In humans, tissue storage of vitamins D can last for months or even years. Ultimately, vitamin D_3 undergoes its first step of activation, namely, 25-hydroxylation in the liver (Fig. 11.4). Over the years, there has been some controversy over whether 25-hydroxylation of vitamin D_3 is carried out by one enzyme

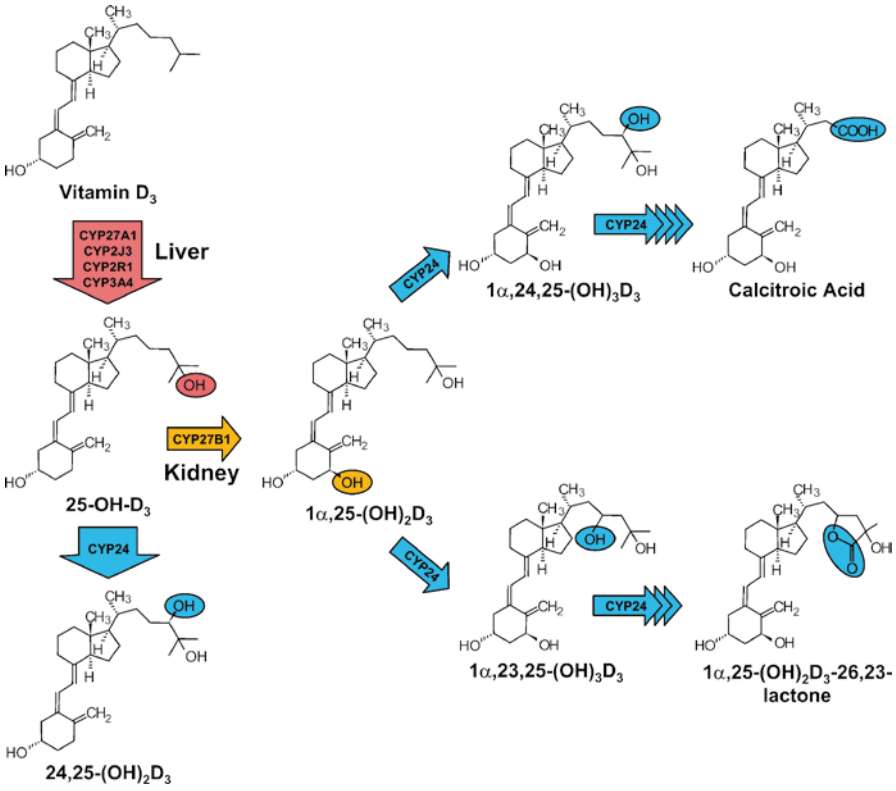


Fig. 11.4 Pathways of vitamin D activation and inactivation. Vitamin D_3 and its chief metabolites are arranged in three pathways: an activation pathway from vitamin D_3 to 1α,25-dihydroxyvitamin D_3 [1α,25-$(OH)_2D_3$] involving the 25-hydroxylase (red) and the 1α-hydroxylase (gold); and two catabolic pathways, the carbon-24 oxidation pathway from 1α,25-$(OH)_2D_3$ to calcitroic acid and the lactone pathway from 1α,25-$(OH)_2D_3$ to the 1α,25-$(OH)_2D_3$-26,23-lactone, both of which involve the 24-hydroxylase (blue). Transport of vitamin D_3 to the liver, transport of 25-hydroxyvitamin D_3 from the liver to the kidney, and transport of 1α,25-$(OH)_2D_3$ from the kidney to the target cell are carried out by the plasma vitamin D-binding protein (also known as Gc protein). Also shown in the figure (with the steps that they are known to carry out) are the cytochrome P450 (CYP) isoforms that are thought to be candidates for the 25-hydroxylase and the two established vitamin D hydroxylases, CYP27B1 and CYP24A1. Note that CYP24A1 is a multifunctional CYP that has been shown *in vitro* to catalyse the formation of the inactive blood metabolite 24,25-dihydroxyvitamin D_3 [24,25-$(OH)_2D_3$]. Note also that vitamin D_2 is an artificial form of the vitamin with an additional double bond between carbons 22 and 23, and an additional methyl group at carbon 24. Vitamin D_2 can be converted by the same enzymes, 25-hydroxylase, 1α-hydroxylase and 24-hydroxylase, into a set of products analogous to the ones shown here for vitamin D_3, except that there is no lactone formation and the carbon-24 oxidation pathway ends at 24,25-dihydroxyvitamin D_2 because of the 22–23 double bond (modified from Prosser and Jones, 2004; with permission)

or two enzymes, and whether this cytochrome P450-based enzyme is found in the mitochondrial or microsomal fractions of the liver (Jones et al., 1998). Currently, only one of these enzymes, the mitochondrial form, has been purified to homogeneity, subsequently cloned from several species and studied in some detail (Andersson et al., 1989; Usui et al., 1990; Cali and Russell, 1991). The cytochrome P450 involved is known as CYP27A1 or P450 c27 because it is a bifunctional cytochrome P450 which in addition to 25-hydroxylating vitamin D_3 also carries out the side-chain hydroxylation of intermediates involved in bile acid biosynthesis. Even though 25-hydroxylation of vitamin D_3 has been clearly demonstrated in cells transfected with CYP27A1, there is still some scepticism in the vitamin D field that a single cytochrome P450 can explain all the metabolic findings observed over the past 2 decades of research. These unexplained observations include:

(a) Using the perfused rat liver, Fukushima et al. (1976) demonstrated *two* 25-hydroxylase enzyme activities: a high-affinity, low-capacity form (presumably microsomal) and a low-affinity, high-capacity form (presumably mitochondrial; CYP27A1).
(b) Regulation, albeit weak, of the liver 25-hydroxylase in animals, given normal dietary intakes of vitamins D after a period of vitamin D deficiency (Bhattacharyya and DeLuca, 1973), is not explained by a transcriptional mechanism since the gene promoter of CYP27A1 lacks a vitamin D responsive element (VDRE) and the hepatocyte lacks the vitamin D receptor (VDR) (Pike, 1991).
(c) No obvious 25-OH-D_3 or 1α,25-$(OH)_2D_3$ deficiency in patients suffering from the genetically inherited disease, cerebrotendinous xanthomatosis, where the gene expressing CYP27A1 is mutated (although a subset of these patients can suffer from osteoporosis, this is more likely due to biliary defects leading to altered enterohepatic circulation of 25-OH-D_3 (Berginer et al., 1993)).
(d) CYP27A1 does not appear to 25-hydroxylate vitamin D_2 (Guo et al., 1993).

There have been a series of claims that several human microsomal cytochrome P450s are able to carry out the 25-hydroxylation of vitamin D_2 or vitamin D_3 or both (Prosser and Jones, 2004). These include CYP2R1, CYP3A4 and CYP2J3. The claims for the physiological relevance of CYP2R1 are particularly pertinent as there is a single report of a human mutation of the *CYP2R1* gene in an individual with rickets (Cheng et al., 2004), and in our hands the enzyme is a 1α-OH-D_2-25-hydroxylase with a high affinity for its vitamin D_2 substrate (Jones et al., 2006 - see also Strushkevich et al., 2008). One thing that the existence of multiple CYPs with 25-hydroxylase activity might explain is the occasional report of 25-hydroxylation of vitamin D_3 in extrahepatic tissues or cells (e.g. in bone, intestine or kidney) (Tucker et al., 1973; Ichikawa et al., 1995). The product of the 25-hydroxylation step, 25-OH-D_3, is the major circulating form of vitamin D_3 and in humans is present in plasma at concentrations in the range of 10–80 ng/mL (25–200 nmol/L) (Hollis, 2005a). The main reason for the extended plasma half-life of 25-OH-D_3 is its strong affinity for DBP (Table 11.1), and the DBP-XO mouse shows accelerated rates of clearance and low 25-OH-D_3 levels (Safadi et al., 1999). Serum levels of 25-OH-D_3 therefore represent a measure of the vitamin D status of the animal *in vivo*.

The circulating metabolite, 25-hydroxyvitamin D_3, is converted to the active form of vitamin D known as calcitriol or 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$). The second step of activation, 1α-hydroxylation, occurs primarily in the kidney (Jones et al., 1998) and there exists a specific mechanism involving the cell-surface receptors, megalin/cubilin, to provide uptake of the substrate in the form of the 25-OH-D/DBP complex by renal proximal tubular cells (Willnow and Nykjaer, 2005; Andreassen, 2006). Megalin-knockout mice show reduced vitamin D metabolite levels and vitamin D deficiency. The synthesis of *circulating* 1α,25-$(OH)_2D_3$ in the normal, non-pregnant mammal appears to be the **exclusive** domain of the kidney. The main evidence for this stems from clinical medicine where patients with chronic renal failure exhibit frank rickets or osteomalacia due to a deficiency of 1α,25-$(OH)_2D_3$ caused by lack of renal 1α-hydroxylase, a situation that is reversed by 1α,25-$(OH)_2D_3$ hormone replacement therapy (Martinez et al., 1996). As the result of a tremendous amount of attention over the past 2 decades, the cytochrome P450, CYP27B1 representing the 1α-hydroxylase enzyme was finally cloned from a rat renal cDNA library by St Arnaud's group in Montreal (St-Arnaud et al., 1997). This discovery was rapidly followed by cloning of cDNAs representing mouse and human CYP27B1 (Fu et al., 1997; Monkawa et al., 1997; Takeyama et al., 1997) as well as the human and mouse genes (Kitanaka et al., 1998; Yamamoto et al., 2005]. It had been known for some time that the kidney mitochondrial 1α-hydroxylase enzyme comprises three proteins – a cytochrome P450, ferredoxin and ferredoxin reductase – all of which are required for activity and is strongly downregulated by 1α,25-$(OH)_2D_3$ and upregulated by PTH as part of the calcium homeostatic loop (Gray et al., 1972; Armbrecht et al., 2003). It has been shown that a similar phosphate homeostatic loop also exists involving fibroblast-like growth factor, FGF-23, seemingly the long-postulated "phosphatonin" hormone which downregulates CYP27B1 enzyme activity presumably at the transcriptional level (Quarles, 2003; Perwad et al., 2005). The promoter for the *CYP27B1* gene appears to contain the necessary regulatory elements (CREs and negative VDREs) necessary to explain the observed physiological regulations of PTH and 1α,25-$(OH)_2D_3$, respectively, at the transcriptional level. Whether additional elements exist to explain the action of FGF-23 remains to be addressed. The human *CYP27B1* gene co-localises to the chromosomal location of vitamin D-dependency rickets type 1 (VDDR-I), a human disease state first proposed to be due to a mutation of the 1α-hydroxylase enzyme over 30 years ago (Fraser et al., 1973). A mouse *CYP27B1* knockout model has also been created by deletion of the *CYP27B1* gene by two independent groups and the 1α,25-$(OH)_2D_3$-deficient model has revealed insights into the regulation of the gene by different stimuli and the different roles of 1α,25-$(OH)_2D_3$ (Dardenne et al., 2001; Panda et al., 2004).

Over the past 20 years, it has been suggested that there are several physiological or pharmacological situations in which an ***extra-renal*** 1α-hydroxylase activity may exist (e.g. Jongen et al., 1984b). Placental 1α-hydroxylase was reported (Ornoy et al., 1978) but has proved to be difficult to purify and there have been suggestions that the activity may be artifactual (Hollis et al., 1989). Before the use of calcitriol and its analogues in clinical medicine, there were occasional reports of anephric individuals given vitamin D_3 or 25-OH-D_3 who exhibited measurable blood levels of metabolite(s) which displaced 1α,25-$(OH)_2D_3$ in receptor binding assays (Barbour et al., 1981;

Lambert et al., 1982; Shultz et al., 1983; Dusso et al., 1988, 1991) suggesting the existence of significant extra-renal 1α-hydroxylase. The concept was given a significant boost when Adams et al. (1986) demonstrated the existence of a 25-OH-D_3-1α-hydroxylase in sarcoid tissue and this poorly regulated enzyme results in elevated plasma 1α,25-$(OH)_2D_3$ levels which in turn cause hypercalciuria and hypercalcemia in sarcoidosis patients. The induction of this 1α-hydroxylase in macrophages by cytokines (e.g. interferon-γ) and other cytokines and growth factors has been demonstrated (Dusso et al., 1997; Stoffels et al., 2006) but its role, if any, in normal macrophages is unknown. The availability of molecular probes for the renal CYP27B1 has made it possible to detect mRNA and immunodetectable protein for this cytochrome in many extra-renal locations (Hewison and Adams, 2005) and this knowledge has given rise to a new concept whereby the extra-renal CYP27B1 is thought to augment circulating 1α,25-$(OH)_2D_3$ with local production of 1α,25-$(OH)_2D_3$ (Holick, 2004, 2007; Hollis, 2005a; Jones, 2007). The locally high concentrations of 1α,25-$(OH)_2D_3$ in certain sites such as skin, prostate and breast are believed to give altered patterns of gene expression, which in turn limit cell growth and lead to tissue-specific differentiation of specific cell types. An important physiological role for the extra-renal 1α-hydroxylase also begins to emphasise the importance of circulating '25-OH-D' levels that provide the substrate for this enzyme as well as the renal enzyme. Lately, there has been a groundswell of support for the idea that the plasma '25-OH-D' concentration is an excellent predictor, superior to the plasma 1α,25-$(OH)_2D_3$ concentration, of the non-classical actions of vitamins D in the health of the immune system, skin, bone, certain epithelial cells, muscle, etc. (Holick, 2004 , 2007; Hollis, 2005a; Jones, 2007).

Irrespective of where 1α,25-$(OH)_2D_3$ is synthesised, as the sole hormonal form of vitamin D_3, it represents the molecule which transduces the biological response inside calcitriol target cells. The physiological functions of 1α,25-$(OH)_2D_3$ (Jones et al., 1998) are usually divided into:

Classical roles which include the regulation of blood calcium and phosphate concentrations by actions at intestine, bone and kidney

Non-classical roles which include cell-differentiation and anti-proliferative actions at various cell lines especially bone marrow (pre-osteoclast and lymphocyte), skin, muscle and intestine

Though most of the classical physiological actions of calcitriol have been known from the early part of this century when dietary vitamin D deficiency was first demonstrated, the non-classical roles have only emerged from more subtle studies involving experiments probing the mechanism of action of calcitriol at the molecular level (Pike, 1991; Stumpf, 1995; White, 2004) and from studies of the vitamin D receptor knockout mouse (Yoshizawa et al., 1997).

All of these physiological functions of calcitriol are believed to be achieved through a steroid hormone-like mechanism involving a nuclear vitamin D receptor that specifically regulates the transcription of vitamin D-dependent genes coding for proteins that in turn regulate cellular events such as intestinal calcium transport and cell division (Haussler et al., 1998) (Fig. 11.5). In the classical steroid hormone model, 1α,25-$(OH)_2D_3$ enters the cell by traversing the plasma membrane in a free form and

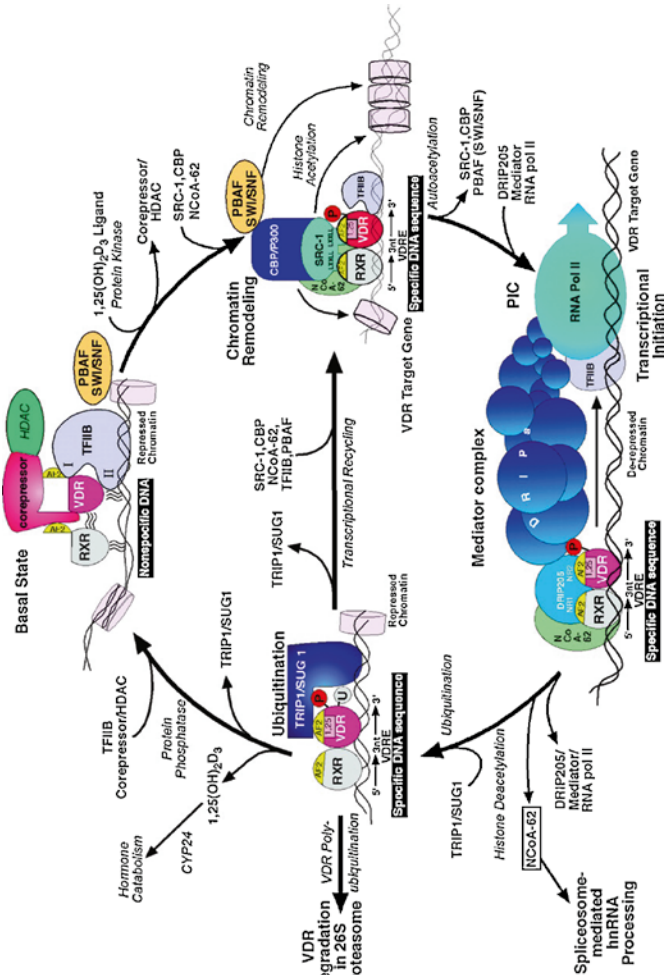


Fig. 11.5 Model of the transcriptional mechanism of action of 1α,25-$(OH)_2D_3$ – this enters the picture at 1 o'clock interacting with the unliganded VDR-retinoid X receptor basal complex and locating the vitamin D-responsive element containing vitamin D-dependent gene. The initial steps in activation involving conformational changes in the VDR and recruitment of coactivators are shown in the 3 o'clock complex. At this point, a SWI/SWF chromatin remodeling complex effects changes in chromatin allowing recruitment of the transcription initiation complex, including attraction of DRIPs shown at 6 o'clock. Active transcription of the vitamin D-dependent gene occurs. The complex at 9 o'clock is involved in turning over the VDR and recycling the rest of the transcriptional machinery. Note that the ligand 1α,25-$(OH)_2D_3$ is also subject to degradation by CYP24A1, an inducible cytochrome *P*450 found in the target cell (from Whitfield et al., 2005, with permission)

binds strongly to the VDR inside the nucleus ($K_d = 2 \times 10^{-10}$ M). In the paracrine/autocrine model, 25-OH-D enters the cell via megalin/cubulin and calcitriol is formed in the cell as illustrated in Figure 11.6. The liganded or occupied VDR specifically targets only vitamin D-dependent genes by interacting with a specific sequence found upstream of the vitamin D-dependent gene. The sequence, known as a vitamin D responsive element, is a tandem repeating oligonucleotide of six base pairs containing a 3-nucleotide spacer which is situated normally around 400–500 base pairs upstream of the 5′ end of the vitamin D-responsive gene. A consensus VDRE, AGGTCAnnnAGGTCA, is found in the rat and human osteocalcin genes, the rat calbindin-9K gene and the mouse osteopontin gene, whereas more complex elements are found in the collagen type I gene and the pre-pro-PTH gene where they play a negative or suppressive role. Further research has shown that VDR requires a heterodimeric partner called the retinoid X receptor (RXR) and a plethora of other transactivators, termed a DRIP complex, in order to transactivate genes (Rachez et al., 1999). The current model suggests that occupation of the ligand-binding domain of the VDR component triggers a protein conformational change in the AF-2 domain of the C-terminus of the VDR (Whitfield et al., 2005) which allows recruitment of positive transcription factors and/or shedding of transcriptional inhibitory factors which lead to increased formation of a transcription initiation complex and an increased rate of gene transcription. Figure 11.5 gives some idea of the complexity of this mechanism and the number of specific and general transcription factors involved. Knowledge of the widespread distribution of the VDR protein in different tissues besides the classical targets of bone, intestine and kidney has encouraged researchers to look for other vitamin D-dependent processes. This was reinforced by the reports of disparate effects of calcitriol and its analogue changes brought about in biological systems *in vitro* and non-classical tissues *in vivo*. Though it is not always clear if these effects are physiological or pharmacological, these observations have certainly broadened our view of the ultimate role of vitamins D. The molecular approach has also strengthened the case that calcitriol is more than just a calcemic hormone. VDRE-containing genes now number around several hundreds suggesting that vitamin D_3 through 1α,25-$(OH)_2D_3$ and VDR may regulate many physiological processes besides intestinal calcium absorption, bone resorption and bone matrix protein formation (Lin and White, 2004; White, 2004). The consensus view stemming from VDR-knockout models and gene chip array experiments is that skin differentiation, immunosuppression, muscle differentiation, blood pressure regulation and osteoclast recruitment are all processes significantly modulated by calcitriol.

24-Hydroxylation of both 25-OH-D_3 and 1α,25-$(OH)_2D_3$ has been shown to occur *in vivo* to give rise to 24*R*,25-$(OH)_2D_3$ and 1α,24,25-$(OH)_3D_3$, respectively (Holick et al., 1972, 1973). The importance of this step has been immersed in controversy since it had been claimed that 24-hydroxylated metabolites might play a role in (a) bone mineralisation; and (b) egg hatchability (Henry and Norman, 1978). Experimental evidence favours a different function for 24-hydroxylation: inactivation of the hydroxylated vitamins D molecules.

This concept comes from four main lines of evidence:

(i) Levels of 24,25-$(OH)_2D_3$ do not appear to be regulated, reaching >100 ng/mL in hypervitaminotic animals (Jones et al., 1987).

(ii) There is no apparent 24,25-$(OH)_2D_3$ receptor similar to VDR within the orphan steroid receptor superfamily stemming from the Human Genome Project.
(iii) Synthesis of calcitriol analogues blocked with fluorine atoms at various carbons of the side chain (e.g. 24F_2-1α,25-$(OH)_2D_3$) results in molecules with full biological activity *in vivo*.
(iv) 24-Hydroxylation of hydroxylated vitamins D appears to be the first step in a degradatory pathway demonstrable *in vitro* (Makin et al., 1989; Reddy and Tserng, 1989) (Fig. 11.4), which culminates in a biliary excretory form, calcitroic acid observed *in vivo* (Esvelt et al., 1979) or 1α,25-$(OH)_2D_3$-26,23-lactone.

The 25-OH-D_3-24-hydroxylase was originally characterised as a P450-based enzyme over 20 years ago and in 1991 the cytochrome P450 species was purified and cloned and named CYP24A1 (Ohyama et al., 1991). The enzyme appears to 24-hydroxylate both 25-OH-D_3 and 1α,25-$(OH)_2D_3$, the latter with a tenfold higher efficiency (Tenenhouse et al., 1988; Ohyama and Okuda, 1991), though in the complete absence of DBP this substrate discrimination is less evident. However, since the circulating level of 25-OH-D_3 is ~1,000 times higher than 1α,25-$(OH)_2D_3$, the role of the enzyme *in vivo* is not clear. The enzyme, particularly the renal form that appears to be expressed at high constitutive levels in the normal animal, may be involved in the inactivation and clearance of excess 25-OH-D_3 in the circulation (Jones et al., 1987). On the other hand, the 24-hydroxylase may be involved in target cell destruction of 1α,25-$(OH)_2D_3$ (Lohnes and Jones, 1992). 1α,25-$(OH)_2D_3$ is a very good substrate for the 24-hydroxylase. Using a variety of cell lines representing specific vitamin D target organs (intestine: CaCo2 cells; osteosarcoma: UMR-106 cells; kidney: LLC-PK1 cells; keratinocyte: HPK1A and HPK1A-ras), a number of researchers have shown that 24-hydroxylation is the first step in the C-24 oxidation pathway, a five-step, calcitriol inducible, ketoconazole-sensitive pathway which changes the hydroxylated vitamin D molecules to water-soluble truncated products such as calcitroic acid (Fig. 11.4) (Makin et al., 1989; Reddy and Tserng, 1989). In most biological assays, the intermediates and truncated products of this pathway possess lower or negligible activity. Furthermore, many of these compounds have little or no affinity for DBP making their survival in plasma tenuous at best. PCR studies of CYP24A1 have led to detection of CYP24A1 mRNA in a wide range of tissues corroborating the earlier studies reporting widespread 24-hydroxylase enzyme activity in most, if not all, calcitriol-target cells. Additional studies have shown that mRNA transcripts for CYP24A1 are virtually undetectable in naive target cells not exposed to 1α,25-$(OH)_2D_3$ but increase dramatically by a VDR-mediated mechanism within hours of exposure to 1α,25-$(OH)_2D_3$ (Shinki et al., 1992). In fact, the promoters of both human and rat CYP24A1 genes possess a double VDRE that has been shown to mediate the calcitriol-dependent induction of CYP24A1 enzyme in both species (Ohyama et al., 1996). It is therefore attractive to propose that not only is 24-hydroxylation an important step in inactivation of excess 25-OH-D_3 in the circulation but it is also involved in the inactivation of 1α,25-$(OH)_2D_3$ inside target cells. As such, one can hypothesise that C-24 oxidation is a target-cell attenuation or desensitisation process that constitutes a molecular switch to turn off calcitriol responses inside target cells (Lohnes and Jones, 1992) (see Fig. 11.6). The development of CYP24A1 knockout animals (St-Arnaud,

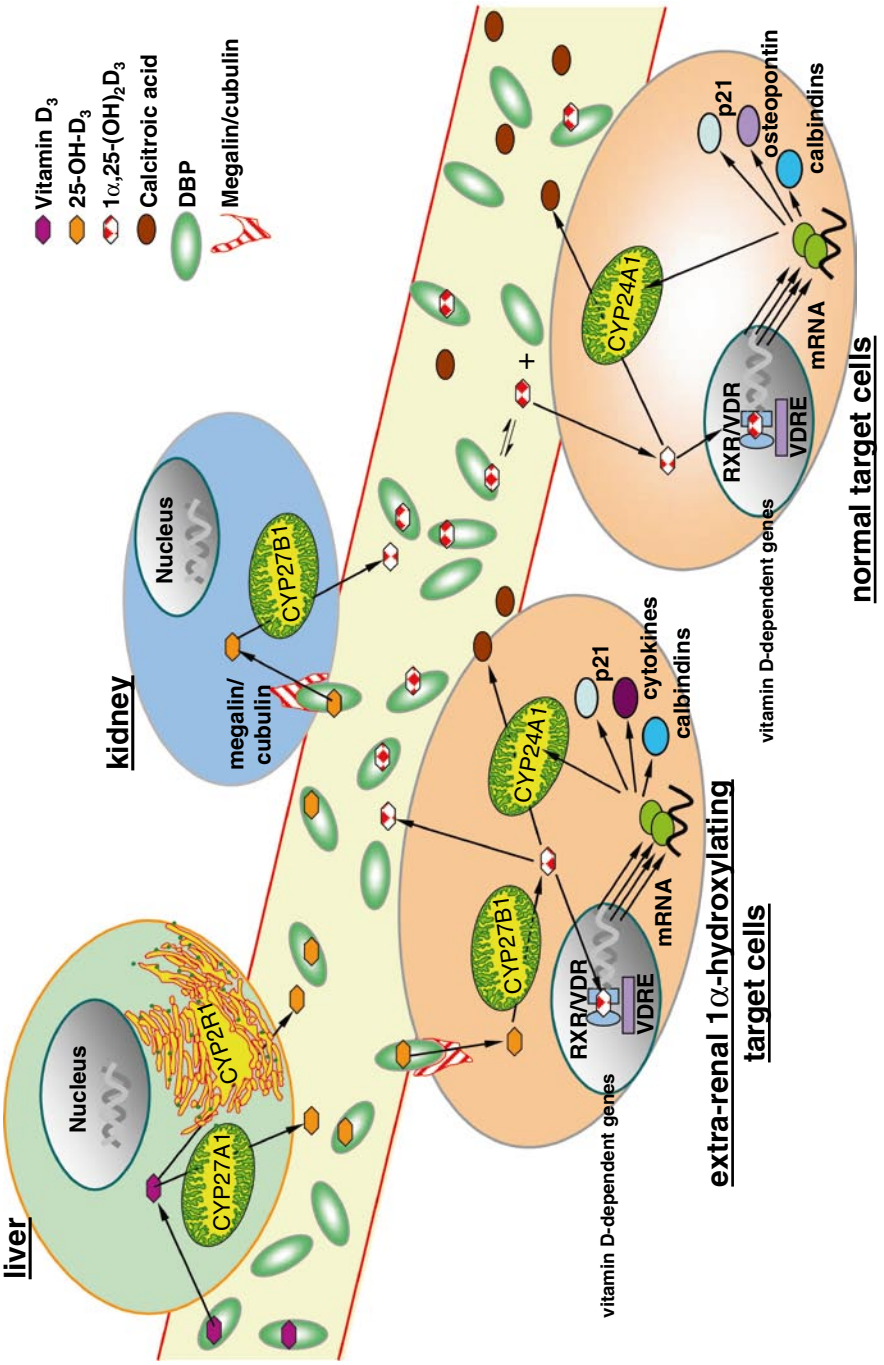

Vitamin D3
25-OH-D3
1α,25-(OH)2D3
Calcitroic acid
DBP
Megalin/cubulin
liver
Nucleus
CYP27A1
CYP2R1
kidney
Nucleus
megalin/
cubulin
CYP27B1
+
extra-renal 1α-hydroxylating
target cells
CYP27B1
CYP24A1
RXR/VDR
VDRE
mRNA
p21
cytokines
calbindins
vitamin D-dependent genes
normal target cells
CYP24A1
RXR/VDR
VDRE
mRNA
p21
osteopontin
calbindins
vitamin D-dependent genes

Fig. 11.6 Roles of extra-renal 1α-hydroxylase (CYP27B1) and target cell CYP24 in vitamin D action – this figure depicts most components of the vitamin D signal transduction pathway. Vitamin D_3 (upper left side of the diagram) is carried to the liver to be activated by one of a series of 25-hydroxylases, the major ones being the microsomal CYP2R1 and the mitochondrial CYP27A1. The resulting 25-OH-D_3 is then carried on DBP to the kidney, where it is hydroxylated by the classical renal CYP27B1 and then put back into the circulation as 1α,25-$(OH)_2D_3$. The 1α,25-$(OH)_2D_3$ then enters and acts upon vitamin D target cells at the level of gene transcription through a VDR-mediated mechanism (cell at bottom right). Note that according to this theory, 1α,25-$(OH)_2D_3$ arrives at the target cell bound to DBP but its entry into the cell is a function of the free pool of 1α,25-$(OH)_2D_3$ in equilibrium with protein-bound 1α,25-$(OH)_2D_3$. In hypervitaminosis D, some investigators argue that saturation of the DBP displaces a greater amount of free 1α,25-$(OH)_2D_3$, thereby increasing gene expression and causing hypercalcemia and other toxicity symptoms. Another aspect of our current understanding of the vitamin D signal transduction system is the concept of the extra-renal 1α-hydroxylase theory, where some target cells express the membrane proteins megalin and/or cubulin to concentrate the 25-OH-D_3/DBP complex and also express the protein CYP27B1 to 1α-hydroxylate the substrate 25-OH-D_3. It is believed that in this way, certain target cells (e.g. macrophages and monocytes) boost 1α,25-$(OH)_2D_3$ production by augmenting the circulating 1α,25-$(OH)_2D_3$ levels produced by the kidney. Although the theory remains unproven, many of its facets are consistent with the observed health advantages of vitamin D and with emerging epidemiological data. Note that all target cells turn on transcription of and express CYP24A1 to attenuate the 1α,25-$(OH)_2D_3$ signal, in the process giving calcitroic acid which enters the bloodstream and is excreted in the bile (from Jones, 2008; with permission)

1999) resulting in hypercalcemia, hypercalciuria, nephrocalcinosis and premature death in 50% of null animals seems to support this hypothesis. On the other hand, surviving animals have unexplained changes in bone morphology which could suggest an alternative role for 24-hydroxylase in bone mineralisation (St-Arnaud, 1999) though double knockouts lacking both CYP24A1 and VDR do not exhibit this bone phenotype (St-Arnaud et al., 2000). Surviving CYP24A1-null animals have been shown to possess much reduced ability to clear a bolus dose of [1β-^{3}H]1α,25-$(OH)_2D_3$ from their circulation as compared to normal wild-type littermates (Masuda et al., 2005). There is thus no evidence for efficient non-CYP24A1 backup excretory systems for calcitriol catabolism. Calcitroic acid, the final water-soluble product of 1α,25-$(OH)_2D_3$ catabolism, is probably not synthesised in liver because C-24 oxidation does not occur in hepatoma cells and therefore must presumably be transferred from target cells to liver via some plasma carrier. Though calcitroic acid has been found in various tissues *in vivo* (Esvelt et al., 1979; Esvelt and DeLuca, 1981), details of its transfer to bile have not been elucidated.

Many other vitamins D metabolites have been reported over the years (see Table 11.1) but most are not much more than metabolic idiosyncrasies with little biological importance. The analyst should be familiar with a few of these because they can become quite abundant in certain biological samples, particularly those from animals given large doses of vitamins D, their metabolites or analogues. Included in these is 25,26-$(OH)_2D_3$ which was the first di-hydroxylated metabolite to be identified back in the late 1960s (DeLuca et al., 1970) and yet its origins are still the most poorly understood. This metabolite is readily detectable in the plasma of animals given large doses of vitamin D_3 and it retains strong affinity for DBP (Table 11.1) (Horst, 1979). However, its biological activity is inferior to other endogenous vitamins D metabolites and it is presumed to be a minor catabolite. The knowledge that CYP27A1 is involved in vitamin D_3 activation; and that 26(27)-OH-D_3 and 1α,26-$(OH)_2D_3$ are formed from vitamin D_3 and 1α-OH-D_3, respectively, in CYP27A1 transfection systems (Guo et al., 1993); when taken together suggest that 26(27)-hydroxylation maybe a consequence of errant side-chain hydroxylation by CYP27A1.

The most abundant 26-hydroxylated metabolite appearing *in vivo* is the 26,23-lactone derivative of 25-OH-D_3. 25-OH-D_3-26,23-lactone accumulates in the blood of hypervitaminotic animals *in vivo* because of its extremely strong affinity for DBP (Horst, 1979). The route of synthesis of this metabolite is depicted in Fig. 11.7 and research indicates that 26-hydroxylation follows 23-hydroxylation in this process (Yamada et al., 1984; Prosser and Jones, 2004). The site of this synthesis is probably extra-hepatic suggesting the existence of extra-hepatic 26-hydroxylation. Evidence (Akiyoshi-Shibata et al., 1994; Beckman et al., 1996) that CYP24A1 carries out 23- as well as 24-hydroxylation of certain analogues, coupled with the evidence that 1α,25-$(OH)_2D_3$-26,23-lactone is absent from the blood of CYP24A1-null mice given 1α,25-$(OH)_2D_3$ (Masuda et al., 2005), raised the likelihood that CYP24A1 is also involved in 26,23-lactone formation. The final proof that this is indeed the case came from mutagenesis studies where human CYP24A1, which is predominantly a 24-hydroxylase biosynthesising calcitroic acid, was turned into a

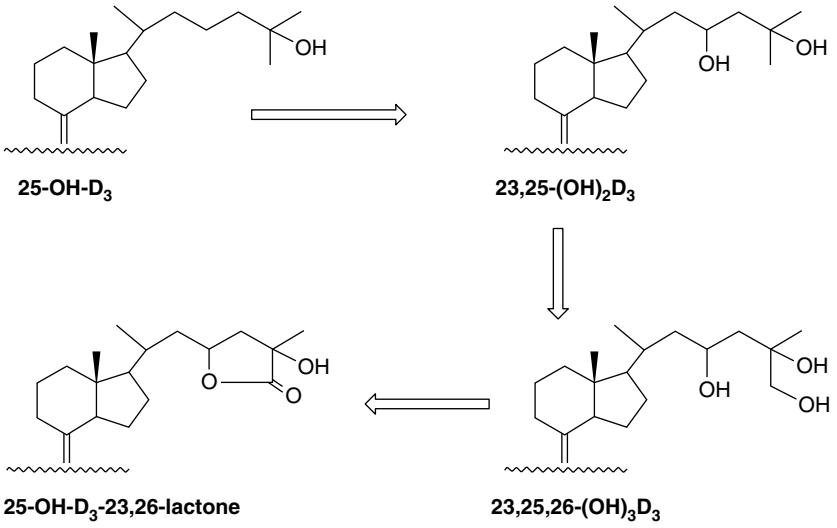


Fig. 11.7 Biosynthesis of 25-OH-D_3–26,23-lactone (only C and D rings illustrated)

23-hydroxylase biosynthesising 26,23-lactone by a single amino acid A326G residue change (Prosser et al., 2007). The biological role of 25-OH-D_3-26,23-lactone or its 1α,25-$(OH)_2D_3$ counterpart, if any, are currently unknown although 23(S)-25-dehydro-1α,25-$(OH)_2D_3$, a synthetic analog has been used in the treatment of Paget's Disease (Ishizuka et al., 2005). Thus, it is presumed that 26,23-lactone formation represents a back-up degradatory pathway for 25-OH-D_3 and/or 1α,25-$(OH)_2D_3$.

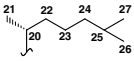
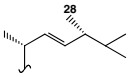
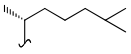
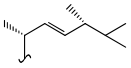
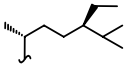
In summary then, knowledge of the biochemistry establishes that 1α,25-$(OH)_2D_3$ is the most important metabolite but occurs in plasma in sparingly low concentrations and its level is not a particularly significant indicator of disease, except for frank vitamin D deficiency rickets. Since this is a rare event, the clinical utility of 1α,25-$(OH)_2D_3$ assay is limited. In contrast, the assay of '25-OH-D', which is the circulating precursor and substrate for renal (endocrine) and extra-renal (paracrine or intracrine) CYP27B1 enzymes, is extremely useful (Holick, 2004; Zerwekh, 2004; Hollis, 2005a). Many epidemiological studies have established the utility of plasma '25-OH-D' assay by showing that concentrations strongly correlate inversely with the risk of mortality in the general population (Melamed et al., 2008a), incidence of various types of cancer (e.g. prostate, breast and colon cancers), multiple sclerosis, peripheral arterial disease and diabetes (Schwartz and Hulka, 1990; Grant and Garland, 2002, 2004; Holick, 2004; Schwartz and Skinner, 2007; Hayes and Acheson, 2008; Melamed et al., 2008b; Smolders et al., 2008). The analyst must be aware of the more obscure vitamin D_3 metabolites such as 24,25-$(OH)_2D_3$, 25,26-$(OH)_2D_3$, the two 26,23-lactones and calcitroic acid; these are all metabolites not routinely measured in clinical laboratories in the twenty-first century but must be removed to make assay of the common metabolites more specific. Moreover, a

student of vitamins D analysis should also be aware that research studies are not confined to the analysis of clinical blood samples and often require the separation and analysis of a wider range of vitamins D metabolites from a variety of different sources (animal tissues, cultured cell models and biochemical or broken cell extracts). Consequently, the analytical procedures described here are not confined to those used in clinical studies but are intended to provide a comprehensive introduction to the variety of techniques used in this field.

11.2.3 Pro-Drugs and Analogues

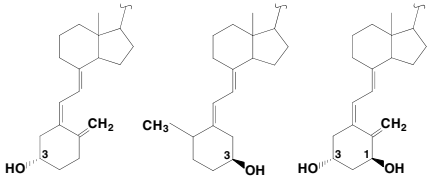
Table 11.2 lists some of the most important pro-drugs of vitamin D_3. All of these compounds require one or more *in vivo* metabolic steps to become biologically active. As vitamin D_2 is mainly derived by *in vitro* irradiation of the plant sterol, ergosterol, and is difficult to detect in plasma from humans eating non-vitamin D_2-fortified food, it can be regarded as an artificial form of vitamin D_3 or a pro-drug.

Table 11.2 Vitamins D pro-drugs

Prodrug [ring structure][a]	Side chain structure	Company	Status	Possible target diseases	Mode of delivery	Reference
1α-OH-D_3 [3]		Leo	In use Europe	Osteoporosis	Systemic	Barton et al., 1973
1α-OH-D_2 [3]		Genzyme	In use USA	Secondary Hyperparathyroidism	Systemic	Paaren et al., 1978
Dihydrotachysterol[b] [2]		Duphar	Withdrawn	Renal failure	Systemic	Jones et al., 1988
Vitamin D_2 [1]		Various	In use USA	Rickets Osteomalacia	Systemic Systemic	Fraser et al., 1973
1α-OH-D_5 [3]		None	In trial USA	Cancer	Systemic	Mehta et al., 2000

[a] Structure of the prodrug D nucleus (secosterol ring structure).
[b] the prodrug is DHT_2 but DHT_3 also exists

Prodrug Nucleus



[1] [2] [3]

Although vitamin D_2 has a chemically modified vitamin D_3 side chain, it is still able to undergo the same series of activation steps, giving rise to 25-OH-D_2, 1α,25-$(OH)_2D_2$ and 24,25-$(OH)_2D_2$. Two of the other pro-drugs, 1α-OH-D_3 and 1α-OH-D_2, were synthesised in the early 1970s (Barton et al., 1973; Paaren et al., 1978) as alternative sources of calcitriol and 1α,25-$(OH)_2D_2$, respectively. 1α-OH-D_5 has shown promising anti-proliferative effects on breast cancer cells (Mehta, 2004).

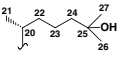
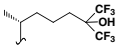
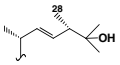
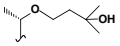
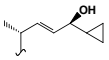
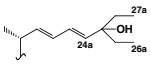
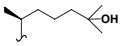
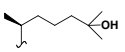
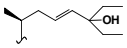
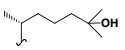
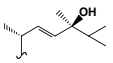
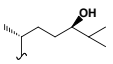
The penultimate compound in the list, dihydrotachysterol$_2$ (DHT_2 – AT10 or Tachyrol), has had a complex history as a pro-drug. Originally, it was considered that, as the rotation of the A ring in this molecule brings the 3-OH into the position normally occupied by the 1α-OH, a single *in vivo* 25-hydroxylation would produce a 'pseudo' 1α,25-dihydroxylated metabolite, which would mimic the effect of calcitriol (Jones et al., 1988). However, more modern studies of the metabolism of this compound has made the position less clear cut, demonstrating an extra-renal *in vitro* 1-hydroxylation of 25-OH-DHTs to 1α,25-$(OH)_2$DHTs and 1β,25-$(OH)_2$DHTs (Schroeder et al., 1993). All four of these metabolites have greater binding to the chick intestinal VDR than 25-OH-DHTs (1α > 1β) (Schroeder et al., 1994).

The discovery of the non-classical effects of calcitriol, not involving the regulation of calcium homeostasis, and the increasing appreciation of its involvement in many biological systems (reviewed by Jones et al., 1998; Christakos et al., 2003; Holick, 2003; Sutton and MacDonald, 2003; DeLuca, 2004; Holick, 2004; Lips, 2006) has led to increasing development of chemical derivatives or analogues of calcitriol (Bouillon et al., 2005; Masuda and Jones, 2006) which do not cause significant hypercalcaemia. One example from many is calcipotriol (Calverley, 1987; Binderup and Kragballe, 1992) which has found considerable clinical success in the treatment of psoriasis by topical administration. Table 11.3 lists the structures of some of the promising analogues of calcitriol already approved by government agencies and/or in development by commercial companies or university research groups. As the number of such analogues is well into the thousands, Table 11.3 lists only a sample, primarily to illustrate the types of analogues produced, the worldwide location of companies involved and the wide spectrum of diseases which these analogues are intended to benefit.

The first generation of calcitriol analogues included molecules with fluorine atoms placed at metabolically vulnerable positions in the side chain and resulted in the production of highly stable and potent 'calcaemic' agents such as 26,27-F_6-1α,25-$(OH)_2D_3$. Attention has also focussed on introducing features that make the molecule more susceptible to clearance such as calcipotriol (MC903), where a C22 = C23 double bond, a 24-hydroxyl and a cyclopropane ring were introduced into the side chain or as in 22-oxacalcitriol (OCT), where the 22-carbon has been replaced with an oxygen atom. Both these modifications have given rise to useful analogues, which are now in clinical use for the treatment of psoriasis and hyperparathyroidism, respectively.

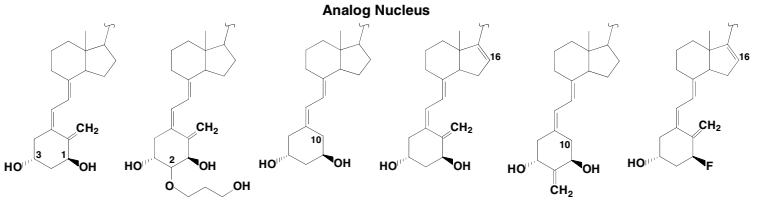
The C24 position is another favourite site for modification, and numerous analogues contain 24-hydroxyl groups (e.g. 1α,24(*S*)-$(OH)_2D_2$ and 1α,24(*R*)-$(OH)_2D_2$ – Strugnell et al., 1995). Other analogues contain multiple changes in the side chain in combination, including unsaturation, 20-epimerisation, 22-oxa replacement, homologation

Table 11.3 Analogues of 1α,25-$(OH)_2D_3$ (from Jones, 2008 with permission)

Analog [ring structure][a]	Side chain structure	Company	Status	Possible target diseases	Mode of delivery	Reference
Calcitriol, 1α,25-$(OH)_2D_3$ [3]		Roche, Duphar	In use worldwide	Hypocalcemia Psoriasis	Systemic Topical	Baggiolini et al., 1982
26,27-F_6-1α,25-$(OH)_2D_3$ [3]		Sumitomo-Taisho	In use Japan	Osteoporosis Hypoparathyroidism	Systemic Systemic	Kobayashi et al., 1982
19-Nor-1α,25-$(OH)_2D_2$ [5]		Abbott	In use USA	Secondary Hyperparathyroidism	Systemic	Perlman et al., 1990
22-Oxacalcitriol (OCT) [3]		Chugai	In use Japan	Secondary Hyperparathyroidism Psoriasis	Systemic Topical	Murayama et al., 1986
Calcipotriol (MC903) [3]		Leo	In use worldwide	Psoriasis Cancer	Topical Topical	Calverley, 1987
1α,25-$(OH)_2$-16-ene-23-yne-D_3 (Ro 23-7553) [6]		Roche	Pre-clinical	Leukemia	Systemic	Baggiolini et al., 1989
EB1089 [3]		Leo	Clinical trials	Cancer	Systemic	Binderup et al., 1991
20-epi-1α,25-$(OH)_2D_3$ [3]		Leo	Pre-clinical	Immune diseases	Systemic	Calverley et al., 1991
2-methylene-19-nor-20-epi-1α,25-$(OH)_2D_3$ (2MD) [7]		Deltanoids	Pre-clinical	Osteoporosis	Systemic	Shevde et al., 2002
BXL-628 (formerly Ro-269228) [8]		Bioxell	Clinical trials	Prostate Cancer	Systemic	Marchiani et al., 2006
ED71 [4]		Chugai	Clinical trials	Osteoporosis	Systemic	Nishii et al., 1993
1α,24(S)-$(OH)_2D_2$ [3]		Genzyme	Pre-clinical	Psoriasis	Topical	Strugnell et al., 1995
1α,24(R)-$(OH)_2D_3$ (TV-02) [3]		Teijin	In use Japan	Psoriasis	Topical	Morisaki et al., 1975

[a]Structure of the analog nucleus (secosterol ring structure).

Analog Nucleus



[3] [4] [5] [6] [7] [8]

in the side chain or terminal methyl groups. The resultant molecules such as EB1089 and KH1060 are attracting strong attention from researchers because of their greatly increased potencies *in vitro* and are being studied as potential anti-cancer and immunomodulatory compounds, respectively.

Few attempts thus far have been made to modify the calcitriol nucleus (see also Section 11.5). The Roche compound, 1α,25-$(OH)_2$-16-ene-23-yne-D_3, reported to be an anti-tumour compound *in vivo*, possesses a D-ring double bond. The A-ring substituted 2-hydroxypropoxy derivative, ED-71, has been tested as an anti-osteoporosis drug. The Abbott compound, 19-nor-calcitriol, lacks a C19 methylene group and is similar to the *in vivo* active metabolite, 1α,25-$(OH)_2DHT_3$, formed from DHT_3 (Schroeder et al., 1994) which retains biological activity even though the C19 methylene group has been replaced with a C19 methyl. Many other compounds have been developed with rigid or altered *cis*-triene structures (Okamura et al., 1995) or modifications of the 1α-, 3β- or 25-hydroxyl functions, not for the purpose of developing active molecules for use as drugs but in order to allow for the establishment of minimal requirements for biological activity in structure–activity studies *in vitro* (Calverley and Jones, 1992; Bouillon et al., 1995).

11.3 Analysis

11.3.1 Introduction

The analysis of vitamins D and their metabolites is worthy of attention for many reasons. Firstly, the development of increasing numbers of efficacious calcitriol analogues places increasing burdens on the analyst as the need to develop methods for the measurement of these compounds in biological tissues (e.g. Kissmeyer and Binderup, 1991; Kissmeyer et al., 2000) becomes of increasing importance. Besides the need to understand the *in vitro* metabolism of these analogues (e.g. for 22-oxocalcitriol (OCT) Masuda et al., 1996; Kamao et al., 2003), which often gives insight into the *in vivo* metabolism (e.g. for OCT – Ishigai et al., 1998a), the measurement of their concentrations in biological fluids provides important pharmacokinetic information (e.g. Kissmeyer and Mortensen, 2000). Measurement of the concentration of metabolites of vitamins D gives an insight into the aetiology, pathogenesis and treatment of diseases involving disturbances of calcium and phosphate metabolism as well as the other areas of physiology in which vitamins D and their metabolites are increasingly implicated. Apart from the need to ensure purity, stability and potency in drug formulations, such as multi-vitamin tablets, injectable solutions and new calcitriol analogue preparations, there is an increasing requirement to determine the concentrations of vitamins D and metabolites in foodstuffs both natural and those fortified with added vitamins D. Animal feeds also may contain added vitamins D and if these are to be used on any major scale in the beef and dairy industry there will be a need for accurate and precise methods for measuring vitamins D and their metabolites in bovine tissues and milk.

In this chapter, we intend to consider carefully the use of LC and LC-$(MS)^n$ methods, as this is the area where most development has taken place over the last 20 years, although we emphasise that GC-MS still has an extremely valuable and complementary role, particularly in the area of structural elucidation of metabolites of calcitriol analogues (e.g. Araya et al., 2003; Reddy et al., 2007), as well as a means of method validation. The need for sample preparation depends upon the matrix studied as well as the method of quantitation used. LC systems allow the separation of unchanged sulphates and glucuronides of vitamins D and their metabolites but, if required (i.e. for GC-MS), the aglycones can be liberated by enzyme hydrolysis (e.g. treatment of urine with β-glucuronidase and solvolysis with acidic ethyl acetate prior to extraction (Higashi et al., 2002a) and bile after RP-LC separation of conjugates for subsequent GC-MS (Higashi et al., 2000). Minimal pre-purification is required for concentrated samples of synthetic vitamin D compounds during chemical synthesis whereas analysis of vitamins D in foodstuffs involves saponification, extraction and/or concentration prior to quantitation. The analysis of trace amounts of metabolites of vitamins D, such as $1\alpha,25\text{-}(OH)_2D_3$, requires extraction of picogram quantities, purification by LC before quantitation by radioligand assay or sensitive MS-MS systems in order to guarantee accurate results.

Like all steroids, vitamin D and its metabolites are usually analysed in three stages, all of which are dependent on each other. These three stages are

1. Extraction of the metabolite of interest from the biological matrix
2. Separation of the metabolite of interest from other metabolites of vitamin D, other steroids or non-specific material which may interfere in stage 3
3. Quantitation

Clearly, the specificity of stage 3, the method of quantitation chosen for a particular metabolite or analogue, will have a profound influence on the necessity and extent of pre-purification required before quantitation. In some recently developed mass spectrometric assays, where stages 2 and 3 are combined and, in addition, stage 3 is highly specific, minimal preparation may be necessary (Guo et al., 2006). The development of immunoassays for steroid hormones has been extremely successful and many immunoassays are now available which can be carried out directly on plasma or on sample plasma extracts (Wheeler, 1993 and in this book – Chapter 4). Unfortunately, the development of immunoassays for vitamins D and their metabolites has not been, inexplicably, so successful and, such immuno- and other binding assays usually require pre-purification before quantitation in order to achieve acceptable accuracy. Stage 3 does not end when an acceptable value for the concentration of the analyte or analytes concerned is obtained. It is also necessary to show that the procedure used for quantitation, taken in conjunction with any preparatory purification, is precise (that is to say replicate measurements are close to the mean value – a between-batch coefficient of variation (CV) of less than 10% is acceptable with within-batch CVs being less than 10% and that the analytical result is accurate. Accuracy implies that the measured concentration of the analyte is solely due to the analyte itself and not to closely related compounds or non-specific interference. The assessment of accuracy is extremely difficult since there are seldom

definitive methods available for vitamins D metabolites which enable this parameter to be measured. A number of devices have been used which purport to assess accuracy, such as addition of pure analyte and subsequent demonstration of quantitative recovery and serial dilutions of sample. In some instances, such procedures do demonstrate deficiencies in the original analytical result but even if recovery of pure standard is quantitative and/or dilution experiments are satisfactory, accuracy is not always guaranteed. It seems to us that the only satisfactory method available is by comparison to a definitive method which is generally accepted to be isotope dilution mass spectrometry (e.g. Coldwell et al., 1990). Although there are a number of LC methods, using tandem mass spectrometry (e.g. Vogeser et al., 2004; Maunsell et al., 2005) it is not entirely clear whether the specificity of these methods is as good as that of GC-MS methods. Such LC-MS-MS methods are becoming increasingly available for vitamins D metabolites, offering an alternative to GC-MS, and will be discussed later on in this chapter. The use of such 'reference' methodology is also subject to criticism in that the 'definitive' GC-MS or LC-MS(-MS) procedure may itself be flawed. It may be necessary in stage 3 to demonstrate the identity of the analyte which can be done by a number of physico-chemical means (such as ultraviolet (UV) and/or infrared spectra, nuclear magnetic resonance (NMR), mass spectrometry etc.). These techniques have been reviewed in previous chapters and will be dealt with later on in this chapter only in so far as they pertain to the solution of this problem in the field of vitamin D. There are a number of reviews of vitamin D methodology which have been published over the last 30 years (Seamark et al., 1981; Bikle, 1983; Horst, 1985; Porteous et al., 1987; Clemens,1990; Holick, 1990; Horst et al., 1990; Jones et al., 1992; Makin et al., 1995; Yeung and Vouros, 1995; Hollis, 1997; Luque de Castro et al., 1999; Jones and Makin, 2000; Zerwekh, 2004; Hollis and Horst, 2007).

11.3.2 Extraction

11.3.2.1 Liquid–Liquid

Vitamins D and their metabolites, like all other steroid hormones, are relatively hydrophobic molecules, carried in plasma as mentioned above to a specific binding globulin (DBP), but also non-specifically to albumin, and are found in tissues bound to a specific or nuclear receptor protein. The majority of these molecules are neutral, although metabolism in the side chain may lead to the formation of carboxylic acids (e.g. calcitroic acid, Makin et al., 1989) and acidic conjugates (sulphates and glucuronides) of metabolites of vitamins D must also be considered (e.g. Higashi et al., 1999a, b). All metabolites are extractable into organic solvents which must also disrupt any protein binding of the analyte in order to achieve satisfactory extraction. The efficiency of extraction depends upon the solvent or solvent mixture used and also upon the metabolite under consideration. Solvent systems for extraction fall into two basic categories (see Table 11.4):

Table 11.4 Solvent extraction procedures for vitamins D and metabolites (modified from Jones and Makin, 2000)

Solvent[a]	Metabolite	Reference
MeOH/$CHCl_3$ (2:1)	General metabolites of D_3 in *S. glycophyllum*	Bligh and Dyer (1957)
		Curino et al. (1998)
		Curino et al. (2001)
EtAc/CycloHx (1:1)	General	Bouillon et al. (1980)
MeOH/CH_2Cl_2 (2:1)	General	Lambert et al. (1981)
Hx/IPA (1:2)	General	Parviainen et al. (1981)
EtOH	D, 25-OH-D	Belsey et al. (1971)
EtOH/H_2O (9:1)	General	Belsey et al. (1974)
Hx(differential resolubilisation in MeCN)	25-OH-D_3	Lukaszkiewicz et al. (1983)
Hx (post-saponification)	D	Thompson et al. (1982)
		Lu et al. (2007)
Ether	25-OH-D	Delvin et al. (1980)
MeOH–H_2O (Sep-Pak)	25-OH-D	Dabek et al. (1981)
CH_2Cl_2	24,25-$(OH)_2D_3$	Eisman et al. (1976)
	1,25-$(OH)_2D_3$	
CH_2Cl_2 on Extrelut	24,25-$(OH)_2D_3$	Mason et al. (1979)
	1,25-$(OH)_2D_3$	
MeOH/IPA (4:1)	25-OH-D	Turpeinen et al. (2003)
(modified for 25OH-D with	1,25-$(OH)_2D$	
extra Hx extraction)	24,25-$(OH)_2D$	Maunsell et al. (2005)
Solvents up to 1999	General	Luque de Castro et al. (1999)
IPA	25-OH-D	Ortiz-Boyer et al. (1998)
	1,25-$(OH)_2D$	
	24,25-$(OH)_2D$	
MeCN/pption/extraction with EtAc	25-OH-D_2	Higashi et al. (2001)
	25-OH-D_3	
Hpt (post-saponification)	D_3	Staffas and Nyman (2003)
CH_2Cl_2/Hx (1:5)	25-OH-D	Granado-Lorencio et al. (2006)

[a]MeOH, methanol; $CHCl_3$, chloroform; EtAc, ethyl acetate; Hx, hexane; IPA, iso-propyl alcohol; EtOH, ethanol; H_2O, water; CH_2Cl_2, methylene dichloride (dichloromethane); MeCN, acetonitrile; Hpt, heptane; pption, precipitation.

1. *Total Lipid Extraction*: for example, methanol/chloroform/water (2:1:0.8) (Bligh and Dyer, 1957) or ethanol/water (9:1) (e.g. Hollis, 2005a)
2. *Selective Lipid Extraction*: for example, ether, ethyl acetate/cyclohexane (1:1), hexane, dichloromethane or hexane/isopropanol (1:2)

Though solvents in the second class are desirable to minimise contamination of the vitamin D analyte extract, they pose problems in that, being selective, they inevitably provide efficient extraction for one particular metabolite or group of metabolites (e.g. the dihydroxylated metabolites) and poorly extract those with different polarity. The selective nature of these solvents must be recognised and extrapolation to other analytes must not be undertaken without full evaluation of the efficiency of extraction.

When simultaneous analysis of several vitamin D metabolites with a wide range of polarities is required, a total lipid extraction (Bligh and Dyer, 1957) may be necessary. The high efficiency of this technique for total lipid extraction (i.e. methanol/chloroform, 2:1 v/v) is probably due to the formation of a monophasic dispersion of the sample in extracting solvent, followed by return to the classic two-phase system by the addition of extra chloroform and saturated KCl. All hydroxylated non-acidic vitamin D metabolites can be extracted quantitatively by this technique. Inevitably, because of its efficiency of extraction, it has the disadvantage that it may also extract other lipid-soluble components which may have to be removed prior to quantitation – particularly if this is to be carried out by LC where lipid overloading of the column may be a problem. Substitution of methylene chloride for chloroform avoids the known carcinogenicity of chloroform (although methylene chloride is hepatotoxic) and reduces evaporation times by taking advantage of the lower boiling point of methylene chloride. The use of ethanol as a rapid method of extraction of vitamin D metabolites and simultaneous disruption/precipitation of protein binding becomes increasingly impractical as sample volumes exceed 1 mL. The Bligh and Dyer (1957) method is particularly useful for total extraction from cell culture medium, where lipid content is low and thus subsequent removal of unwanted lipid to avoid LC column overloading is not a significant issue.

Two-phase liquid–liquid extraction of samples is the preferred choice of sample preparation in the majority of applications. Vitamins D and their photoisomers are relatively nonpolar and can be extracted with hexane and other low-polarity solvents, provided that the matrix is broken down by saponification, etc. Acid hydrolysis must be avoided because of the tendency to dehydrate the 1-hydroxyl and give rise to isomerisation. In the biochemical areas of analysis, all types of saponification are avoided, chiefly because of the concern about the stability of vitamins D metabolites but also because of the lack of convenience. More polar solvents such as modified hexane mixtures, cyclohexane/ethyl acetate (1:1) (Bouillon et al., 1980) hexane/isopropanol (1:2) (Parviainen et al., 1981) or hexane/isopropanol/*n*-butanol (93:3:4) (Jongen et al., 1981), are employed for extraction of metabolites of vitamin D and to break protein-bound vitamins D complexes. Horst et al. (1981) use ether followed by methanol/methylene chloride (1:3) in a lengthy but ingenious attempt to analyse a series of vitamin D_2 and D_3 metabolites simultaneously. Care must be taken when using ether since this solvent is highly flammable but may also contain peroxides that can react with the *cis*-triene of vitamins D. Methylene chloride is frequently used for the extraction of 24,25-$(OH)_2D_3$ and 1α,25-$(OH)_2D_3$ from plasma. Alvarez and de Mazancourt (2001) deproteinised plasma with ethanol and then extracted with hexane/methylene chloride (90:10) in an LC method for retinol, tocopherol and 25-OH-D_2 and 25-OH-D_3. Though the use of methylene chloride was originally as a two-phase system with plasma (Eisman et al., 1976), one modification is to adsorb the plasma onto Kieselguhr packed in a column (commercial version Extrelut, Merck, Darmstadt, Germany) and elute by passing methylene chloride through the column (Mason et al., 1979). A total of 80 to 90% extraction of both dihydroxylated metabolites is obtained, and the amount of lipid extracted is so small as to make additional clean up steps unnecessary. A similar methodology

has been adopted for purification of 1α,25-$(OH)_2D_3$ prior to radioimmunoassay (Scharla et al., 1984). Methanol/IPA (4:1) has been used for serum extraction prior to a RP-LC-UV (265 nm) method for 25-OH-D_3 (Turpeinen et al., 2003) and after further extraction with hexane for an LC-MS-MS procedure for 25-OH-D_2 and 25-OH-D_3 in serum (Maunsell et al., 2005). There is an excellent summary of solvent extraction procedures used up to 1999 for vitamins D and metabolites in Luque de Castro et al. (1999).

Fatty acids, which have been shown to interfere in a number of saturation analyses, may also be extracted to varying degrees by solvents mentioned above but can be removed from the organic extract by washing with alkaline buffer (usually phosphate, pH 10.5) (Bishop et al., 1980) or extracting from alkaline medium. This procedure does not, however, remove neutral fats and it may be necessary to carry out further purification procedures in order to remove them; as an example, saponification of foodstuffs may be required (e.g. Heudi et al., 2004).

Table 11.4 lists some of the common solvents used for liquid–liquid extraction.

11.3.2.2 Solid-Phase Extraction (SPE)

The use of solvent extraction carries with it the obvious hazards associated with relatively large amounts of solvents which may be toxic or flammable. In addition, the safe disposal of chlorinated solvents may pose difficulties and thus other methods of extraction of vitamin D and its metabolites have been sought. A major advance in this area was the introduction of solid-phase microparticulate cartridges which were first introduced for the analysis of steroids in 1980 (Shackleton and Whitney, 1980). The use of an inexpensive, disposable (though many of these cartridges can be reused many times if suitably washed between use), gravity flow (systems are also available to increase flow rates by the use of pumps) minicolumns as a penultimate step prior to LC serves several useful purposes:

1. It protects the expensive LC column from lipid, particulate matter, etc.
2. It eliminates remaining neutral and highly polar lipid contaminants, further reducing the lipid load applied to the LC.
3. It can be used to fractionate the vitamin D metabolites into major classes for subsequent analysis using different solvent systems.
4. It can serve as a support for solid-phase extraction.

Minicolumns can have disadvantages too:

1. Batch-to-batch and manufacturer-to-manufacturer variability requiring frequent calibration checks.
2. The minicolumn sometimes provides unwanted resolution of vitamins D_2 and D_3 metabolites, particularly when using Sephadex-LH-20 and hydroxyalkoxypropyl Sephadex (HAPS), giving misleading results when using radioactive vitamin D metabolites as internal standards unless larger fractions are collected to include both vitamins D_2 and D_3 metabolites.

3. Losses can occur without achieving any substantial purification of the sample.
4. Adsorbents used in the minicolumns can be dirty and introduce interference into subsequent analyses.

It is fair to say that many of the initial disadvantages of minicolumns have now been overcome with the introduction of prepacked cartridges, particularly those based upon the syringe-type minicolumn. On balance then, the advantages of such cartridges outweigh the disadvantages and their introduction has revolutionised sample preparation, streamlining the procedures greatly and in some clinical assays eliminating the need for LC altogether (Reinhardt et al., 1984). Cartridges have largely replaced the use of liquid–liquid extraction since they can act as a solid-phase extraction surface in the same way that Extrelut was used in the early 1980s. Cartridges can also be used as a convenient and simple separation tool to resolve vitamin D and its metabolites. They can be used for a combination of solid-phase extraction and separation procedures (Hollis 1986, 1997). Figure 11.9 shows such a separation using a SIL cartridge. These cartridges may be reversed- or straight-phase or a combination of both but are usually based upon microparticulate silica which may be modified in various ways. The material is prepacked into polypropylene syringes (e.g. Bond Elut, Analytchem.) or cartridges (Sep-Pak, Waters). For extraction of vitamins D, their analogues and metabolites, C18- or ODS-bonded silica cartridges are the method of choice. Sep-Pak C18 is the reversed-phase analogue of the silica cartridge and uses a bonded octadecasilane (C18) phase. It can also be used to fractionate plasma in the reverse manner to that described by Adams et al. (1981). Dabek et al. (1981), Turnbull et al. (1982) and Fraher et al. (1981) have developed methods for 25-OH-D_2 and 25-OH-D_3 using Sep-Pak C18 for both extraction and preliminary purification of the sample. Prior to use of the cartridge, protein must be removed and in the above methods, this has been effected usually by shaking with methyl cyanide (acetonitrile), acidified methanol or ammonium sulphate–ethanol. The method of Dabek et al. (1981) utilises the properties of methanol both as an extraction solvent and as a mobile phase. However, these procedures do not extract the parent compound, vitamin D_3. The method of Hollis and Frank (1985), using ammonium sulphate–ethanol, and that of Axelson (1985), using pentylamine, do remove vitamin D_3 from serum or plasma.

Further investigation into the utility of ODS-bonded silica cartridges has led to a procedure that has been described as "phase-switching" (Hollis, 1986). This procedure uses a concept by which solid-phase extraction and subsequent normal-phase separation of vitamin D metabolites can be achieved on a single cartridge. This study by Hollis (1986) demonstrated that the type of ODS-microsilica packing used, greatly affects the resolution achieved with respect to metabolites of vitamin D. This concept is displayed in Fig. 11.8. Use of a fully end-capped ODS-silica cartridge results in little resolution between 24,25-$(OH)_2D_3$ and 1α,25-$(OH)_2D_3$ (Fig. 11.8A). However, using a partially end-capped ODS-silica material (ODS-OH), excellent resolution of these two important vitamin D_3 metabolites is achieved (Fig. 11.8B) (Hollis and Kilbo, 1988), but it will be noted that by using ODS-OH cartridges, the resolution previously achieved between 25-OH-D_3 and 24,25-$(OH)_2D_3$ is lost. However, such a system has proved very useful in the purification of

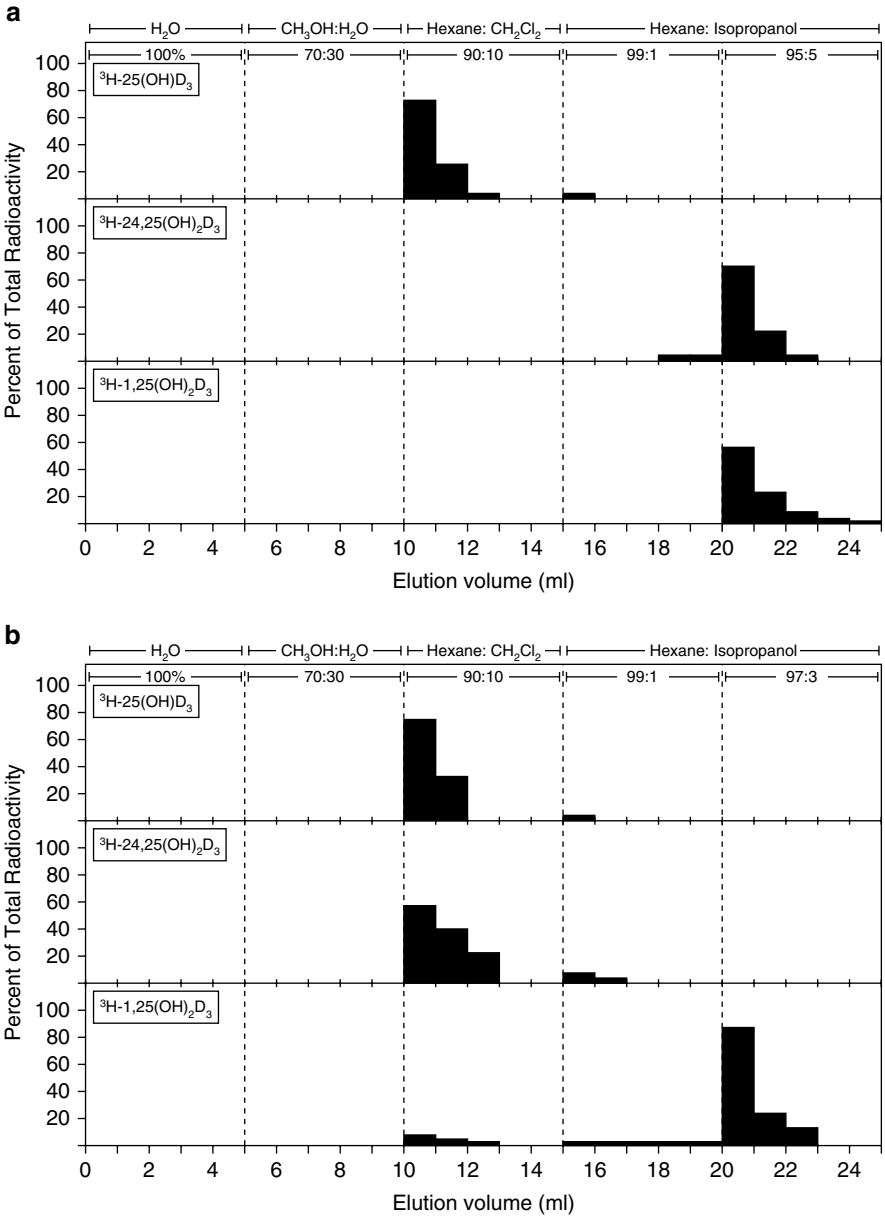


Fig. 11.8 Elution of radiolabelled vitamin D_3 metabolites from BOND ELUT cartridges. (**a**) ODS-BOND-ELUT, (**b**) ODS-BOND-ELUT-OH (from Hollis and Frank, 1985; Hollis, 1986; with permission)

1,25-$(OH)_2D_3$ prior to radioligand assay (Reinhardt et al., 1984) and is now available as part of a commercial assay (Diasorin, Ltd., Wokingham, Berks., UK). Other reversed-phase type silica cartridges (-NO_2) have been used to separate 25-OH-D_3

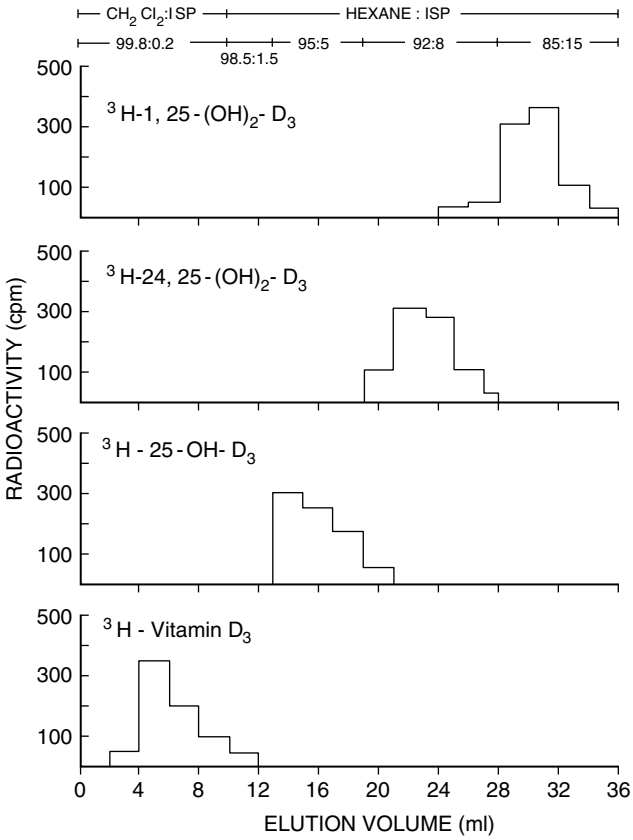


Fig. 11.9 Elution profiles of radiolabelled vitamin D_3 and its metabolites from a silica BOND ELUT cartridge. Each bar represents the amount of radioactivity in 2 mL eluent during batch elution with various mixtures of IPA in methylene dichloride or hexane (from Hollis, 1986; with permission)

and $1\alpha,25\text{-}(OH)_2D_3$ (Jongen et al., 1984b). Straight-phase cartridges can thus be used to provide a convenient and simple method for preliminary separation of metabolites into different polarity fractions (Fig. 11.9). Today, further analysis is mainly accomplished by the use of LC.

Sep-Pak SIL cartridges (Waters Associates, Milford, MA) use high-efficiency silica particles and offer minimal variability. These cartridges have been used by Hollis et al. (1981) to demonstrate the lack of vitamin D sulphate in human milk and by Adams et al. (1981) to fractionate classes of vitamin D metabolites in human plasma. Evaluations of the use of Sep-Pak SIL as a means of preliminary fractionation of vitamin D and its metabolites have been published (Adams et al., 1981; Redhwi et al., 1981). Hollis et al. (1981) demonstrated the use of these cartridges for the rapid separation of vitamin D and vitamin D sulphate. The plastic coating of Sep-Pak is made of virgin polyethylene with minimal or zero amounts of plasticisers. Early

versions of the Sep-Pak SIL were not completely devoid of UV-absorbing impurities and as a result numerous interfering peaks were introduced into the sample during "purification". Leaching may still occur from Sep-Paks with the use of chlorinated hydrocarbons. Bond Elut cartridges on the other hand are encased in surgical-grade polypropylene and leaching of plastics into the solvents has not been a problem. This issue takes on great importance when using subsequent sensitive analytical techniques such as LC or LC-MS. In our hands, the separation on a Sep-Pak cartridge is inferior to that achieved using the higher-quality microsilica supplied by Analytichem International as Bond Elut cartridges (Fig. 11.9).

Many other types of cartridge are now available and a wide variety is listed in the latest catalogues of manufacturers/suppliers. These are suitable for polar and non-polar extractions and cation and anion exchange. -CN cartridges are useful for the separation of metabolites containing oxo groups and Bond Elut NH_2 has, for example, been used for the separation of 25-OH-D_3 and 1α,25-$(OH)_2D_3$ (Kao and Heser, 1984), and 25-OH-D_3, 24,25-$(OH)_2D_3$ and 1α,25-$(OH)_2D_3$ (McGraw and Hug, 1990). These cartridges can also be useful for the removal of excess derivatising reagent and in two methods using fluorescent triazoline derivatives, Bond Elut PSA primary/secondary amine (Shimizu et al., 1992) and Baker NH_2 (Jordan et al., 1991) cartridges were used for just this purpose This does emphasise the point that it is important to evaluate cartridge performance carefully before adoption, as cartridges from different sources may have different characteristics.

'High-performance' TLC, using microparticulate silica has been described (Thierry-Palmer and Gray, 1983) and indeed TLC may well provide a satisfactory solution to many separatory problems in countries where LC is not available (e.g. Justova and Starka, 1981). TLC also has the advantage that it is capable of dealing with large numbers of samples by batch processing whereas LC is sequential. Areas on the TLC plate which contain the analyte of interest can be removed, the analyte eluted with solvent and subsequently analysed (e.g. Slominski et al., 2006). TLC procedures may in some cases attain the same resolution as LC but analyte elution may lead to the introduction of non-specific interference into the subsequent quantitation procedure (Slominski et al., 2006, ascribed interference in the NMR spectrum to contamination from the TLC support material). If in a particular application TLC does seem to offer advantages, the TLC material should be carefully examined to ensure that it does not interfere with the final quantitation step. The use of paper chromatography using modified Bush (1961) systems as a means of purification after ether extraction of plasma and prior to a calf thymus radioreceptor assay for 1α,25-$(OH)_2D$ has been described (van Hoof et al., 1993, 1998, 1999, 2001). It is, however, difficult to follow the arguments advanced for this choice and thus the use of paper chromatography for this purpose is not recommended.

An assay for 1α,25-$(OH)_2D_3$ used Extrelut-1 columns adding serum diluted with TRIS buffer, the eluate being eluted directly onto a Sep-Pak SIL column (Wildermuth et al., 1993). Most attention has been paid in the last 20 years to the extraction of vitamin D analytes from plasma or serum and when other biological matrices are under consideration, care must be taken not to assume that methods of efficient extraction from plasma will be applicable. Extraction from incubation

media, for example, may well be better effected by the use of solvent and the problem of extraction from milk or other foodstuffs may be even more difficult. The possible presence of vitamins D and their metabolites as esters of sulphuric or glucuronic acids or fatty acids has already been mentioned and these conjugates may require saponification/hydrolysis prior to extraction (Holick et al., 1992; Chen et al., 1993; Tanner et al., 1993). Such hydrolytic procedures, unless very carefully controlled, may give rise to the production of artefacts since the vitamin D structure is labile and subject to isomerisation and oxidation.

It must be emphasised that suitable precautions to avoid destruction must be taken throughout the extraction and pre-purification procedures and, for example, it is suggested that all glassware should be silanised by treatment with 1% dimethyldichlorosilane in toluene prior to use. This avoids adsorption and loss of vitamin D metabolites, particularly when solvents are removed. Vitamin D metabolites should not be stored in a dry state for long periods except under nitrogen and it is recommended that small volumes of solvent such as methanol are added if storage is necessary. Exclusion of ultraviolet light is also useful and use of amber glass or storage in the dark are also recommended.

Over the last 20 years, SPE has emerged as a common sample preparation technique prior to online LC-MS analysis. This methodology seeks to remove phospholipids, which may contribute to ion suppression or matrix effects in downstream LC-MS analyses, enrich analytes under investigation and act as a pre-purification step which ultimately enhances the lifetime of the analytical column. Oasis brand HLB (hydrophilic–lipophylic balanced) SPE cartridges manufactured by Waters are particularly suited to the extraction of vitamin D metabolites from a variety of matrices because of their specific composition of hydrophilic (*N*-vinylpyrrolidone) and lipophilic (divinylbenzene) monomers, in addition to a high degree of reproducible recovery. Typical applications have included the pre-purification of vitamins D metabolites from plasma, prior to LC-MS analysis (Higashi et al., 2001a, 2002a; Vogeser et al., 2004; Murao et al., 2005a). The availability of the HLB column chemistry in 96-well plate formats has opened up the possibility of automated high-throughput analyses, which may become an important component of assays of vitamins D and their metabolites (see flowchart depicted later in Fig. 11.27). Oasis HLB in cartridge form has already been used in an automated assay for 24,25-$(OH)_2D_3$ and 25-OH-D_3 (Mata-Granados et al., 2004). Supercritical fluid extraction of vitamins D_2 and D_3 using carbon dioxide has also been described (Gamiz-Gracia et al., 2000).

11.3.2.3 Immunoaffinity Columns

A successful approach to the clean and specific extraction of a steroid analyte from biological material was described more than 20 years ago in a method for the measurement of oestradiol (Gaskell and Brownsey, 1983). This procedure involved linking a specific oestradiol antibody to Sephacryl thus immobilising the antibody and producing an affinity column. The production of antibodies to vitamin D and its metabolites

has not been as successful as that for other steroid hormones and thus this approach has only found limited use in the vitamin D field in immunoassays for 25-OH-D_3 and 1α,25-$(OH)_2D_3$ (Kobayashi et al., 1993a, 1995, 1997a, b, c; Withold et al., 1995; Fraser et al., 1997). However, care must be exerted here as the purity of the immunoextraction depends on the cross-reactivity of the antibody used for extraction and other compounds may also be extracted and interfere in the subsequent analysis (e.g. 1α,25-$(OH)_2D_3$-26,23-lactone in an RIA using immunoextraction for 1α,25-$(OH)_2D_3$ – Hollis, 1995). As binding to the immunoabsorbent occurs in the aqueous phase, solvent extracts of the matrix must be re-dissolved in aqueous media (e.g. 10% ethanol in water) before application to the column containing the immunosorbent (Kobayashi et al., 1995). A commercially available immunoextraction system for 1α,25-$(OH)_2D_3$ in plasma de-lipidates with dextran sulphate and magnesium chloride before application to cartridges contain immobilised monoclonal antibody (Fraser et al., 1997). After washing, the bound analyte is eluted from the column. Ideally, the specificity of the antiserum used for extraction should be complementary to that of the antibody (or other binding protein) used in the saturation analysis. There appears to have been little further development in this area since 2000 but use of IAC remains a valuable means of extraction, provided satisfactory antibodies can be produced.

11.3.2.4 Internal Standards

When making any quantitative measurement of the concentration of a steroid, some form of internal standard is necessary to take account of the losses which occur during extraction and purification, and assays for vitamin D and its metabolites are no exception. The more purification steps there are in the analysis, the greater is the need for an internal standard. The choice of an internal standard must satisfy the same criteria used for any steroid assay, namely: the standard used must not occur in the matrix being analysed, it does not separate from the analyte (i.e. has similar structure to the analyte) during the extraction and purification process and it can be distinguished from the analyte in the final quantitation procedure. For assays involving some kind of saturation analysis, which includes immunoassay, the only satisfactory internal standard is the analyte itself or chemically related compound – radiolabelled, usually with tritium (e.g. Levan et al., 1994). This, like all internal standards, is added to the matrix at the start of the analytical procedure. The amount that is added in terms of mass should not interfere in the final quantitation step but there should be sufficient radioactivity to enable an accurate assessment of recovery to be made prior to saturation analysis. If the labelled compound used as an internal standard is the same as that used in the assay procedure, it may be sufficient simply to add extra radioactive counts immediately prior to assay if insufficient radioactivity has been recovered. It is of course important to try to ensure, as far as possible, that the internal standard, when added to the matrix, is distributed in the same way as the analyte. This is usually achieved by the addition of the standard, dissolved in a small volume of ethanol and incubation at 37°C for 1 h, which is normally sufficient to ensure that the standard is

distributed onto any binding protein, although again it may not always be true for vitamin D itself which is probably more tightly bound as part of the lipoproteins. A similar approach is usually adopted when assessing the recovery of added analyte as part of the method validation process. For 25-OHD, there are potential problems here, which need investigation (Carter et al., 2007).

For chromatographic assays using LC or GC, it is sufficient, though not always desirable, to add relatively large amounts of a chemical analogue of the analyte of interest (e.g. Shimizu et al., 1995; Shimada et al., 1997; Ishigai et al., 1998b) which is added in the same way as described for radiolabelled material. However, the amount of standard which is added, although considerably more than the amount used for radiolabelled standards, should not be vastly in excess of the amount of analyte expected. When measuring vitamin D_3 and its metabolites, it has been possible to use a combination of radiolabelled material for the pre-MS separation and vitamin D_2 as an internal standard for the GC (e.g. Seamark et al., 1980a), which is found in very small amounts in human body fluids in the UK. When measuring metabolites of vitamin D analogues, it may be possible to adopt a similar approach (e.g. Schroeder et al., 1994). LC assays, using UV detection as an end point, clearly require that the internal standard absorbs at the wavelength monitored for quantitation together with all the attributes described above. A recent LC-UV method for serum 25-OH-D (Lensmeyer et al., 2006a) uses laurophenone as an IS – no explanation for this choice seems to have been given and it is hard to justify, as the structures of the analytes and the internal standard are significantly different, although the retention time of the IS is appropriate. The fact that it seems to work is fortuitous. For the purposes of analyses using mass spectrometry, the ideal standard is a stable isotope(usually deuterium)-labelled analyte with at least three deuterium atoms in the molecule (e.g. Oftebro et al., 1988; Coldwell et al., 1990, 1995; Tomiyama et al., 1994), although internal standards with four, five or six deuterium atoms are often used (e.g. Kissmeyer and Sonne, 2001; Higashi et al., 2001a, b, 2002c; Maunsell et al., 2005; Murao et al., 2005b; Guo et al., 2006). It is also possible to use one deuteriated labelled metabolite as an internal standard for the measurement of another metabolite (i.e. $[^2H_6]$24,25-$(OH)_2D_3$ in the measurement of 25,26-$(OH)_2D_2$, as described in Coldwell et al., 1990). Murao et al. (2005a) have described an LC-MS-MS assay for 1α-OH-D_3 in rat plasma using $[^2H_4]$-1α-OH-D_3 as the internal standard. A recent assay for 25-OH-D_2 and 25-OH-D_3 (Saenger et al., 2006) used, as an internal standard, $[^2H_3]$-labelled Δ^9-tetrahydrocannabinol (THC). Deuterium labels are often inserted by acid-catalysed exchange and are therefore susceptible to loss under acid conditions. Tables 11.5 and 11.6 summarise (*inter alia*) the use of s isotope-labelled internal standards in a selection of GC-MS and LC-MS methods. Insertion of ^{13}C label in the steroid nucleus offers a more stable labelled internal standard but thus far no such $[^{13}C]$-labelled internal standard has been used in the analysis of vitamins D or their metabolites, although a mixed $[^{13}C,^2H_3]$-labelled 25-OH-D_3 was used as an internal standard in an LC-MS-MS procedure proposed as a candidate reference method for serum 25-OH-D_3 (Vogeser et al., 2004). Non-isotope labelled chemical analogues are still used as internal standards (e.g. 24-OH-D_4) as an IS in an LC-MS-MS assay for 25-OH-D_2

and 25-OH-D_3 – Higashi et al. (2001b) and 24-nor-OCT in an LC-MS assay for OCT – Ishigai et al. (1998b).

11.3.3 Separation by Liquid Chromatography

11.3.3.1 Introduction: Purification Prior to LC

One of the most common problems with LC is the presence of particulate matter in the samples for injection. If unchecked, this inevitably leads to clogging of the injector or, worse, the inlet frit of the column. Changing the inlet frit is a procedure to be avoided by an inexperienced chromatographer, since, unless great care is taken, disturbance of the column bed may occur and column efficiency reduced. Filtering of samples before injection is recommended. There are available for this purpose commercial filtration kits which use 0.45-μm filters made of solvent-resistant materials (e.g. Teflon or PTFE) and suction or centrifugation to facilitate solvent flow through the filter. Alternatively, samples can be transferred to 5 mL conical screw-capped vials (Reactivial – Pierce Chemical Co., Rockford, IL) and centrifuged at low speed (500 g for 5 min), although this is not always satisfactory. As an extra precaution against contamination of the analytical column, guard columns can be inserted in series between the injection loop and the column.

LC analysis of vitamins D and their metabolites is now the method of choice, and over the last 20 years tremendous improvements in LC instrumentation have been made – pump technology can now generate precise isocratic and gradient solvent systems allowing highly reproducible LC profiles. Automatic injection systems and the incorporation of solid-phase cartridges into these systems improve consistency of injection and no longer require pre-LC SPE extraction. Improvements have been effected in detection sensitivity and column packing technology, allowing detection of as little as one nanogram of a vitamin D metabolite under ideal UV detection conditions. Extra column peak broadening has been minimised by the use of eddy-free flow connectors and injectors, and the use of minimum dead-space tubing. Modern LC equipment is now marketed as integrated computer-controlled systems (e.g. the Alliance system marketed by Waters Corporation, MA 01757-3696, USA) that can easily be connected to a suitable MS or MS-MS system. Increasingly, LC-MS or LC-MS-MS systems are routinely used.

11.3.3.2 Columns

The objective of all good LC analyses is to maximise resolution R, which is defined by the equation:

$$R = \frac{1}{4}\underbrace{\left[\frac{\alpha - 1}{\alpha}\right]}_{\text{Selectivity}}\underbrace{\left[\sqrt{N}\right]}_{\text{Plates}}\underbrace{\left[\frac{k'}{1+k'}\right]}_{\text{Retention}}$$

The value of *N* is determined by the size and nature of the particles used to make the column and by the quality of the packing procedures. Most columns used for analysis of vitamins D and metabolites use 3–10 μm particle sizes, which should give values of *N* in excess of 10,000 per column. The selectivity factor (α) and capacity factor (κ′) can be altered by changing the chemical characteristics and strength of the solvent (in straight phase = polarity), respectively. Examples of the effect of increasing R by changing α and κ′ are given below.

The nature of the analysis to be carried out and the design of the LC system may be important considerations in the selection of the type and size of the column to be used, as they may influence *N*. Some practical suggestions in this context are:

1. Use a column size appropriate to the volume of injection, weight of solute, etc.
2. Use small volume injections since these are theoretically best for sharp peaks (i.e. large *N*). However, practical considerations of sample handling may dictate a compromise to a larger injection volume.
3. Use a mobile phase as the sample solvent to avoid peak artefacts.
4. Standardise injection volumes for more reproducible results, since changes in injection volume will influence retention time and, hence, peak height/area ratio. If peak areas are used for quantitation instead of peak heights, changes in injection volume should have little effect.

While a 25 × 0.21 cm microbore or ultrabore LC column may be ideal for analysis of repeated 2–5 mL containing microgram quantities of a synthetic vitamin D metabolite using UV detection or a MS detector, it is not ideal for the analysis of 25-OH-D_3 in human plasma. Here, the samples may still contain considerable amounts of lipid and a column with high lipid-loading capacity is required. Some of the newer 3-μm particle columns (8 × 0.63 cm Zorbax) are ideal for the LC of biological samples since they are very tolerant of large injection volumes (100–200 μL) and large lipid loads (i.e. whole extract from 2–5 mL of plasma) and yet still give minimal sample loss and effects of extra-column band broadening are small.

There are basically two different types of column packing material: straight-phase (SIL-based material) and reversed-phase (SIL with bonded hydrophobic material – e.g. ODS (octadecasilane) C18). Several microparticulate (3–6 μm) spherical silicas are available (Lichrospher, Zorbax-SIL and Spherisil). The advantage of these spherical particles is that they pack more uniformly than if they were irregularly shaped and thus have less tendency to settle with time and pressure.

Mathews et al. (1974) were the first to report reversed-phase LC of vitamins D metabolites using the pellicular packing ODS-Permaphase and a gradient of methanol and water. With the advent of the microparticulate bonded phases, such as μ-Bondapak C18 and Zorbax-ODS, analysts have successfully used these reversed-phase systems for many vitamin D compounds but the use of such systems has not been widely adopted for LC separations other than for vitamins D_2 and D_3. This has, however, changed with the advent of LC-MS systems, which require the use of reversed-phase solvents for ionisation (*vide infra*) and some loss of chromatographic resolution has been sacrificed because of improved detector resolution.

One application of ODS cartridges and LC columns is during the purification of calcitroic acid from tissue extracts. Here, the ionisation properties of the weak acid group can be minimised and the molecule retarded by its interaction with the C18 hydrophobic surface. An example of this interaction during sample preparation and quantitation by LC has been described (Makin et al., 1989). Mobile phases for calcitroic acid are rich in water and contain a small percentage of acetic acid or other acid to lower pH (acetonitrile/water/gla. acetic acid (40:60:1)). Figure 11.10 shows an example of reversed-phase LC using ODS for the resolution of

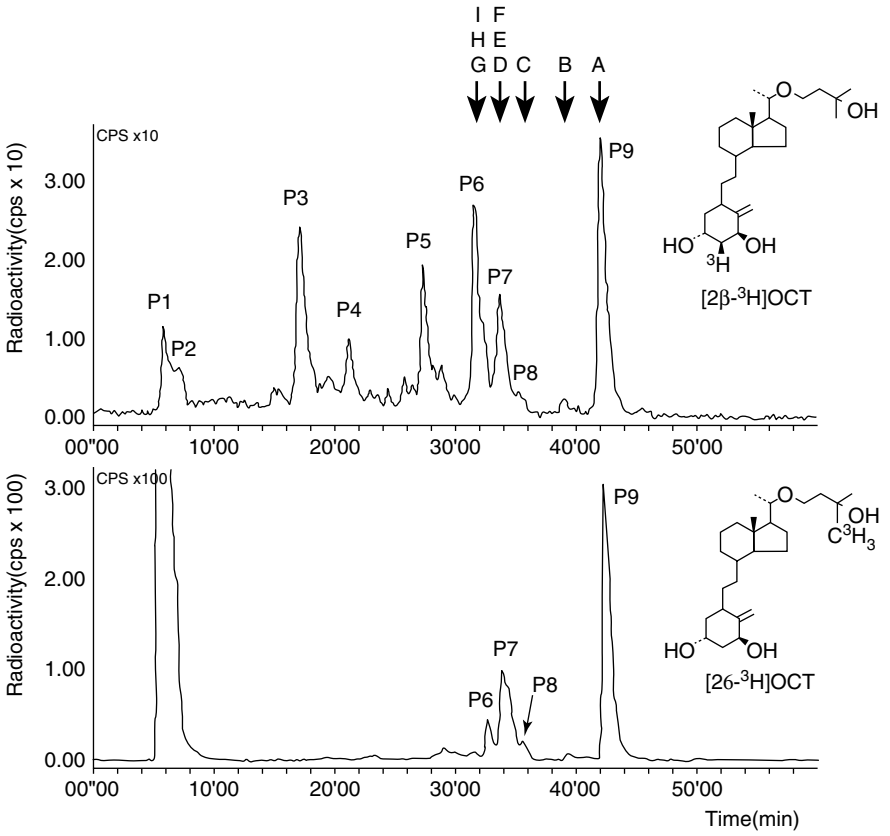


Fig. 11.10 LC-radioactivity detection profile of plasma metabolites of [^{3}H]OCT (22-oxa-1α,25-$(OH)_2D_3$) after administration to rats. [^{3}H]OCT was labelled at two different positions, at C2β (*upper panel*) and at C26 (*lower panel*) as illustrated in the formulae given and were administered iv at a dose of 10 mg/Kg to rats. *Arrows* (A–I) indicate the retention times of synthetic standards in the same system (A – OCT, B – pre-OCT, C – 24-oxo-OCT, D – 20-oxo-hexanor-OCT, E – 24*S*-OH-OCT, F – 24*R*-OH-OCT, G – 20*S*-OH-hexanor-OCT, H – (25*R*)-26-OH-OCT, I – (25*S*)-26-OH-OCT). LC was carried out under the following conditions: column – YMC-Pak ODS-A A-313 (6 × 250 mm), mobile phase – (A) THF–H_2O (1:9, v/v), (B)–THF–CH_3OH–H_2O (2:1:2, v/v/v) – (B) 0% at 0 min., 75% at 20–50 min, 100% at 50.1 min. Flow rate 1 mL/min. Radioactivity detection (counts/s) (from Ishigai et al., 1998a; with permission)

metabolites of 22-oxacalcitriol (OCT) (Ishigai et al., 1998a), illustrating the good resolution of the more polar metabolites (P1-P%) and the poor resolution of the less polar compounds (P6–P9). These less polar peaks can more easily be resolved by straight-phase LC (Masuda et al., 1996) but of course this lack of resolution in the reversed-phase systems required for ionisation in LC-MS systems can largely be overcome in an LC-MS system as the mass spectrometer has greatly enhanced detection specificity.

There has been limited use of other packings. Straight-phase bonded packings offer excellent recoveries but often the solvent strength has to be weakened significantly in comparison to that for silica in order to conserve adequate retention. μBondapak-CN and Zorbax-CN have been used with some success (Rosenthal et al., 1980) for the separation of hydroxylated metabolites of vitamins D from those containing aldehyde or oxo groups. One good example of this is the separation of 25-OH-D_3-23,26-lactone from 24,25-$(OH)_2D_3$ referred to above. Zorbax-CN can be used to provide baseline resolution, the lactone being strongly retarded by this packing and emerging much after the 24,25-$(OH)_2D_3$ (Cunningham et al., 1990); contrast this with other means of resolution – methylene chloride:isopropanol mixtures where the lactone co-migrates with 25-OH-D_3. The -CN packing has proved to be very useful in the identification of a number of oxo compounds found on the pathway from 1α,25-$(OH)_2D_3$ to calcitroic acid (Lohnes and Jones, 1987), in the purification of metabolites of 25-OH-DHT_3 (Jones et al., 1988) and in the resolution of metabolites of a synthetic analogue, KH 1060 (Dilworth et al., 1997). The reversed-phase C18 column (3.3 × 0.46 cm) normally used in an LC-MS assay for serum 25-OH-D was replaced with a 5-dinitrobenzoyl-(*R*)-phenylglycine column (25× 0.46 cm) in order to resolve the 3-epimer of 25-OH-D_3 present in serum samples from infants (Singh et al., 2006). Although the method for 25-OH-D_3 described by Lensmeyer et al. (2006a), which uses a (25 × 0.46 cm) 5-μm Cyanopropyl column (at 50°C) has been criticised (Schmidt, 2006) for its apparent failure to remove this 3-epimer interference, the authors have however published LC chromatograms illustrating the separation of 25-OH-D_3 (though not 25-OH-D_2) and the 3-epimer (Lensmeyer et al., 2006b) confirming the original publication (Lensmeyer et al., 2006a).

11.3.3.3 Eluting Solvents

Isopropanol–hexane mixtures provide good solubility for the vitamins D and their metabolites, and have been widely applied in straight-phase LC systems for the resolution of a variety of compounds ranging in polarity from photo-irradiation mixtures of vitamins D (see Fig. 11.3) to 1α,25-$(OH)_2D_3$. Isocratic mixtures of 2.5% isopropanol in hexane separate vitamins D_2 and D_3 from 24-OH-D_2, 24-OH-D_3 and 25-OH-D_3 (see Fig. 11.11). Isopropanol/hexane (10:90) provides resolution of most of the known metabolites of vitamin D_3 in a single chromatographic run (see Fig. 11.12) and can be useful for vitamin D_2 metabolites. These early SP separations can now be achieved on a single 25 × 0.46 cm or 25 × 0.6 cm columns

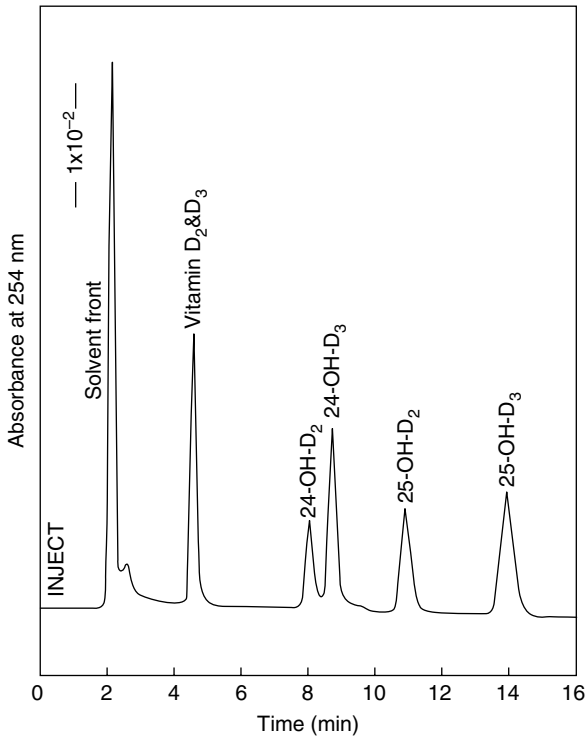


Fig. 11.11 LC separation of vitamins D_2 and D_3, 24-OH-D_2, 24-OH-D_3, 25-OH-D_2 and 25-OH-D_3, on Zorbax SIL. Solvent system was 2.5% IPA in Hx (from Jones and DeLuca, 1975; with permission)

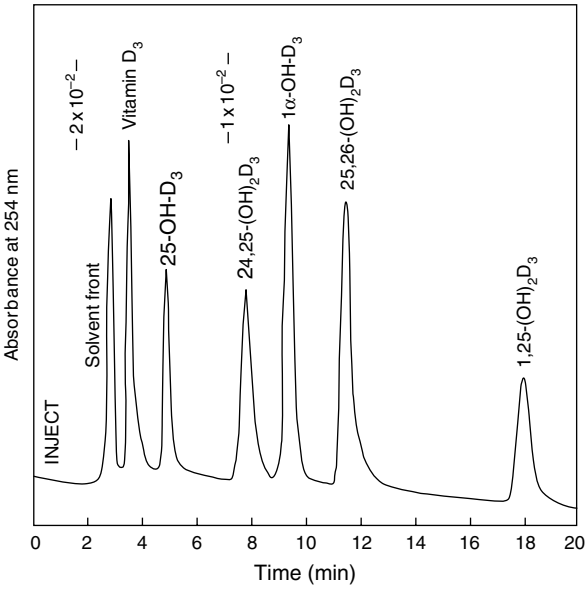


Fig. 11.12 LC separation of vitamin D_3 and its metabolites on Zorbax SIL. Solvent system was 10% IPA in Hx (from Jones and DeLuca, 1975; with permission)

using similar solvent systems. The use of ternary solvent systems, incorporating methanol into the standard isopropanol–hexane system (Jones, 1980) greatly minimises peak tailing, particular with 24*R*,25-$(OH)_2D_3$. Figure 11.13 shows the results of using a hexane/isopropanol/methanol (87:10:3) solvent mixture on the separation of 25-OH-D_3, 24*R*,25-$(OH)_2D_3$ and 1α,25-$(OH)_2D_3$. The polarity of the eluent can be reduced while keeping the proportion of isopropanol–methanol the same.

Ikekawa and Koizumi (1976) described a gradient solvent system (0.2–6% methanol in methylene chloride) to resolve a mixture of dihydroxy metabolites on Zorbax-SIL (25 × 0.21 cm). This solvent system gives a higher theoretical plate number (N) than does the isopropanol–hexane–methanol mixture under otherwise identical conditions. However, the low viscosity of methylene chloride–methanol mixtures can lead to bubble problems with some solvent delivery systems. Replacing some of the methylene chloride with hexane and using a hexane/methylene chloride/methanol (90:10:10) mixture overcomes the viscosity problem but may lead to poor miscibility and difficulties with sample solubility. The ternary hexane–methylene chloride–methanol system (Jones, 1980) beautifully separates metabolites of vitamins D_2 and D_3 but not the vitamins themselves.

Figure 11.14 illustrates the influence of small changes in solvent composition (α) on the resolution of 1α,25-$(OH)_2D_2$ and 1α,25-$(OH)_2D_3$ using the same SP column (Zorbax-SIL, 25 × 0.62 cm) while keeping the retention time approximately the same. One further solvent system that should be mentioned here is a methylene chloride/isopropanol (96.5:3.5) mixture which separates 25-OH-D_3-26,23-lactone and 24,25-$(OH)_2D_3$ (Horst, 1979). The lactone runs with a retention time almost identical to 24,25-$(OH)_2D_3$ in isopropanol–hexane mixtures but elutes with 25-OH-D_3 in a solvent rich in methylene chloride.

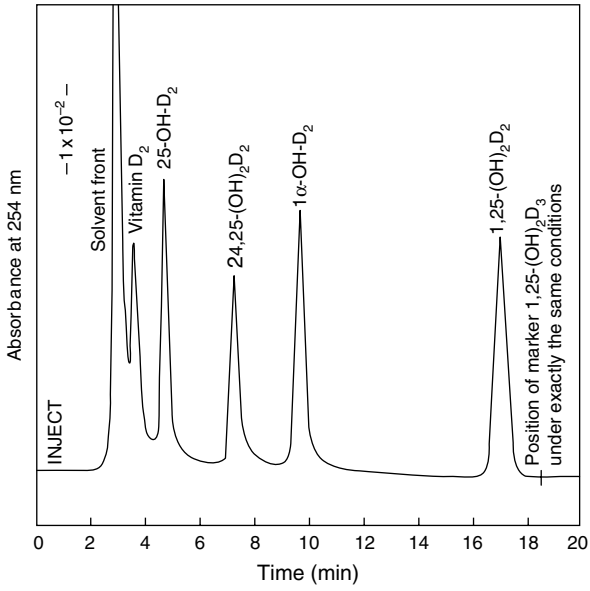


Fig. 11.13 LC separation of 25-OH-D_3, 24(*R*),25-$(OH)_2D_3$, and 1α,25-$(OH)_2D_3$ on Zorbax-SIL. Solvent system was Hx/IPA/MeOH (87:10:3) (from Jones, 1980; with permission)

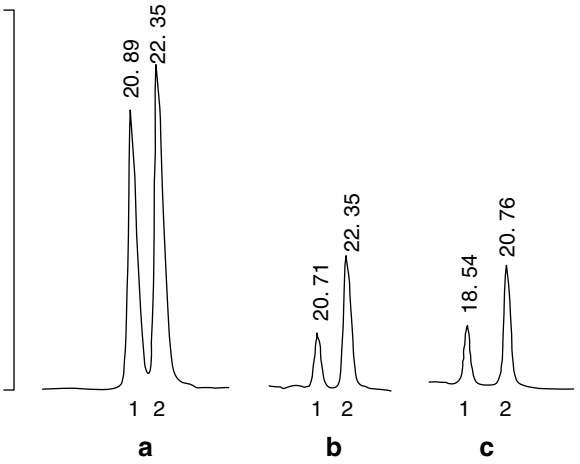


Fig. 11.14 LC separation of 1α,25-$(OH)_2D_2$ (peak 1) and 1α,25$(OH)_2$D3 (peak 2) by changing solvent selectivity (α). Column was Zorbax-SIL and solvent systems were (a) Hx/IPA (85:15), (b) Hx/IPA/MeOH (87:10:3) and (**c**) Hx/EtOH/$CHCl_3$ (80:10:10). Retention times in minutes are given above each peak (from Jones, 1980; with permission)

As increasing use is made of the mass spectrometer as a detector and tandem LC-MS systems become more widely used, attention has necessarily shifted to the use of RP LC systems, which use the water-based (methanol–water or acetonitrile–water) solvents required for ionisation to occur in the interface between the LC column and the MS. Addition of other components to the solvent mixture can enhance the ionisation, as can pre-column derivatisation, and thus careful consideration must be given to solvent composition when using these LC-MS systems.

Improvements in the separation of other epimeric steroids have been reported by including cyclodextrins in the mobile phase and this has been applied to the separation of substituted triazoline adducts (6α- and 6β-) of pro-vitamin D sulphate and glucuronide using methyl-β-cyclodextrin (Shimada et al., 1994). Separation of the 3-epimers of 24,25-$(OH)_2D_3$ formed *in vitro* by 3α- and 3β-hydroxysteroid dehydrogenases from *Pseudomonas testosteronii* was effected by the use of inclusion RP-chromatography, incorporating γ-cyclodextrin (4 mM) in the eluting solvent (MeCN/water 11:9). Similar separation was achieved using a YMC chiral column γ-CD (Higashi et al., 2004).

11.3.4 Analysis Using LC Detection and/or Quantitation

11.3.4.1 Ultraviolet

LC separations have usually been monitored by the use of ultraviolet detectors since all vitamin D_3 metabolites have an absorption maximum of 264 nm (see Fig. 11.3) with molar extinction coefficients in the region of 18,000. This detection is not necessarily highly specific since many other non-vitamin D compounds also absorb at this wavelength. Care must be taken therefore when utilising UV absorption after LC separation as a means of quantitation as an uncritical reliance

upon LC with UV absorption as a method of analysis may still lead to erroneous results, presumably because of the presence of non-specific interference. LC methods may well provide high-quality separation between vitamin D metabolites but this does not necessarily mean that other UV absorbing compounds are removed from the analyte of interest. The advent of photodiode array detection, linked to LC separation, has greatly improved the ability to detect the presence of non-vitamin D interfering compounds. In addition, monitoring the UV spectrum of eluting compounds can be of considerable value when studying the metabolism of vitamin D analogues with specific and/or characteristic UV spectra (Jones et al., 1988). Figure 11.15 shows the use of the photodiode array detector to monitor the separation of metabolites of 24,25-$(OH)_2D_3$ formed during *in vitro* kidney perfusion. The presence of metabolites can be immediately detected because they all possess the characteristic UV absorption spectra of vitamin D metabolites (see Fig. 11.3b). Figure 11.16 illustrates the value of photodiode array detection in ascertaining the purity of what appears to be a single LC peak. Contour plots (see Fig. 11.17) can also be obtained which are extremely valuable in the assessment of the homogeneity of the emerging peak. LC systems can of course be automated and can be programmed to inject automatically and, when connected to a fraction collector, collect peaks at pre-programmed retention times. Reliance on retention times during analysis of large numbers of samples is dangerous as slight changes in retention times do occur unless careful control of temperature of the column and consistency of the eluting solvent is maintained – even so, retention times can be influenced by the presence of peaks with similar retention times and by the presence of large amounts of lipid or other extraneous material at the injection site.

The normal plasma concentration of 25-OH-D_3 (around 20 ng/mL, 50nmol/L) is sufficiently high to enable it to be visualised by UV detectors attached to the end of LC columns and there have been methods devised using LC with UV detection for the measurement of this metabolite of vitamin D using relatively small amounts of plasma (reviewed by Porteous et al., 1987 ; Jones et al., 1992). Other metabolites, with the possible exception of vitamin D itself, do not circulate in sufficient concentration to be susceptible to measurement in this way. However, a note of caution should be sounded at this point since the measurement of 25-OH-D using a single straight-phase separation followed by UV absorption at the maximum (264 nm) does not always provide sufficient specificity and it may be necessary to utilise a reversed-phase LC separation in addition (e.g. Jones, 1978). An alternative approach, which provides greatly enhanced specificity and improved sensitivity, is to interpose a simple chemical reaction – treatment of vitamin D and its non-1α-hydroxylated metabolites, dissolved in a suitable chlorinated solvent, with hydrochloric acid gas leads to a quantitative isomerisation to the isotachysterol isomers (Seamark et al., 1980b, c; Agarwal, 1990). These isomers have nearly twice the extinction coefficient at the maximum (251 nm) than do the vitamin D metabolites at their maximum (264 nm).

Electrochemical methods have been used in the past as a detection system for LC of vitamins D and their metabolites and some methods were listed in Jones and

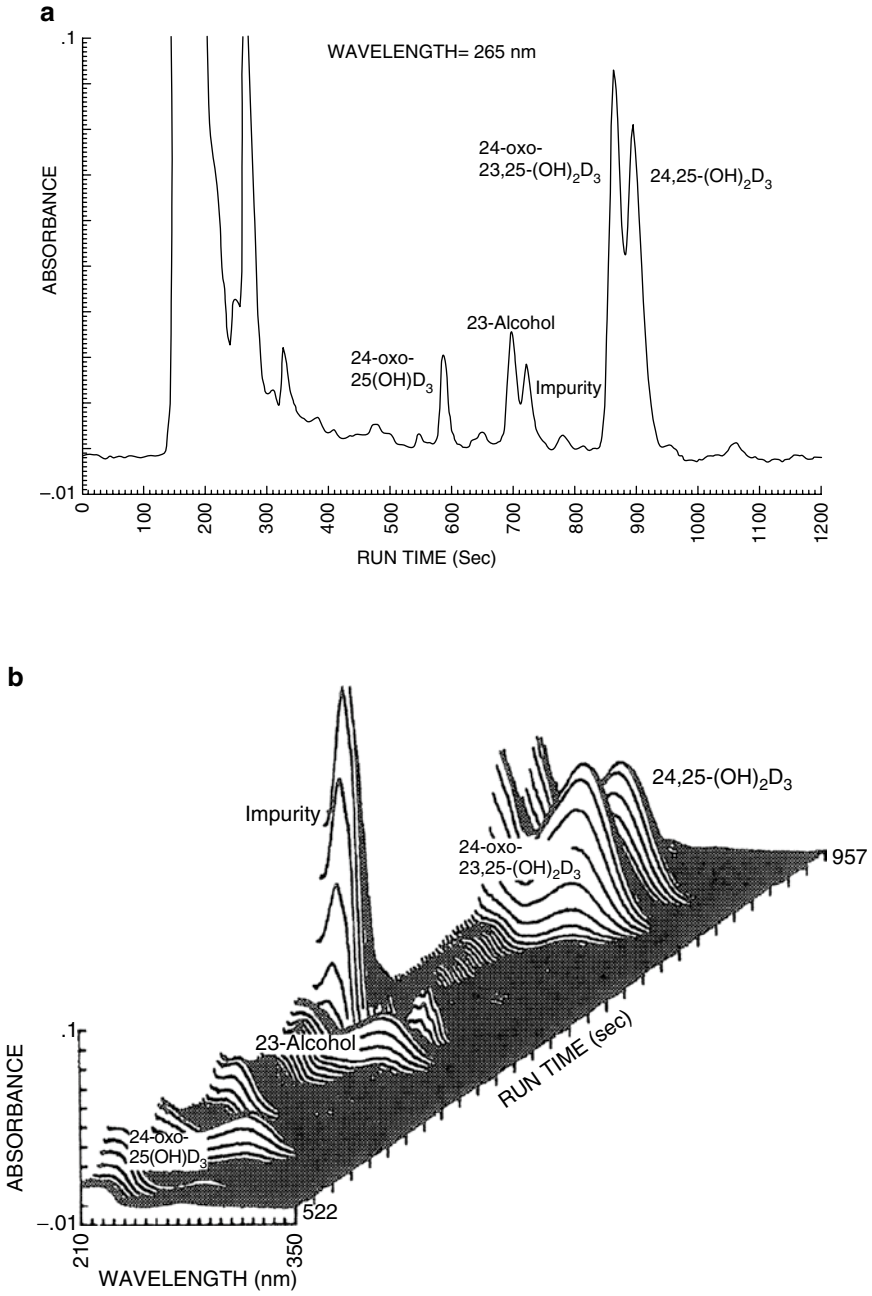


Fig. 11.15 LC separation of metabolites of 24,25-$(OH)_2D_3$ formed in the perfused rat kidney using a photodiode array detector to identify the metabolites. The *upper* (a) section shows the UV absorbance at 265 nm against retention time, whereas the *lower* (b) section attempts to give a 3D picture, monitoring wavelength (210–350 nm) against retention time and absorbance, thus allowing identification of LC peaks with the classical UV spectrum. The impurity, which absorbs strongly at 225 nm, does not have a vitamin D-like spectrum (from Jones et al., 1984; with permission)

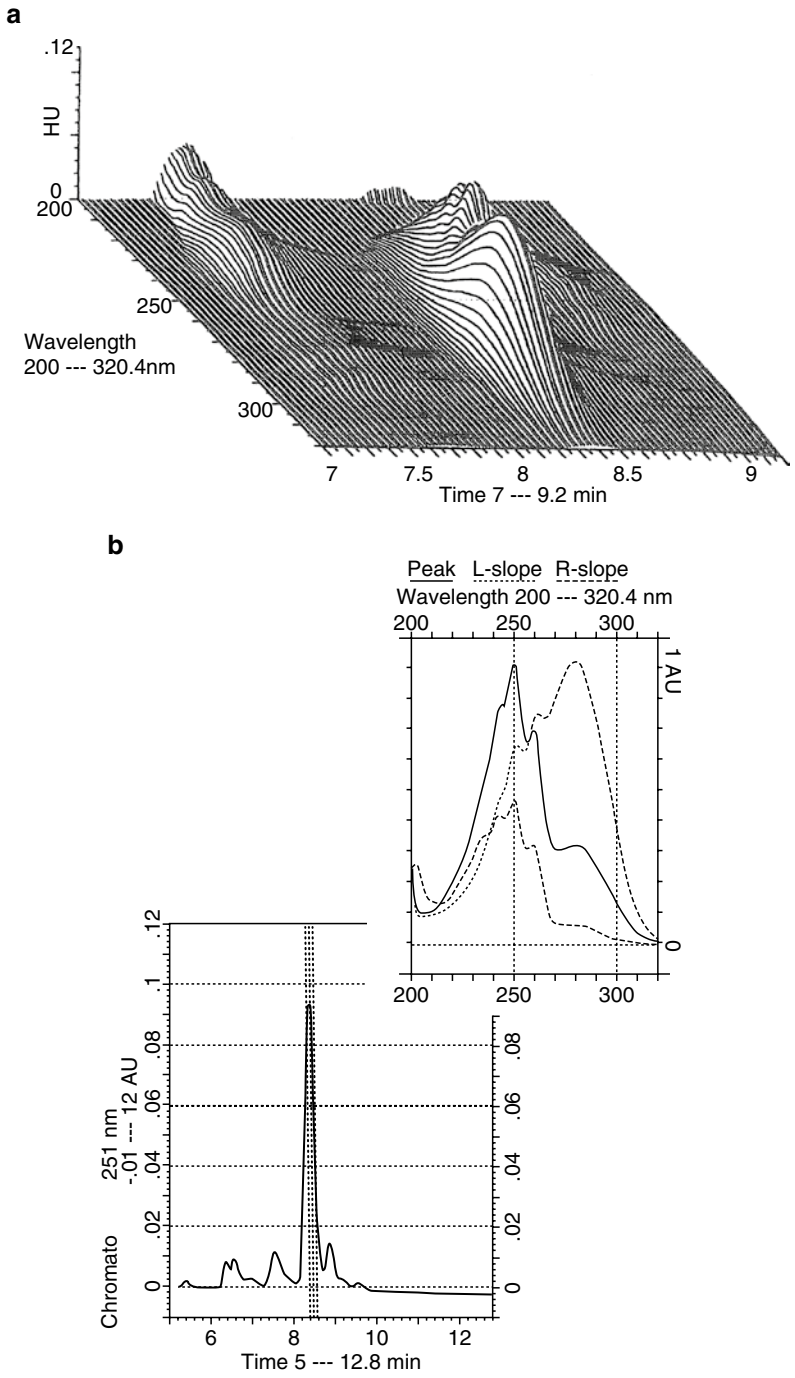


Fig. 11.16 Use of photodiode array detection after LC separation of metabolites of 25-OH-DHT$_3$ formed in HD-11 cells. The 3D picture illustrated in (**a**) suggests that what appears to be a single peak is in fact composed of two unresolved compounds with very different spectra. This can more easily be appreciated in (**b**), which shows the HPLC peak, monitored at 225 nm, and the UV spectra obtained from the leading edge of the peak, the apex and the trailing edge (from Makin et al., 1995; with permission)

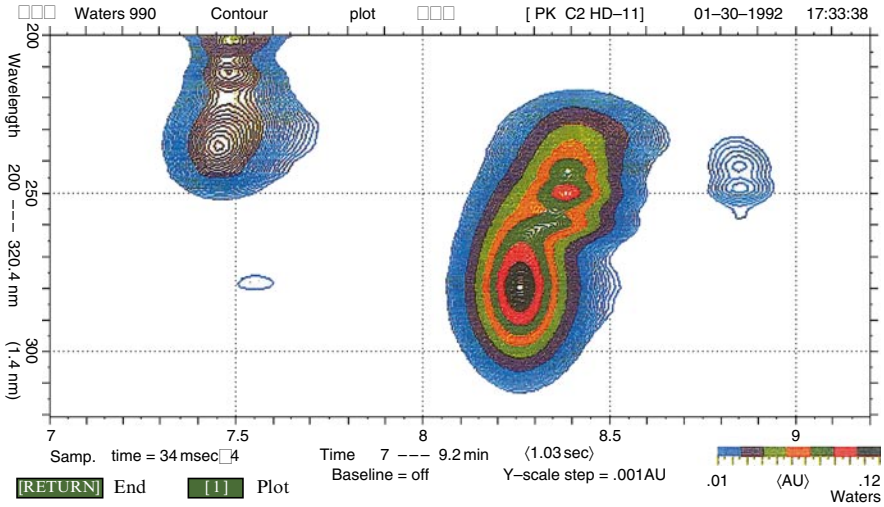


Fig. 11.17 Photodiode array – contour plot. This shows a contour plot of the same spectrum as that in Fig. 11.16(b), from which it can be seen clearly that the major peak, eluting between 8.1 and 8.6 min is composed of two components, one with a UV maximum at around 280 nm on the leading edge and another with three UV maxima on the trailing edge. In contrast, smaller, more symmetrical and uncontaminated peaks are seen at 7.47 and 8.85 min (from Makin et al., 1995; with permission)

Makin (2000). Since the first edition of the present book, only one method applied to the measurement of 25-OH-D_3 and 24,25-$(OH)_2D_3$ in human plasma has been published (Masuda et al., 1997) and there appear to be no publications on this since then. LC with electrochemical detection has, however, been applied to the measurement of vitamin D_3 in fish (Ostermeyer and Schmidt, 2006) and in dairy products, infant formulae and fortified milk (Perales et al., 2005a, b).

The specificity of some LC methods has been assessed by the collection of the peak of interest and demonstration that it contains a mass spectrum which accords with that of the expected compound. This, however, may lead to a false sense of security and it has been shown (Holmberg et al., 1984) that such procedures do not necessarily provide a measure of specificity. The use of MS for the confirmation of peak identity following UV detection by single-wavelength or multi-wavelength detectors has been extended in the twenty-first century with the emergence of $LC\text{-}MS^{(n)}$ technology where peaks can be assayed in tandem by both UV and mass detectors. LC-UV and LC-fluorescence methods up to 2001 have been reviewed by Shimada et al. (2001). While modern developments in LC methods for vitamins D metabolite assays tend to use LC-MS or LC-MS-MS, LC-UV methods for routine use continue to be developed for 25-OH-D_2 and 25-OH-D_3 at least (Lensmeyer et al., 2006a), with good correlation with the results from LC-MS-MS, CPBA and RIA methods.

11.3.4.2 Radioactivity

Several manufacturers (e.g. Wallac-Berthold, Beckman, Packard) have developed sensitive radioactivity monitors to measure radiolabelled compounds separated by LC systems by passing the eluent through a flow cell placed between two photomultiplier tubes. Early models were bulky and used a spiral Teflon tube between two plates as a flow cell with a pulsatile low-pressure pump and a post-LC column mixer to mix eluent with scintillation fluid. The flow cell had low tritium counting efficiency and residence time was short. The system was unreliable since it tended to get blocked when incompatible scintillation fluid/eluent solvents gelled in the flow cell. Modern systems use standard LC pumps to add to the eluent an application-specific scintillation cocktail customised to the requirements of LC (clear, low viscosity and flow optimised) which reduced detector instability. These new generation detectors also used smoothing algorithms incorporated into the computer-based software, which increased sensitivity and produced a very sensitive and reliable detector. An example of the use of such a detector is illustrated in Fig. 11.18.

Although most instruments use the scintillation fluid-based mixer approach, some instruments utilise 300-μL flow cells containing a solid glass yttrium salt-coated scintillator with a tritium efficiency of 2–5%. This of course obviates the need for liquid scintillant and also reduces peak broadening. Eventually absorption of lipids onto the scintillator requires replacement of the flow cell. ^{14}C efficiencies are in the region of around 80%.

11.3.4.3 Fluorescence

In order to enhance the sensitivity of detection of peaks emerging from an LC column, other forms of detection have been investigated and one, fluorescence detection, seems to provide significant improvements in sensitivity. Vitamins D and their metabolites lack native fluorescence and in order to utilise this methodology some pre-column derivatisation is required. A number of methods for 25-OH-Ds utilising the formation of fluorescent triazoline adducts have been described. These adducts provide a degree of specificity since they recognise the conjugated *cis*-triene structure of vitamin D and form a bridge across the 6–19 carbons (see Fig. 11.19). The value of substituted 1,2,4-triazoline-3,5-dione (TAD) as a reagent for the protection of the vitamin D *cis*-triene structure during chemical synthesis or modification has been known for many years (Aberhart and Hsu, 1976). By linking TAD to fluorescent or UV absorbing moieties, adducts can be formed with vitamin D and its metabolites which can be used to enhance the sensitivity and specificity of detection after LC separation. TAD is not the only compound which can form such adducts with vitamin D but a study of a number of such dienophilic compounds showed that phenyl-4-TAD was the reagent which most readily formed the appropriate adduct (Vreeken et al., 1993). The development and synthesis of a number of derivatives of TAD and the nature of their reaction with vitamin D and its metabolites

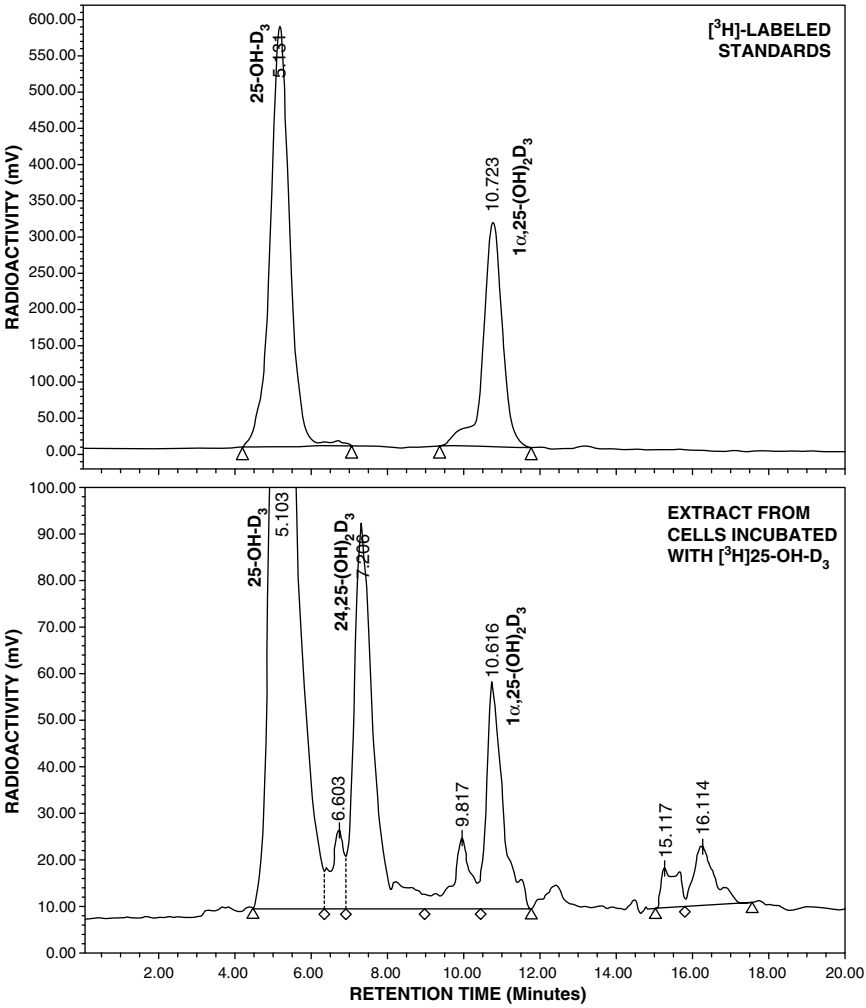
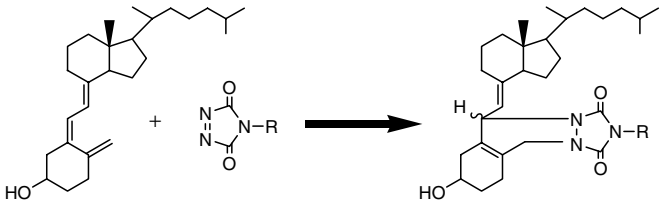


Fig. 11.18 Use of an in-line radioactivity detector (Wallac-Berthold) to detect 3H-labelled metabolites of [^{3}H]25-OH-D$_3$ produced in the human lung cancer cell line SW900

have been the subject of a number of investigations (Shimada et al., 1991; Shimizu et al., 1991, 1993; Vreeken et al., 1993; Wilson et al., 1993). Two methods for the measurement of 25-OH-D$_3$ using LC with fluorescence detection have been published, both procedures using pre-column formation of fluorescent adducts with derivatives of TAD. 25-OH-D$_3$ could be detected in serum at a concentration of 0.25 nmol/L (Jordan et al., 1991) using as a derivatising agent 3(1-pyrenyl)propyl-4-TAD. The second method (Shimizu et al., 1992), preliminary details of which were published in 1991 (Shimizu and Yamada, 1991), used DMEQ-4-TAD (see Fig. 11.19) and assayed 25-OH-D$_3$ and 24,25-(OH)$_2$D$_3$ using 0.2 and 2 mL plasma,

respectively. Both these methods require extraction, use of Sep-Pak SIL or C_{18} cartridges, formation of the appropriate adduct and reversed-phase LC. Excess reagent was removed prior to LC using Baker NH_2 or Bond Elut PSA cartridges. This method has also been used for the fluorimetric measurement of $1\alpha,25\text{-}(OH)_2D_3$ in human plasma (Shimizu et al., 1997). The method of Shimizu et al. (1992) interpolated a extra straight-phase LC separation after extraction and before derivatisation, a step which was not found to be necessary by Jordan et al. (1991). TAD



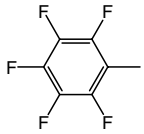
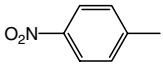
R group	*Usage*	*Reference*
 Phenyl (P-TAD)	Static/dynamic FAB LC-MS-thermospray	Yeung et al. (1992)
	Characterization of metabolites of $1,25(OH_2)D_3$-16-ene	Yeung et al. (1993)
	Structural studies on EB1089	Yeung et al. (1995)
	Detection of $24,25(OH_2)D_3$ glucuronides & $25\text{-}OHD_3$-sulfate.	Weiskopf et al. (2001)
	$25\text{-}OHD_3$ in saliva	Higashi et al. (1999)
	D metabolites in serum	Higashi et al. (2007)
 Pentafluorophenyl	LC-MS using particle beam (-) ionization for Ro24-2090	Aronov et al. (2008)
		Wang et al. (1996)
 4-nitrophenyl (NP-TAD)	LC-APCI(-) of $25\text{-}OHD_3$	Higashi et al. (2003)

Fig. 11.19 (continued on next page)

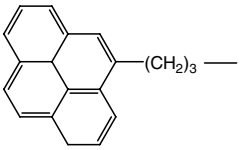
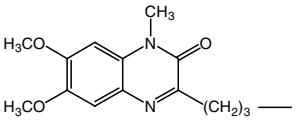
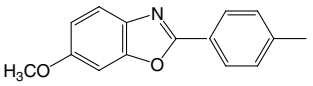
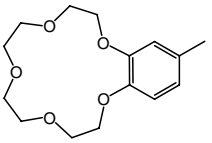
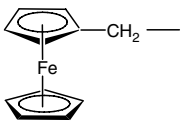
	LC-fluorescence of 25-OHD_3	Jordan et al. (1991)
3-(1-pyrenyl)propyl		
	LC-fluorescence and LC-MS-MS of vitamin D_3 metabolites.	Shimizu and Yamada (1991) Shimizu et al (1993)
2-(6,7-dimethoxy-4-methyl)-3,4-dihydroxyquinoxalinyl)ethyl (DMEQ-TAD)	Characterisation of 25-OHD_3 in plasma & monoglucuronides of D_3 & 25-OHD_3 in rat bile.	Shimizu et al. (1992) Shimizu et al. (1997) Wang et al. (1997) Higashi et al. (2001b)
	1α-OHD_3 in plasma	Higashi et al. (2001b)
	Urine metabolites of D_3	Higashi et al. (2001a) Shimada et al. (1995) & (1996) Higashi (2006)
	Vitamins D_2 & D_3 & 25-OHD_2 & 25-OHD_3 in breast milk	Kamao et al. (2007)
	LC-fluorescence of 7-dehydrocholesterol,	Shimada & Mizuguchi (1992)
4-[4-(6-methoxy-benzoxazolyl)-phenyl] (MBO-TAD)	LC of vitamin D_3 metabs. & conjugates	Shimada et al. (1994)
	LC-MS-MS of $24,25(OH)_2D_3$ in plasma	Higashi et al. (2001a)
	LC-ESI-MS	Wilson et al. (1993)
Benzo-15-crown-5-		
	LC-ESI-MS-MS of 1α-OHD_3	Murao et al. (2005a)
Ferrocenylmethyl (FM-TAD)		

Fig. 11.19 Examples of derivatives of 1,2,4-triazoline-3,5-dione (TAD) used in the measurement of vitamins D, analogues and metabolites

adducts can be formed on either side of the vitamin D molecule and thus the hydrogen on C6 can be either α or β. The two isomers are separated in the reversed-phase LC system used by Shimizu et al. (1992) but not in the system used by Jordan et al. (1991). Details of the formation of TAD adducts with vitamins D metabolites and analogs and the formulae of some of the TAD derivatives used are given in Fig. 11.19. LC systems for the separation of TAD adducts of pro-vitamin D, vitamin D and 25-hydroxyvitamin D_3 and their glucuronide and sulphate conjugates have been described (Shimada et al., 1994).

11.3.5 Analysis Using Saturation Analysis and Immunoassay

The use of UV absorption and fluorescence detection attached to the end of an LC column for quantitation has already been described above. LC-MS and GC-MS system are discussed below. While GC is seldom used for quantitative measurement of vitamins D and their metabolites today and is still not suitable for routine use, LC and LC methods are increasingly finding their way into routine laboratories, perhaps because of the difficulties encountered in getting consistent analytical results from simpler binding assays (DeLuca, 2004). However, the majority of the routine assays for vitamin D metabolites still rely on some form of competitive protein binding. These assays for vitamin D and its metabolites rely upon some form of saturation analysis using plasma binding proteins, intracellular receptor proteins or antibodies as the binding protein. The majority of such assays utilise radiolabelled metabolite, although there has been a description of an enzyme-labelled assay for plasma 25-OH-D_3 (Kobayashi et al., 1991, 1993a). In simplistic terms, all these assays rely upon the competition between labelled and unlabelled analyte for a binding protein. Comprehensive reviews of such methods have been published (Seamark et al., 1981; Porteous et al., 1987; Jones et al., 1992, Makin et al., 1995, Jones & Makin, 2000). The specificity of such assays is dependent upon a number of different parameters, the most important of which being the degree of specificity of the binding protein. The lower the specificity of the binding protein, the more important some form of pre-purification becomes. This requirement for pre-purification is very well illustrated in the binding assay for 25-OH-D_3 where a non-LC method usually gives much higher values than those assays utilising an LC separation step prior to quantitation (Makin et al., 1991).

Knowledge of precision and accuracy of routine assays is extremely important since without this information it is not possible to assess the significance of differences in values nor can there be any certainty that it is indeed the analyte of interest which is being measured. Surveys previously carried out have demonstrated quite clearly that many of the assays for 25-OH-D and 1α,25-$(OH)_2$D, the two most commonest assays in routine use, have poor precision (Mayer and Schmidt-Gayk, 1984; Jongen et al., 1984a) and these results were confirmed nearly 10 years later (Makin et al., 1991) although it is clear that advances in methodology published for 1α,25-

$(OH)_2D_3$ have greatly improved the situation. Surprisingly though, it does appear that the inter-laboratory precision of measurement of 25-OH-D is worse than that for 1α,25-$(OH)_2D_3$, even though 1α,25-$(OH)_2D_3$ is present in plasma at a concentration approximately 1,000 times lower than 25-OH-D. Assays for vitamins D metabolites which may be suitable, and indeed may have been developed, for use in research laboratories, may not always be easily transferred to the routine clinical laboratory without careful training. Our experience suggests that clinicians utilising results of analyses of vitamins D metabolites in human body fluids should take great care before assuming that the results they are provided with are accurate and precise. They should also understand exactly what the assay used is measuring.

The majority of saturation analysis methods for 25-OH-D assay utilise plasma DBP as the binding protein. Normal human or rat plasma can be used as the source of the binding protein without any purification and methods have been described using pregnancy plasma in which the concentration of DBP is higher than normal. Two radioimmunoassays for 25-OH-D have been described (Bouillon et al., 1984; Hummer et al., 1984) but no normal ranges were given and a further immunoassay (Hollis and Napoli, 1985) has been described using antibodies raised against a pentanor-vitamin D_3 C22 acid-bovine serum albumin complex and this method forms the basis of a widely-used commercially available kit. This method has now been modified to use a ^{125}I-labelled tracer (Hollis et al., 1993). This immunoassay cross-reacts with the majority of vitamin D metabolites but can be used as a crude means of measuring 25-OH-D since that is the metabolite present in human plasma in highest concentration. Such an assay may be expected to be susceptible to inaccuracy at low and high concentrations of 25-OH-D where further metabolites such as 24,25-$(OH)_2D_3$, 25,26-$(OH)_2D_3$ and 25-OH-D_3-26,23-lactone, all with good affinity for DBP, might be expected to accumulate in the bloodstream. A similar 'group-specific' antibody has been raised using 1α-hydroxy-25,26,27-trinor-vitamin D_3-24-oic acid (Kobayashi et al., 1994a). One enzyme immunoassay has been described (Kobayashi et al., 1993a) in which antibodies were raised to 25-OH-D_3-3-hemisuccinate-BSA and 25-OH-D_3-hemiglutarate-BSA. The enzyme label was β-glucuronidase linked to 25-OH-D_3 via the hemisuccinate and the immunoassay used the heterologous antibody raised to the 25-OH-D_3-3-hemiglutarate-BSA which was also used for immunoaffinity extraction. Normal values quoted for this method were in agreement with that of other workers (Porteous et al., 1987).

Methods available for the measurement of other metabolites of vitamins D are usually saturation analyses using D binding protein requiring extensive pre-purification, although a number of immunoassays have been developed for 24,25- and 25,26-$(OH)_2D_3$, one using an antibody raised against a conjugate of 24,25-$(OH)_2D_3$ with BSA (Hummer and Christiansen, 1984) and the others utilising antibodies raised against conjugates of 1,25-$(OH)_2D_3$ (Fraher et al., 1980) or 1α-hydroxy-cholacalcioic acid with BSA (Hummer et al., 1985), utilising the lack of specificity of antibody binding. Very little work has been carried out in the development of assays for these metabolites since they circulate in human plasma in relatively low concentrations and have as yet not been shown to have any significant physiological

functions. The major calcium homeostatic metabolite of vitamin D, 1α,25-$(OH)_2D_3$, has, on the other hand, been the subject of numerous studies attempting to develop accurate and precise methods for its measurement. Saturation analysis techniques using chick intestinal receptor-binding proteins have been widely described and a considerable amount of work has been carried out in developing immunoassays for this metabolite. A comprehensive list of these methods is given in a number of reviews (Porteous et al., 1987; Jones et al., 1992). An important method utilised for the measurement of plasma 1α,25-$(OH)_2D$ (Reinhardt et al., 1984; Reinhardt and Hollis, 1986) involves the use of the calf thymus VDR (Reinhardt et al., 1982) as the binding protein after separation on small prepacked microparticulate silica cartridges (Hollis, 1986). This methodology seems to be extremely accurate, in that it gives values which are close to those obtained by gas chromatography-mass spectrometry (Oftebro et al., 1988) and also are precise in that intra-laboratory variations are low (Makin et al., 1991). A very comprehensive study of this methodology has been undertaken by Masuda et al. (1991, 1993). The calf thymus assay has an added advantage in that it does not discriminate between 1α,25-$(OH)_2D_2$ and 1α,25-$(OH)_2D_3$ unlike the chick intestinal receptor which has a lower affinity for 1α,25-$(OH)_2D_2$ than for 1α,25-$(OH)_2D_3$ (Jones et al., 1980; Hollis, 1984). Interestingly, a recent study (Seiden-Long and Vieth, 2007) compared the results from the IDS-EIA with a calf thymus-based assay, getting good correlation, even though the manufacturers of the IDS kit indicate that their assay underestimates 1α,25-$(OH)_2D_2$.

An immunoassay for 1α,25-$(OH)_2D$ using a monoclonal antibody (Mawer et al., 1990) has also been developed and this antibody also does not discriminate between 1α,25-$(OH)_2D_2$ and 1α,25-$(OH)_2D_3$. Most of the antisera for 1α,25-$(OH)_2D_3$ immunoassay are raised by conjugation of the hapten via C3 or on the side chain (mainly via C25) to bovine serum albumin or other immunogenic protein. Experience in the steroid field would suggest that the specificity of the antiserum depends upon the exposed groups on the hapten–protein complex. It would seem therefore that the best approach would be to link the 1α,25-$(OH)_2D_3$ molecule to the protein via a position on the nucleus which exposes both he important A ring and the side chain. Kobayashi et al. (1993b) linked 1α,25-$(OH)_2D_3$ via the steroid 11α-position to BSA and obtained antiserum with high affinity (K_a = 0.34–3.3 × $10^{-10}M^{-1}$) with less than 2% cross-reactivity to a wide variety of vitamin D_3 metabolites (D_3, 25-OH-D_3, 24,25-$(OH)_2D_3$, 25(*R* & *S*)26-$(OH)_2D_3$, 1α,24,25-$(OH)_3D_3$ and 1α,25-$(OH)_2$-D_3-26,23-lactone). Further studies on these polyclonal antibodies were described by Kobayashi et al. (1994c). Other methods of linking 1α,25-$(OH)_2D_3$ to the immunogenic protein via 11α-position (Kobayashi et al., 1994b) and via carboxylic acid groups on the side chain (Gregorio et al., 2007) have been studied.

Most of the saturation analyses used for vitamin D metabolites utilise radiolabelled standards, mostly high specific activity tritium labelling, although a modification of a previously published method replacing tritium labelling with ^{125}I-histamine labelling has been reported (Hollis et al., 1993) and this method is now available as a commercial kit. Synthesis of ^{125}I-labelled derivatives of

1α,25-$(OH)_2D_3$ have also been described (Tanabe et al., 1991). Some reports of enzyme-labelled immunoassays (Kobayashi et al., 1993a; Lind et al., 1997; Higashi et al., 1998) have been published but only a few have been successfully applied to the routine analysis of plasma vitamin D metabolites (Higashi et al., 1999a), although there are commercial enzyme immunoassays assays available for 25-OH-D and 1α,25-$(OH)_2D$ (see Table 11.8). The assay described by Armbruster et al. (2000) may in fact be the basis of the kit supplied by Immundiagnostik AG, although it is not referenced as such. It is of course not necessary that the labelled metabolite be the same as the analyte, and in some cases it is not always possible to obtain labelled analyte. Methods have been described where the analyte displaces another metabolites, usually 1α,25-(OH)-D, from the binding protein (e.g. Fraher et al., 1980). These procedures work well provided the binding affinity of the analyte is not too dissimilar from that of the labelled metabolite.

There are other procedures which have been described which are worthy of mention here – in particular, the interesting procedure of Manologas (1986) which used a cytoreceptor assay for the measurement of 1α,25-$(OH)_2D$. This assay relies upon the incubation of analyte with intact rat osteosarcoma cells and its affect upon the uptake of radiolabelled 1α,25-$(OH)_2D_3$. A number of modifications to the original published procedures were found to be necessary to prevent erroneous results (Nicholson et al., 1985). This procedure is not widely used but represents a novel approach to the problem of vitamin D analysis. Two other methods for the measurement of plasma/serum 1α,25-$(OH)_2D$ concentration have been published. The first (Koyama et al., 1992) uses a monoclonal antibody to the VDR (Dame et al., 1986). After extraction and Sep-Pak SIL separation of the appropriate fraction, immunoassay is carried out using VDR from pig intestine, biotinylated monoclonal anti-pig VDR and BSA. [^{3}H]1α,25-$(OH)_2D_3$ was used as a label. The free and bound were separated by addition of avidin-sepharose (Reinhardt et al., 1984).

The second method (Wildermuth et al., 1993) is an interesting approach to homogeneous immunoassay using the scintillation proximity principle (Hart and Greenwald, 1979). In this assay, an antiserum raised in sheep (Clemens et al., 1978) coupled to 1α,25-$(OH)_2D_3$-BSA is incubated with scintillation material contained within beads which are linked to anti-sheep IgG. [^{3}H]1α,25-$(OH)_2D_3$ is used as the labelled antigen. The theory is that only the label bound to the antibody, which is then bound to the anti-sheep IgG, is sufficiently close to the scintillant to cause scintillation and thus the count rate reflects the bound fraction. Separation of free and bound label is therefore not necessary. While this method is interesting and convenient, it still suffers from all the disadvantages which may be associated with this antiserum and specificity is probably compromised to some degree by the lack of LC separation prior to quantitation although the Sep-Pak SIL separation may compensate to some degree. The inter- and intra-assay precision are reasonable but the sensitivity for 1α,25-$(OH)_2D$ (27 pg/mL) is very close to the bottom of the normal range (32–80 pg/mL) quoted. In fact, this procedure has not been used since its introduction in 1993.

11.4 Mass Spectrometry

11.4.1 Introduction

The early difficulty of linking the LC output directly to MS systems meant that the use of MS was off-line and suitable volumes of the eluates had to be collected, derivatised and applied to the MS, usually via GC-MS. However, in the early 1990s methods of directly linking the LC to the mass spectrometer (LC-MS) began to be developed and there were a few descriptions of the use of thermospray LC-MS for vitamin D metabolites. Takamura et al. (1991) described the use of thermospray LC-MS to identify the presence of vitamin D_2 in the shiitake mushroom. Watson et al. (1991) used thermospray LC-MS to measure 1α,25-$(OH)_2D_3$ in mitochondrial incubations and also examined a plasma extract but concluded that, while sensitivity was reasonable, the precision which would be attained using this technique was inadequate. Although considerable advances in LC-MS technology have been made since then, it would seem that at the present time direct online LC-MS analysis of underivatised (but *vide infra*) vitamins D metabolites may have sufficient sensitivity for assays of 25-OHDs in human plasma but may lack sufficient sensitivity for the analysis of all vitamins D analogues and metabolites. LC-MS does, however, offer the advantage of direct analysis of vitamin D analogues or conjugates, unstable in GC systems, which have been the subject of an LC-MS study (Vicchio et al., 1993) using static FAB looking at sulphates and glucuronides of vitamin D_3. It is clear today that LC-MS, monitoring only the pseudo-molecular ion or dehydrated fragments thereof, often provides very little extra information beyond that already obtained from photodiode array detection and retention time and, if use for specific quantitation is envisaged, it is worth investing the extra money to acquire LC-MS-MS, which is discussed below (see Section 11.4.3).

GC-mass spectrometry has proved to be of considerable value both in providing a means of measuring, with high specificity and sensitivity, concentrations of metabolites for which no binding protein is available, providing target values which can be used in external quality assurance schemes, and also providing valuable information about the structure of unknown metabolites isolated from *in vitro* incubation media and from body fluids after formation *in vivo*. Methods are available for the mass spectrometric analysis of intact steroid conjugates using dynamic FAB (Gaskell, 1990) and conjugates of vitamin D_3 have also been examined by static FAB-MS (Jardine et al., 1984). However, this technique has not been generally applied to the study of biological fluids or incubation media although FAB-MS has been used to provide evidence for the presence of a monoglucuronide of 1α,25-$(OH)_2D_3$ in rat bile (Litwiller et al., 1982) and so far MS investigations applied to biological fluids have been confined to unconjugated vitamin D metabolites. The majority of early methods employed electron impact (EI) ionisation and have monitored positive ions produced using low-resolution spectrometers. With EI, it is a general finding that as the molecular weight of the the metabolite rises, the intensity of ions of high mass/charge ratio tends to decrease. In these circumstances, it would be

expected that other forms of ionisation such as CI, monitoring negative and/or positive ions, would be valuable, providing a measurement of the molecular weight and possible enhancement of sensitivity. Because mass spectra produced by such soft ionisation techniques do not show many fragmentation peaks, CI has only limited value as a means of identifying unknown steroids. However, negative ion CI, has been used, for example, as supportive evidence in the identification of vitamin D_3 in the skin of UV-irradiated rats (Okano et al., 1979).

Analytes can be loaded directly into the ion source of a mass spectrometer by direct probe, which can then be slowly heated to volatilise the steroid. Alternatively, analytes can be injected onto a GC or LC column which is interfaced to the MS (GC-MS and LC-MS). For non-volatile compounds, probe insertion may be the only way of getting the sample into the mass spectrometer. However, direct probe insertion requires that the sample be reasonably pure since the only separation which can be achieved prior to mass spectrometry relies upon the temperature at which the sample volatilises. Use of GC or LC prior to the MS increases specificity and also presents the sample to the spectrometer in a more concentrated form than the diffuse peak obtained with the probe – it is therefore more sensitive. Few novel GC-MS methods have been described over the last 20 years although a simple method for 25-OH-D_3 involving on-column dehydration has been described (Coldwell et al., 1995) – see Fig. 11.20. The increased sensitivity of high-resolution GC-MS has also been demonstrated by its application to the measurement of hexafluorocalcitriol in plasma with a sensitivity of 2 pg/mL (Komuro et al., 1994) and for the detection of 1α,24-$(OH)_2D_2$ in human serum from a subject taking vitamin D_2 (Fig. 11.21). The major use of GC-MS has been its application as a means of structural determination or confirmation. Thus, GC-MS can be regarded as an off-line means of LC detection and/or quantitation. Developments in the application

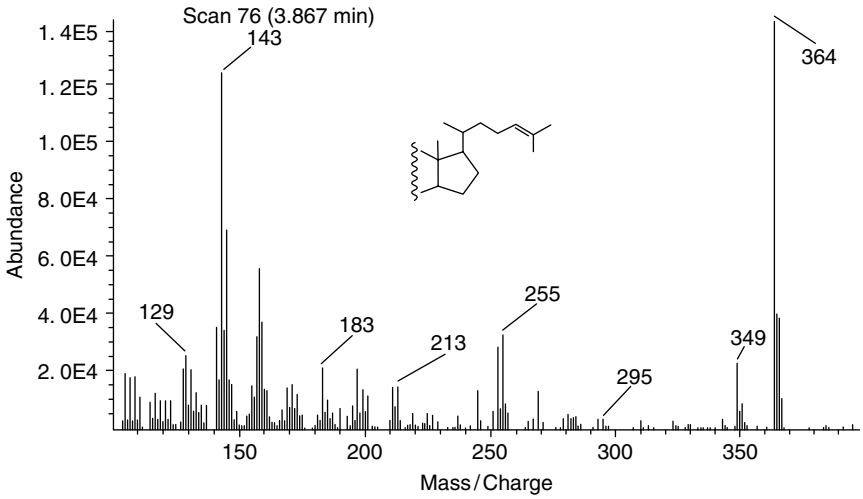


Fig. 11.20 EI(+) mass spectrum of underivatised dehydration product of 25-OH-D_3

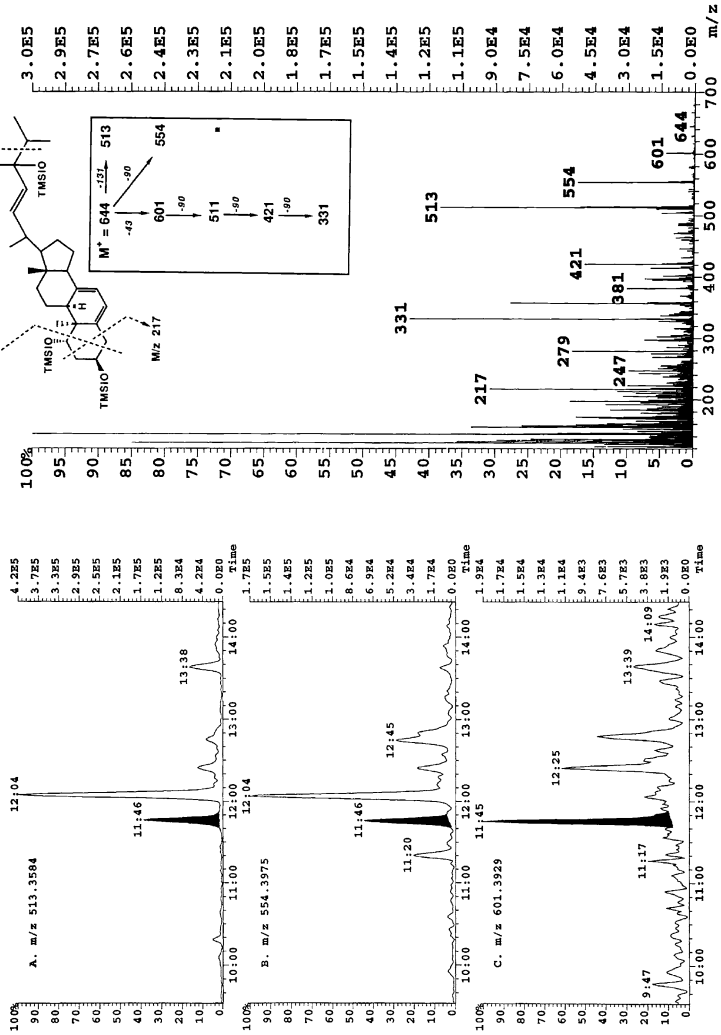


Fig. 11.21 EI(+) mass spectrum, obtained using a high-resolution mass spectrometer, identifying the presence in serum of the per-TMSi ether of 1α,24-$(OH)_2D_2$. High resolution mass fragmentography of an extract of serum from a patient taking vitamin D_2. When injected into the heated zone of the gas chromatograph, vitamin D molecules are cyclized to form pyro- and pyro-isomer. The mass spectral interpretation inserted in this figure has been applied to the pyro-isomer. Ion chromatograms of pertrimethylsilyated 1α,24-$(OH)_2D_2$ monitoring three separate ions, m/z 513.3584 (A). m/z 554.3975 (B), and m/z 601.3929 (C) showing the trace between 9 and 14 min. The peaks the from the pyro-isomer of 1α, 24-$(OH)_2D_2$-MSi are shaded (from Mawer et al., 1998, with permission. Copyright Endocrine Society)

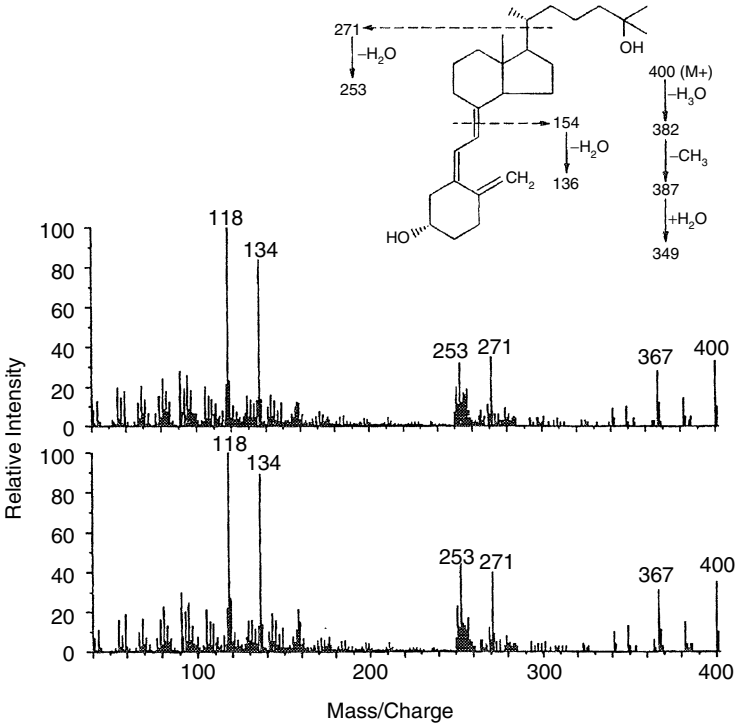


Fig. 11.22 EI(+) mass spectra of underivatised 25-OH-D_3 from Hep 3B cells (*upper panel*) and synthetic compound (*lower panel*) (from Jones & Makin, 2000 with permission)

of mass spectrometry have centred on the connection (interfacing, which includes methods of ionisation) of the LC eluate directly to the mass spectrometer (LC-MS) and the introduction of collision-induced dissociation (CID) and further linking to MS (i.e. LC-MS-MS); these applications are dealt with later in this chapter. Although direct probe MS is seldom used today (Slominski et al., 2006), an EI(+) spectrum of 25-OH-D_3 is illustrated in Fig. 11.22, which shows a comparison between a standard and 25-OH-D_3 obtained from *in vitro* incubation studies. These spectra should be compared with those obtained by GC-MS of the trimethylsilyl ether derivative of 25-OH-D_3 illustrated in Fig. 11.23.

11.4.2 Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS was the first MS technique used to develop methods for the measurement of vitamins D and their metabolites in human tissue fluids. The perceived complexity of GC-MS methodology and the widespread use of LC methodology for metabolite separation prior to GC-MS have lead to interest in and development of LC-MS^n

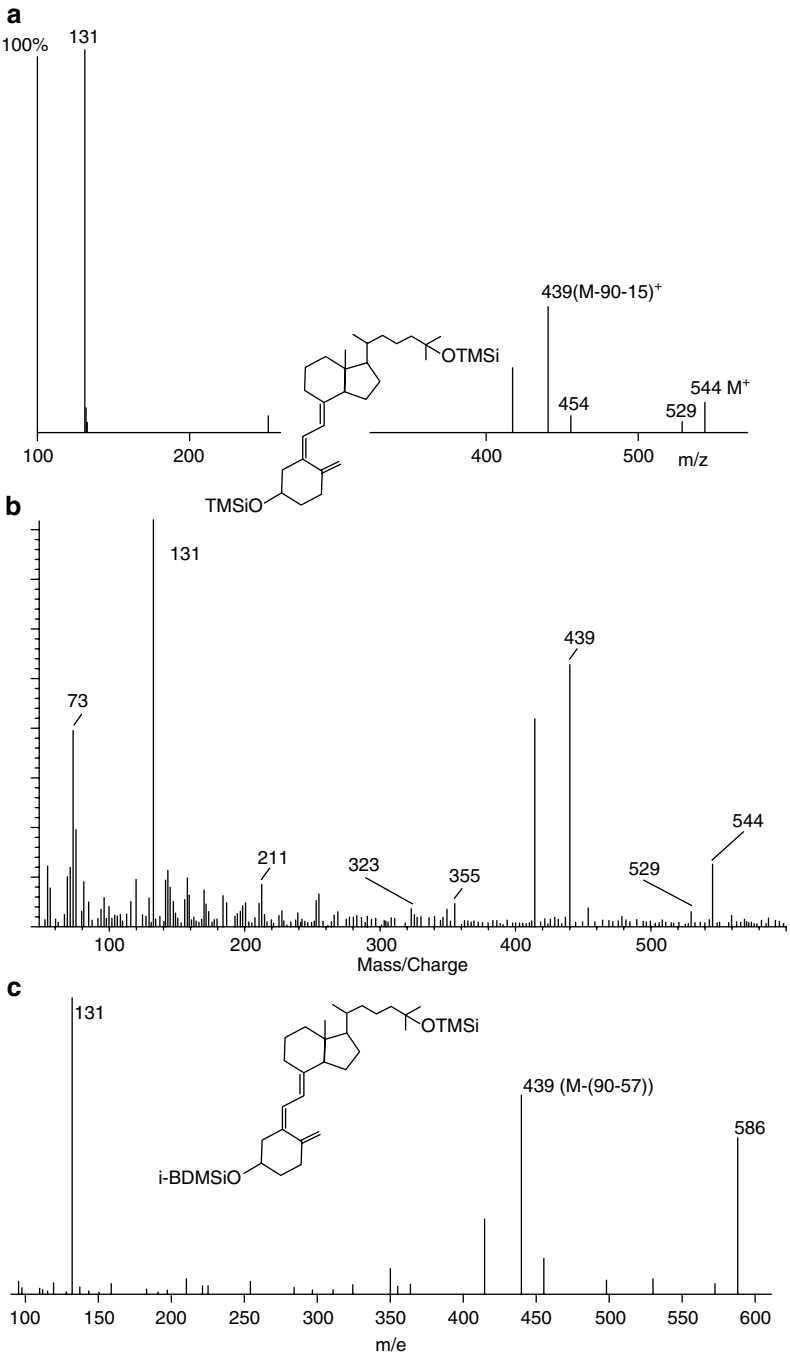


Fig. 11.23 EI(+) mass spectra of 25-OH-D_3-3,25-di-TMSi ether and 25-OH-D_3-3-t-butyldimethylsilyl ether-25-TMSi ether on three different mass spectrometers. EI(+) mass spectra obtained from 25-OH-D_3 as (**a**) per-trimethylsilyl (TMS) ether on a single focusing magnetic sector GC-MS (LKB 2091); (**b**) per-TMS ether on a benchtop quadrupole system tuned to high mass and (**c**) mixed TMS ether/t-butyldimethylsilyl ether on a magnetic sector GC–MS (LKB 2091) (Bjorkhem and Holmberg, 1980 permission) (from Jones & Makin, 2000 with permission)

methodology, which has been increasingly successful. GC-MS development has correspondingly been neglected, even though it is still the method of choice at the present time for structural determination provided the compounds of interest are stable in the gas chromatograph. GC-MS still has value and provides important complementary information to that obtained by LC-MS and -MS/MS, which is discussed below in Section 11.4.3. The early work in applying GC-MS to the analysis of vitamins D and their metabolites was reviewed by Makin and Trafford (1985).

11.4.2.1 B-Ring Closure

A prerequisite of the use of GLC is that the solute should be in the vapour phase during the process of partition between the mobile gas phase and the stationary liquid phase. For compounds of molecular mass in excess of around 200 Da, this is required to be carried out at elevated temperatures – usually in excess of 100°C. Direct probe insertion also requires elevated temperatures in order to volatilise the steroid of interest. At these temperatures, vitamin D and its hydroxylated metabolites undergo thermal rearrangement involving B-ring closure. In naturally occurring steroids (e.g. cholesterol), the 19-methyl attached to C-10 is always β and is *trans* with respect to the 9α-hydrogen. When B-ring closure occurs in the vitamin D series, two isomers are formed both of which are *cis* across the 9–10 carbon–carbon bond (i.e. C19β, 9β, isopyro; and C19α,9α, pyro). These isomers are formed by heating vitamin D in a test tube to around 190°C. Although isomerisation took place rapidly at this temperature and was complete in about 4 h, ring closure has been shown to occur at all temperatures in excess of 125°C (Pelc and Marshall, 1978). It is irreversible and the ratio of pyro to isopyro remains at 2:1. This reaction occurs in the absence of oxygen and it is now thought that the immediate precursor of these isomers is the previtamin (see Fig. 11.24). Therefore, at the temperatures at which GLC is carried out vitamin D and its metabolites are all

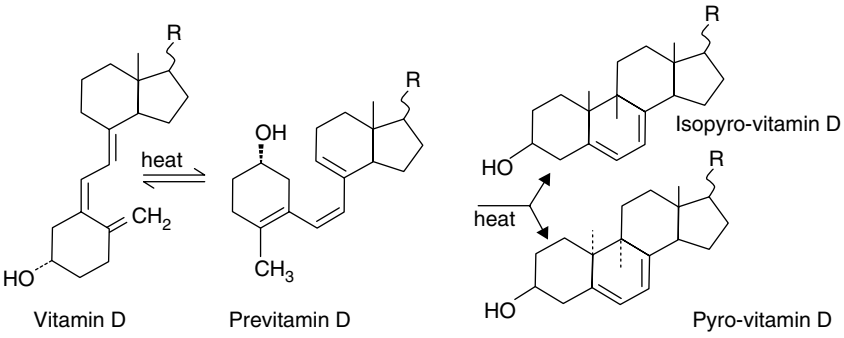


Fig. 11.24 Thermal transformation of vitamins D to *pyro-* and *isopyro-* isomers. Cyclisation occurs in the injection zone of the GC, giving the two isomers in the ratio 2:1 (pyro- :isopyro-). The pro- isomer is usually monitored during GC–MS

converted irreversibly and quantitatively to a 2:1 mixture of pyro and isopyro isomers that separate in all the GLC systems so far examined (Nair et al., 1965; Nair and DeLeon, 1968; Sklan et al., 1973; Seamark et al., 1980a;). If vitamin D metabolites are separated by GC, monitoring the effluent gas by flame-ionisation detection shows that the peak areas of these two peaks are in the correct ratio. However, when the effluent gas is monitored by a mass spectrometer, the peak area ratios are not always in the correct ratio, probably because these two isomers have slightly different quantitative mass spectra. The extent of the difference appears to vary sometimes quite markedly between metabolites. The presence of these two isomers during a GC separation is a very useful additional indication that a secosteroid of the vitamin D type was injected into the system. No GC-MS EI(+) spectra of the unisomerised vitamin D secosteroid have been described and it must therefore always be appreciated that GC-EI(+) mass spectra in this book are always derived from vitamins D derivatives with cyclised pyro- B ring structures, even though the seco-steroid is illustrated. LC-thermospray mass spectra of underivatised vitamin D_2 have been obtained (Takamura et al., 1991). For the majority of vitamin D metabolites, but not for those containing a 1-hydroxyl, it is possible to take advantage of another isomerisation process (formation of isotachysterols), which can be carried out prior to GC separation, which prevents thermal B-ring closure and thus allows single peaks to be produced during chromatography. Such procedures, which have been discussed previously, have the advantage that they do increase the sensitivity of detection of both after GC and also if used with LC-UV.

11.4.2.2 Derivatives for GLC

As with all GLC systems for steroid analysis, the stability and volatility of vitamin D and its metabolites can be increased by the formation of derivatives on the polar hydroxyl groups. Free underivatised hydroxyl groups may give rise to adsorption during chromatography and non-linearity of detector response. If vitamin D and particularly its polyhydroxylated metabolites are injected into a GC without derivatisation, broad peaks are obtained, indicating some degree of adsorption. In addition, dehydration of the 25-hydroxyl can occur giving rise to two extra peaks in addition to the pyro and isopyro isomers. This dehydration is variable and occurs with all hydroxylated steroids (Trafford et al., 1991), but 100% conversion to 25-dehydro derivative can sometimes be achieved by introducing powdered glass at the top of the GC column, thus allowing use of the dehydro peaks for quantitation.

The use of powdered glass to achieve dehydration is not always effective however and more consistent results can be obtained if required using aluminium powder at 400°C. Although it would be expected that pyro and isopyro isomers would still be formed during chromatography, only one major peak is observed for each metabolite during GC-MS probably because the isopyro isomer has only low-intensity ions. The mass spectrum of dehydrated 25-OH-D_3 is shown in Fig. 11.20. Dehydration can occur in a number of ways; for example, loss of the 25-hydroxyl could lead to a double bond between C24 and C25 or between C25 and C26. Use of a high-resolution capillary

column indicates that two isomers are formed during dehydration of 25-OH-D_3 and use of stable isotope-labelled compounds has confirmed that both the expected dehydration products are formed. Dehydration of dihydroxylated vitamin D metabolites also occurs and the products from 24,25-$(OH)_2D_3$ and 25,26-$(OH)_2D_3$ are easily resolved during GC because in 25,26-$(OH)_2D_3$ both the 25- and 26-hydroxyls cannot be removed simultaneously and the remaining hydroxyl makes the product more polar than that from 24,25-$(OH)_2D_3$. Use of dehydration as a simple GC-MS method for plasma 25-OH-D_3 has been described (Coldwell et al., 1995). Modification or derivatisation of vitamins D and metabolites may offer GC- and LC-mass spectrometric advantages and give rise to more intense high-mass ions – a positive advantage in developing specific and sensitive MS assays.

Although the formation of derivatives on the polar hydroxyl groups of vitamin D and metabolites improves stability and volatility, it does not overcome the problem of B-ring closure and the formation of the two pyro and isopyro peaks. The formation of these isomers is quantitative and either of the peaks or both the peaks can be used for measurement (Bjorkhem and Holmberg, 1976; Bjorkhem and Larsson, 1978; Halket and Lisboa, 1978; Zagalak et al., 1978; Bjorkhem et al., 1979), but the separation of a number of metabolites on a single run can be complicated by the multiplicity of peaks. To overcome absorption, resolution and sensitivity difficulties, it is usual to derivatise vitamin D and its metabolites prior to GC. It is usually sufficient to form derivatives of hydroxyls and leave oxo groups underivatised although derivatisation of the latter, to form *N*-*O*-methyloximes, does improve peak shape and resolution. When choosing the derivatives to be used, consideration must be given not only to the GC characteristics of the chosen derivative but also to the MS characteristics. For both identification and measurement of vitamin D metabolites, in common with other steroids, high-mass ions of reasonable intensity are desirable and not all derivatives which give good GC characteristics also provide this. The procedure adopted in our laboratory is to form trimethylsilyl ether derivatives using trimethylsilyl imidazole as the derivatisation reagent (Coldwell et al., 1990), although it must be said that these derivatives have been chosen on the basis of experience with other steroids and no comprehensive study of other derivatives has yet been undertaken. Because the 25-hydroxyl is sterically hindered, it is difficult to derivatise and only two reagents have proved satisfactory for the low levels present in plasma – trimethylsilylimidazole (TMSI) and a mixture of *bis*-trimethylsilyltrifluoroacetamide (BSTFA): trimethylchlorosilane (TMCS) (3:1 v/v). Early work on the GC separation of vitamin D and its metabolites used conventional packed columns with relatively high carrier gas flows, which required some sort of separator system. Such systems are no longer required as capillary columns with low carrier gas flow rates (1–2 mL/min) are now used, which can be linked directly to the mass spectrometer by inserting the end of the column directly into the ion source of the mass spectrometer.

Trimethylsilyl ether derivatives have been widely used for GC-MS; however, as can be seen from Fig. 11.23 they are not always ideal derivatives for mass fragmentography (MF) in the vitamin D field since intensities of ion fragments of high mass are low but they do offer some advantages in structural elucidation. The mass spectra of TMS ethers of 25-hydroxylated vitamin D_3 metabolites all contain a base peak at

m/z 131 (the fragment obtained by the side-chain cleavage between C24 and C25 containing C26, C27, C25-O-TMS). 26-Hydroxylation increases the mass of this peak to *m/z* 219 because of the presence of the extra trimethylsilanol group. Figure 11.23 also illustrates the EI (+) mass spectra obtained for a mixed *t*-BDMS/TMSi derivatives of 25-OH-D_3 in comparison to that obtained from 25-OH-D_3-per-TMSi. One further way of enhancing high-mass ions can be applied to quadrupole mass spectrometers because they show mass discrimination and have electronic means of changing the intensity of high-mass ions. Figure 11.23 also shows the enhancement of high-mass ions that can be achieved using a single benchtop quadrupole system for 25-OH-D_3-per-TMSi. Comparison of the mass spectrum illustrated in Fig. 11.22 with that in Fig. 11.23 shows the value of derivatisation as TMSi ether in that the number of hydroxyls (*n*) can easily be ascertained as the M^+ of the TMSi ether is $n \times 72$ Da higher than the M^+ from the underivatised molecule. In addition, formation of TMSi ethers also enhances the distinction between oxo- and hydroxyl compounds.

An interesting derivative for 24,25-$(OH)_2D_3$ has been described by Lisboa and Halket (1979) who formed a cyclical boronate across the adjacent 24- and 25-hydroxyl groups as previously described for steroids (Brooks and Harvey, 1969; Brooks and Middleditch, 1971; Baillie et al., 1972). Halket et al. (1980) obtained mass spectrometric and retention times for methyl- and *n*-butylboronate-3-methysilyl ether derivatives of 24,25-$(OH)_2D_3$ and found that the use of the cyclic boronate derivatives stabilised the molecule and gave rise to considerably enhanced intensity of ions of high mass/charge ratio. Figure 11.25 compares the normalised mass spectra of 24,25-$(OH)_2D_3$-TMS with that obtained from the methylboronate TMS derivative, showing the greatly increased mass fragments at *m/z* 381 ($[M - 131]^+$), the fragment probably obtained by A-ring cleavage containing C2, C4 and C3-O-TMS and *m/z* 407 ($[M - 90 + 15]^+$). The use of *n*-butylboronate-trimethylsilyl derivatives also has the advantage that these derivatives of 24,25-$(OH)_2D_3$ and 25,26-$(OH)_2D_3$ separate during GC on a non-selective stationary phase (Coldwell et al., 1984), and such derivatives have been used in the development of mass-fragmentographic assays for both these vitamin D metabolites (Coldwell et al., 1985). Vicinal hydroxyls and 1,3-*cis*-diols can both form cyclic boronates and *n*-butyl, *n*-phenyl and *n*-methylboronate of 24,25-$(OH)_2D_3$ can easily be formed. Interestingly, only *n*-butyl and *n*-phenylboronates can be formed with ease from 25,26-$(OH)_2D_3$; *n*-methylboronates appear not to be formed (Coldwell et al., 1984).

Use of other derivatives, such as cyclic boronates, can as illustrated for 24,25-$(OH)_2D_3$ greatly enhance the intensities of higher-mass fragments more suitable for use in MF analysis. It will be noted that dehydration provides intense high-mass ions, also suitable for improved sensitivity of measurement and enhanced specificity. Both mass spectra in Fig. 11.25 were obtained using the pyro peak, since formation of these derivatives does not prevent the thermal cyclisation of the B-ring. Other derivatives have been used in attempts to improve the mass spectral characteristics and studies have been carried out using *t*-butyldimethylsilyl (*t*-BDMS) ether derivatives (Lindback et al., 1987) which have been shown to give greatly enhanced intensity of the $[M - 57]^+$ ion fragment for steroids (Phillipou

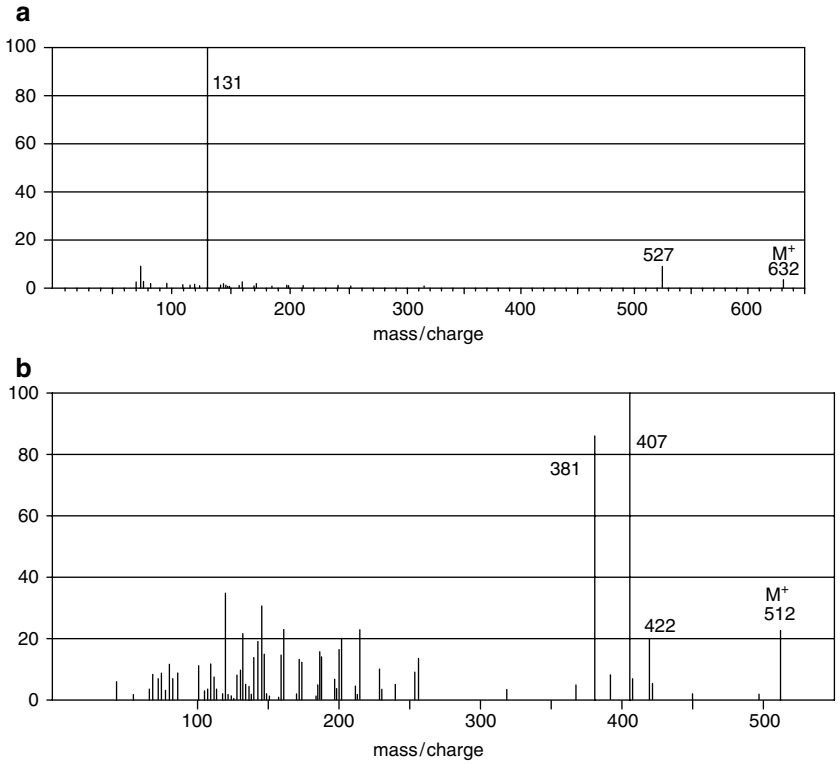


Fig. 11.25 Normalized mass spectra of two derivatives of 24,25-$(OH)_2D_3$ during GC-MS. GC was carried out on a 50m OV-17 capillary column at 260°C. In each case the pyro peak was scanned. (**a**) 24(R),25-$(OH)_2D_3$-tris-trimethylsily ether; (**b**) 3-Trimethylsilyl ether-24,25-methylboronate ester derivative of 24(R),25-$(OH_2D_3$ (Halket et al., 1980)

et al., 1975). A study by Shimada's group (Higashi et al., 2002b) examined the derivatisation of 24,25-$(OH)_2D_3$ by nine different substituted phenylboronic acids as a means of improving sensitivity during LC-APCI(-)-MS. They described a method for determination in human plasma – after deproteinisation with acetonitrile and purification using an OASIS-HLB cartridge, derivatisation with [3-(2-nitro-4-trifluoromethylphenyl)aminophenyl]dihydroxyborane and LC-MS, increasing sensitivity × 200.

Use of GC-MS using these derivatives for the measurement of plasma concentrations has been described for all the major vitamin D metabolites, and a list of these methods has been given in Table 11.5. It will be noted from this table that only one GC-MS method for the important calcium homeostatic hormone, 1α,25-$(OH)_2D_3$, has been described and this method (Bjorkhem et al., 1979) required 20 mL of plasma for analysis. The sensitivity of simple single-focussing low-resolution mass spectrometers is at present too low to measure 1α,25-$(OH)_2D_3$, unless large volumes

Table 11.5 Gas Chromatography-Mass Spectrometry Methods for Analysis of Vitamins D, Analogs, and Metabolities in Biological Fluids published between 1983–1994 – none published after 1994

Analyte	Derivative used	Internal standard	Concentration	
D_2	TMSi	[6,19,19-$^2H_3$]-D_2	136 mg/mL in urine (268 mg/mL after enzyme hydrolysis)	Zagalak et al. (1983)
D_2	TMSi	[3,4-$^2H_2$]-D_3*	120 mg/ml in urine (up to 500 ng/ml after enzyme hydrolysis) 4.5 ± 2.8 ng/ml in serum	Zagalak et al. (1983)
25-OH-D_3	TMSi	[26(27)-$^2H_3$]-25-OH-D_3	21.4 ± 5.3 ng/ml in serum	Zagalak et al. (1983)
25-OH-D_3	TMSi-tBDMS		15.3 ± 1.6 ng/mL	Berlin, et al. (1986a)
			19.2 ± 2.0 ng/mL	Berlin, et al. (1986b)
	TMSi	[26,27-$^2H_6$]-25-OHD_3	2.3-32.6 ng/mL in plasma	Coldwell et al. (1989)
25-OH-D_2	TMSi	D_2	0.6-1.0 ng/mL	Seamark et al. (1980a)
	TMSi	[26,27-$^2H_6$]-25-OHD_3	0.12-6.6 ng/mL in plasma	Coldwell et al. (1989)
24,25-$(OH)_2D_3$	TMSi TMSi-nBBA	Vitamin D_2	0.6-2.9 ng/mL	Seamark et al. (1980a)
24,25-$(OH)_2D_3$	TMSi TMSi-nBBA	[26,27-$^2H_2$]-24,25-$(OH)_2D_r$	1.6 ± 0.6 ng/mL (UK summer) 3.1 ± 1.4 ng/mL (Australian summer)	Coldwell et al. (1984)
	TMSi-nBBA	[6,19,19-$^2H_6$]-24,25-$(OH)_2D_3$	Rat serum (no values but linear response to added 24,25$(OH)_2D_3$)	Tomiyama, et al. (1994)
25,26-$(OH)_2D_3$	TMSi-nBBA	[26,27-$^2H_6$]-25,26-$(OH)_2D_3$	Mean 0.39 ng/mL (UK plasma) mean 0.76 ng/inL range 0.3-1.3 ng/mL (Australian plasma)	Coldwell et al. (1985)
24,25-$(OH)_2D_2$	TMSi-nBBA	[26,27-$^2H_6$]-25,26-$(OH)_2D_3$	ND in normal plasma. 4.1-29.5 ng/mL on D_2	Coldwell et al. (1989)
25,26-$(OH)_2D_2$	TMSi-nBBA	[26,27-$^2H_5$]-25,26-$(OH)_2D_3$	ND in normal plasma 0.85-2.11 ng/mL on D_2	Coldwell et al. (1989)
l,25-$(OH)_2D_3$	TMSi	[26-$^2H_3$]-25,26-$(OH)_2D_3$	Validation of calf thymus assay	Oftebro et al. (1998)
I,25-$(OH)_2D_3$	TMSi	Vitamin D_2	Semiquantitative (three serum samples in range 22.0-38.1 pg/mL). Lower values than calf thymus assay	Poon et al. (1993)
[26,27-F6]-l,25-$(OH)_2D_3$	TMSi	[H_2[26,27-F6]-1,25$(OH)_2D_3$	28 ± 10 pg/mL in 12 volunteers 4 hr after 2μg oral dose	Komuro et al. (1994)

*At a later stage [6,19,19-$^2H_3$]-D_3, [2,2,3,4,4,6-$^2H_6$]-D_3, and [2,2,3,4,4,6,19,19-$^2H_8$]-D_3 were used as internal standards; n-BBA, n-butyl boronate; TMSi, trimethylsilyl ether

of plasma are used, even with careful optimisation of both the derivatisation and separatory procedures prior to MS, and tuning of the mass spectrometer itself by focussing onto reference masses close to those which are to be monitored (Poon et al., 1993). The Bjorkhem et al. (1979) procedure has, however, been used to evaluate a calf thymus assay (Reinhardt et al., 1984) by comparing $1\alpha,25\text{-}(OH)_2D_3$ results obtained by this procedure with those obtained on the same plasma by GC-MS (Oftebro et al., 1988). All these methods used GC-MS with EI(+) and primarily use stable isotope dilution techniques, that is to say, deuterium-labelled analyte was added to the matrix as an internal standard, the analyte purified assuming that no separation between the labelled and unlabelled analyte occurred, the analyte derivatised and GC-MS carried out. The reason for the development of GC-MS or LC-MS-MS procedures for the measurement of vitamin D metabolites, when supposedly adequate and simpler saturation analysis methods are available, can easily be appreciated if these simpler methods are more carefully examined. Target values are obtained by analysis using a reference procedure and at the present time GC-MS is the most definitive methodology available and is widely accepted as the ideal methodology for this purpose and has been used by Lindback et al. (1987) as a reference method for the evaluation of commercial kits. LC-MS-MS procedures are, however, being increasingly proposed as alternative reference methods (see Section 11.4.3).

One suggested disadvantage to the use of GC-MS in comparison to LC-MS-MS is the necessity for GC of derivatisation before chromatography. Although such procedures are not just necessary for chromatographic reasons but also may provide improved mass spectral characteristics and important structural information, they do increase the complexity of pre-GC manipulations, even though the usual derivatisation procedures are very simple. This ‘disadvantage’ of GC-MS may in fact be more apparent than real, as there is increasing dependence on derivatisation during LC-MS (or -MS/MS) to improve sensitivity.

The mass spectrometer can be used as a detector for GC and LC in two ways. The most sensitive method relies on the ability of the MS to focus on specific fragments. There are numerous ways of arranging this depending on the type of mass spectrometer being used. The spectrometer can be focused on a single ion fragment or on different ion fragments at different times. The dwell time on each fragment is directly related to sensitivity. In a single-focusing magnetic sector machine, the focusing on different fragments can be achieved by altering the accelerating voltage so that different ions are sequentially focused on the electron multiplier. Modern laminated magnets now allow rapid alteration of the magnetic field without associated hysteresis effects and focusing can thus be achieved by altering the current through the magnet. Quadrupole mass spectrometers operate on a different principle and focus ions by altering radiofrequency and direct current applied across four rods through the axis of which the ions travel. As mentioned previously, quadrupole mass spectrometers are assuming increased importance in analytical laboratories. The process of mass fragmentography is the most sensitive mode of operation but an alternative approach can be adopted. All modern mass spectrometers are now equipped with sophisticated data acquisition and manipulation computing systems and thus are capable of scanning the eluent from the GC or LC at regular intervals

(one to five scans per second) and storing the complete mass spectrum obtained. At the end of the GC/LC run, data can be recalled and total-ion or single-ion chromatographs can be constructed. Such a system (mass chromatography) is not as sensitive as MF but provides a very valuable method of establishing the ion fragment to monitor for best sensitivity and specificity. Sensitivity and specificity can be improved by the use of high-resolution GC-MS which is achieved by double-focusing instruments which give greatly improved signal-to-noise ratios by careful focusing on specified ions in MF mode. An example of the value of this technology has already been illustrated in Fig. 11.21.

11.4.3 Liquid Chromatography-Mass Spectrometry (LC-MS and LC-MS-MS)

11.4.3.1 Introduction and General Considerations

Just under 20 years ago, LC-$MS^{(n)}$ emerged as a complementary method to GC-MS in the identification and quantitation of vitamin D compounds. Since then, LC-MS technology has dramatically improved and the technique has been applied to the analysis of vitamin D metabolites and analogues by a number of different research groups, revealing both the strengths and weaknesses of this technology (see Table 11.6). Several different interfacing techniques have been tried to separate effectively solute from solvent in LC effluent and then, after suitable ionisation, to transfer this quantitatively to a mass spectrometer.

LC-MS using thermospray was one of the early developments applied to the analysis of phenyl-4-TAD adducts of vitamin D_3, 25-OH-D_3, 1α,25-$(OH)_2D_3$, 24,25-$(OH)_2D_3$ and 1α-OH-D_3 by Vreeken et al. (1993). Both positive and negative ion spectra were examined and simple spectra with protonated and deprotonated molecular ions were seen. These workers reported better signal-to-noise ratios with negative ion spectra in contrast to the work of Watson et al. (1991), who could find no signal when monitoring negative ions. Sensitivity of this procedure was good (1–7 nmol/L) but not sufficient for use in the analysis of serum or plasma. Unlike other procedures, this method utilised post-column formation of the phenyl-4-TAD adduct which was effected by mixing the derivatising reagent in a reaction coil after the LC separation but before MS. Thermospray MS is said to be inherently unstable and Watson et al. (1991) even suggested that this technique might never be suitable for routine plasma vitamin D metabolite measurement. This view, however, was not endorsed by Vreeken et al. (1993) who suggested that their system was sufficiently sensitive for the measurement of vitamin D and several of its metabolites but not, however, for 1α,25-$(OH)_2D_3$, which would still require large volumes of plasma. Fortunately, the introduction of electrospray MS has improved the potential of LC-MS.

Capillary LC-electrospray tandem mass spectrometry has been used to study *in vitro* metabolism of 1α,25-dihydroxy-16-ene-vitamin D_3 in rat kidney with

Table 11.6 LC–MS^n methods for the analysis of vitamins D, metabolites and analogues

Analyte	Method	IS	Reference
Ro 24-2090 (23-24-yne-25OHD3)	LC-particle beam ECNI-MS pentafluorobenzyl-TAD	[26,27-$^2H_6$] analyte	Wang et al. (1996)
22-OCT	LC-ESI(+)-MS-MS	ED94 (25-nor-OCT)	Ishigai et al. (1998)
EB1089	LC-ESI(+) MS-MS	[26a,27a-$^2H_6$] analyte	Kissmeyer et al. (2000)
24,25$(OH)_2D_3$	LC-APCI(+)MS MBO-TAD	[26,27-$^2H_6$] analyte	Higashi et al. (2001a)
25OH-D_2 and -D_3	LC-APCI(+)-MS-MS DMEQ-TAD	25-OHD_4	Higashi et al. (2001b)
1,25-$(OH)_2D_3$	LC-ESI(+) MRM	[26,27-$^2H_6$] analyte	Kissmeyer and Sonne (2001)
1-OHD_3	LC-APCI(+)-MS(-MS) DMEQ-TAD/acetylation	[22,23-$^2H_4$] analyte	Higashi et al. (2002)
25-OH-D_3	LC-ES-API-MRM	[25-^{13}C-$^2H_6$] analyte[a]	Vogeser et al. (2004)
1-OH-D_3	LC-ESI(+)-SRM 4-ferrocenylmethyl-TAD	[22,23-$^2H_4$] analyte	Murao et al. (2005a)
ED-71	LC-ESI(+)-MS-MS	[26,27-$^2H_6$] analyte	Murao et al. (2005b)
25-OH-D_2 and 25-OH-D_3	LC-ESI(+)-MS-MS	[26,27-$^2H_6$]	Maunsell et al. (2005)
1,25-$(OH)_2D_3$			Casetta et al. (2010)
25-OH-D_2 and 25-OH-D_3	LC-APCI-MRM	[$^2H_3$] 25-OH-D_3	Tsugawa et al. (2005)
25-OH-D_3	LC-APPI-MRM	[$^2H_6$] analyte	Guo et al. (2006)
25-OH-D_2 and 25-OH-D_3	LC-APCI-MRM	[$^2H_6$] analyte	Singh et al. (2006)
25-OH-D_2 and 25-OH-D_3	LC-ESI(+)-MRM	[$^2H_3$]Δ^9-THC[b]	Saenger et al. (2006)
D_2, D_3, 25-OH-D_2 and 25-OH-D_3	LC-APCI(+)-MRM	[$^2H_7$]-D_3 and [$^2H_6$]-25-OHD_3	Kamao et al. (2007)
25-OH-D_3	LC-ESI(+)-MS/MS	25-OHD_4	Higashi et al. (2008)
25-OH-D_2 and 25-OH-D_3	LC-APCI(+)-MS/MS	[26,27-$^2H_6$]-25-OHD_3	Chen et al. (2008)
Profile (D_2, D_3, 1,25$(OH)_2D_2$ and 25-OH-D_3, 24,25-$(OH)_2D_3$, 25-OH-D_2 and 25-OH-D_3)	UPLC-ESI(+)-MS/MS	d_6-1,25$(OH)_2D_3$ and -D_3	Aronov et al. (2008)

[a]Synthesised as described by Bjorkhem and Holmberg (1980).

[b]THC, tetrahydrocannabinol, ECNI: electron capture negative ion, TAD: triazoline adducts (see Figure 11.19), ESI electrospray ionisation, APCI: atmospheric pressure chemical ionisation, DMEQ-TAD (see Figure 11.19), API: atmospheric pressure ionisation, SRM: single reaction monitoring, MRM: multiple reaction monitoring, APPI atmospheric pressure photoionisation.

detection limits in the 50–100 pg level (Yeung et al., 1995). Even in this early work, the potential value of LC-MS in the field of vitamin D analysis was recognised useful since most metabolites of vitamin D can be resolved by a suitable combination of LC systems and the added specificity of both derivatisation and subsequent mass spectrometry would be a very powerful and specific means of quantitation.

Both ESI- and APCI-MS have been shown to be selective, reproducible and sensitive techniques, a development which has opened up the possibility of using LC-MS in wide variety of applications. Several groups have reported the use of LC-MS, albeit often with concomitant use of GC-MS, to characterise novel catabolic products of 1α,25-$(OH)_2D_3$ and 20-epi-1α,25-$(OH)_2D_3$ (Kusudo et al., 2003; Masuda et al., 2003, 2004; Astecker et al., 2004; Sawada et al., 2004; Jones et al., 2006), where their molecular weights were discerned from several characteristic ions, including molecular ions ($[M + H]^+$), dehydration products ($[M + H - H_2O]^+$), as well as adductions (see Fig. 11.26; MS1 panel). For each substrate, the molecular weights of the various products were mapped by shifts in the *m/z* ratios of the characteristic ions, as predicted from knowledge of the predicted intermediates in a CYP24A1-catalysed reaction. The presence of several characteristic ions per analyte, as opposed to a single molecular ion, help identify de facto metabolic products by indicating their molecular mass but in most cases provide few characteristic fragments, unlike GC-MS. Since contaminants of the same molecular weight are unlikely to give rise to the characteristic ion 'fingerprint', LC-MS/MS is particularly amenable to studies in the vitamin D field since most metabolites of vitamin D can be resolved by a combination of different LC systems (see Fig. 11.27, left), prior to the final MS analysis – the end result of which is a mass spectrum (Fig. 11.26, middle right) derived from a metabolite peak possessing a characteristic retention time (Fig. 11.26 top left) and the typical vitamin D UV spectrum (Fig. 11.26, top right). It is, however, only the combination of these three attributes, which gives rise to the specificity. Herein lies the problem with LC-MS at the present time. There is no doubt that LC-MS of underivatised metabolites of vitamins D is extremely convenient and may provide an excellent method for the definitive assay of known metabolites, which can be further enhanced by the use of LC-MS/MS with CID (collision-induced dissociation) between M1 and M2, using SRM or MRM. However, the precise nature of the CID fragmentation is often not known and thus impossible to predict.

One of the limitations of ESI- and APCI-MS of vitamin D metabolites is the lack of specificity of the molecular fragments generated by CID (MS2 in Fig. 11.26, bottom right) (Ishigai et al., 1997). In our hands, identical spectra containing A-ring cleavage fragments are observed for most catabolites of 1α,25-$(OH)_2D_3$, indicating that LC-MS yields only limited structural information (Masuda et al., 2003, 2004). This has reinforced the complementary nature of GC- and LC-MS systems, particularly the role of GC-MS in structural elucidation. A number of groups have continued to use GC-MS (Guryev et al., 2003; Astecker et al., 2004; Rahmaniyan et al., 2005), as, together with derivatisation (usually, formation of trimethylsilyl ethers), it provides mass spectra containing valuable structural information. An example of this is given in Fig. 11.33, where four dihydroxyvitamin D_3 epimers, with identical molecular weights, can be distinguished from their mass spectra

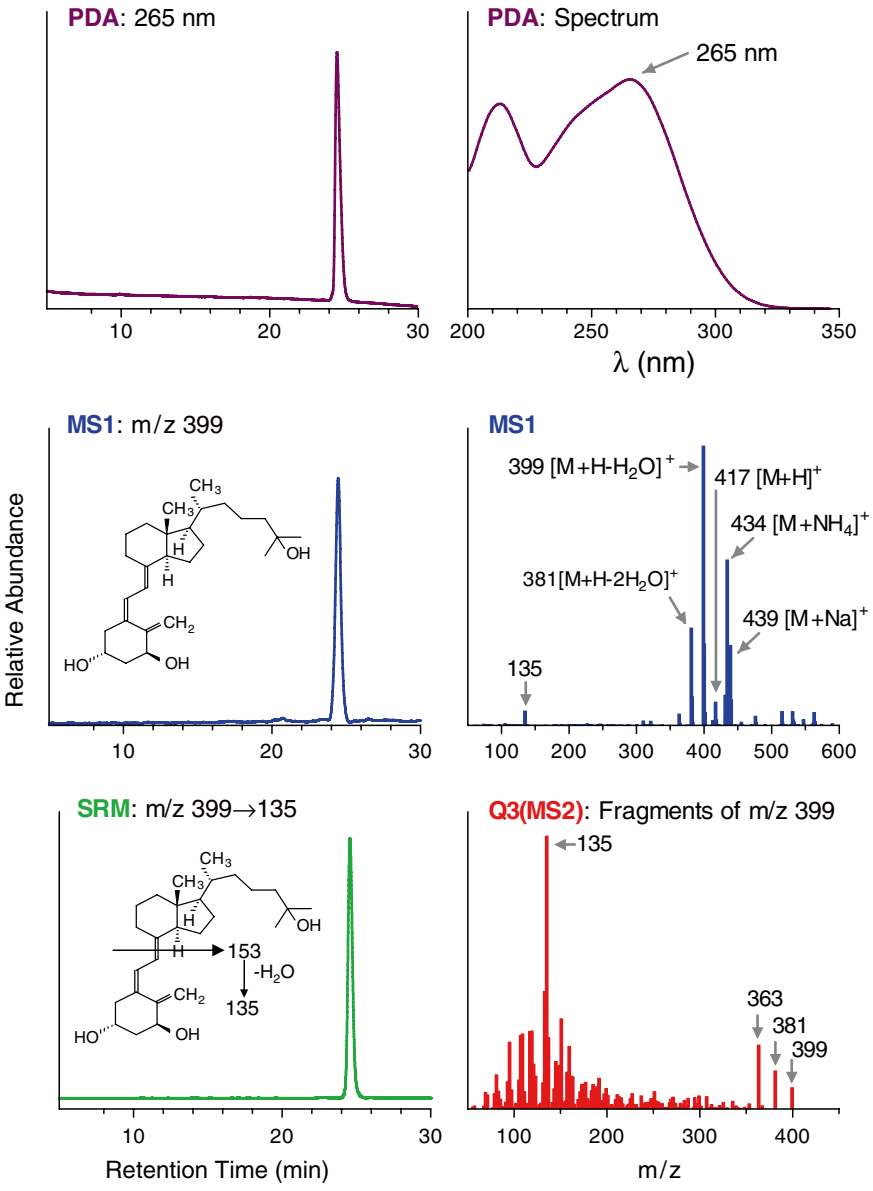


Fig. 11.26 LC-MS analysis of 1α,25-$(OH)_2D_3$. This figure demonstrates several important pieces of information which can be discerned from a single LC–MS run of a selected vitamin D metabolite. For example, 1α,25-$(OH)_2D_3$ can be identified on the basis of its characteristic retention time obtained from the chromatogram, monitoring UV absorption at 265nm (top left panel), and by the characteristic vitamin D cis-triene chromophore obtained from photodiode array spectrophotometry (top right panel). Confirmation of retention time is obtained by monitoring m/z399 (middle left panel) and by single reaction monitoring of the transition m/z399/135 after CID (lower left hand panel).

alone. Examples of the use of GC-MS for structural elucidation of metabolites of vitamins D and analogues are given in Section 11.6.3 below. Astecker et al. (2004) used this technique to identify two groups of positional isomers, each containing metabolites of identical molecular weight: 1α,24-$(OH)_2$-23-oxo-D_3, 1α,23(*S*)-$(OH)_2$-24-oxo-D_3 and 1α,25-$(OH)_2$-24-oxo-D_3: as well as the two trihydroxyvitamin D metabolites: 1α,23,24-$(OH)_3D_3$ and 1α,24(*R*),25-$(OH)_3D_3$. Identification of these products was based upon side-chain fragments resulting from a cleavage at C23/C24 or C24/C25 – fragments which have not been reported by using LC-MS alone. It is possible that LC-MS could be sufficient for the resolution of isomers if the number of isomers were limited, synthetic standards were available and some chromatographic resolution of isomers could be attained (Shinkyo et al., 2004). Continued advances in capillary-LC and UPLC technology are expected to help improve the specificity of the online LC analysis, used for the identification of vitamin D metabolites. In summary, it is clear that current LC-MS procedures are convenient and valuable tools for the quantitation of known metabolites and for the partial identification of unknown metabolites but GC-MS with its more detailed and predictable fragmentation and the added information provided by derivatisation still provides more definitive structural information.

11.4.3.2 Quantitation Using LC-MS-MS

One of the potential advantages of LC-MS is that it can also be used as a quantitative tool. Though GC-MS has been used for the past few decades as the definitive quantitative tool, its dependence on off-line derivatisation, the formation of pyro- and isopyro- isomers during thermo-isomerisation and a requirement for extensive purification prior to analysis when compared to LC-MS make it inherently more inconvenient. Indeed, in our hands despite GC-MS possessing unchallenged sensitivity for vitamin D down into the femtogram range, the technique suffers from

Fig. 11.26 (continued) When coupled directly with a mass spectrometer, the vitamin D metabolite under investigation yields a characteristic MS1 mass spectrum possessing an informative array of ions including a pseudo-molecular ion $[M + H]^+$, dehydration products $[M + H - H_2O]^+$, $[M + H - 2H_2O]^+$ and other adduct ions with ammonia and sodium any of which, when plotted against time, reveal a peak at the characteristic UV retention time. MS2 analysis of CID-induced daughter ions of m/z399 1α,25-$(OH)_2D_3$ show a qualitatively, but not quantitatively, similar spectrum to that of MS1 (bottom right panel) similar mass spectra (f). MS1, MS2 and retention time should therefore be viewed together in order to identify 1α,25-$(OH)_2D_3$. It should be noted that that MS1 and MS2 analysis of many isomers of 1α,25-$(OH)_2D_3$ yield identical MS1 and MS2 spectra to that of 1α,25-$(OH)_2D_3$ itself, making the analysis inconclusive. Owing to the superior fragmentation pattern offered by GC-MS, this technique can act as a valuable complementary tool. The specificity of quantitation using single reaction monitoring (SRM), which can be complemented by multiple reaction monitoring (MRM), is not absolute and, if potential isomers are unresolved by the LC solvent system, then the analysis may be flawed (cf. Singh et al., 2006).

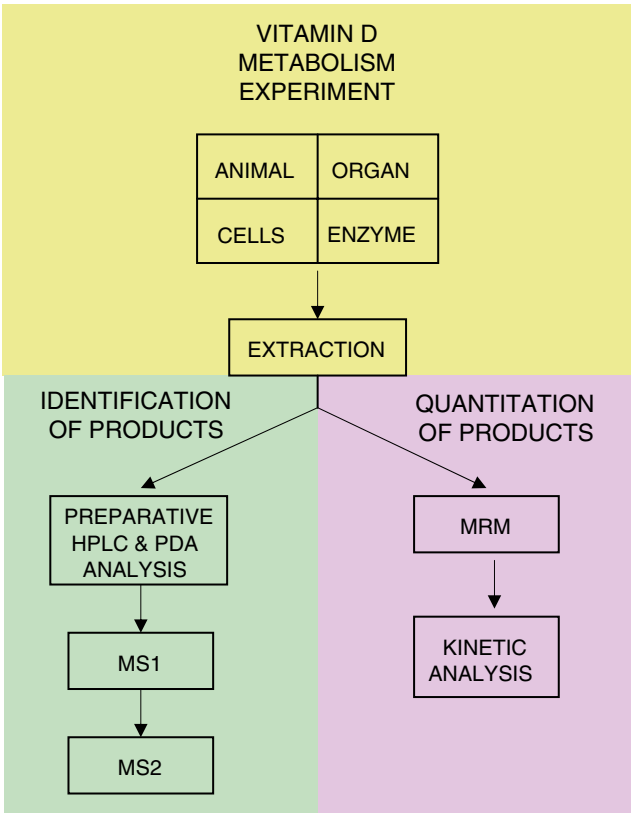


Fig. 11.27 Role of LC-MS/MS in a vitamin D metabolism experimental scheme. This figure illustrates the versatile nature of LC-MS in both qualitative and quantitative applications. LC-MS /MS is a technique which can serve as a critical endpoint in vitamin D metabolic studies, where the main objective is partially to identify (MS1/MS2) or to quantitate SRH (MRM) metabolic products. It should be noted that the methodology incorporates liquid/liquid and solid-phase extraction techniques which facilitate the concentration and partial purification of metabolites from a variety of experimental matrices; ranging from plasma acquired from *in vivo* experiments to reconstituted CYP preparations incubated with vitamin D substrates. Generally, the approach involves a qualitative assessment of metabolites (as shown on the left) which sets the stage for a more quantitative approach incorporating MRM or SRM measurements (as shown on the right). Overall, the LC-MS/MS-based methodology is far simpler and more convenient than steps used in the past which incorporated off-line GC-MS. Simplicity and convenience however do not always deliver the required specificity and precision.

requiring inefficient, laborious and time-consuming pre-purification, derivatisation and loading techniques. The consequence is a much reduced real-life sensitivity. Our rule of thumb is that GC-MS should not even be attempted unless the analyst has between 500 ng and 1 μg of pure vitamin D metabolite. In contrast, although LC-MS and LC-MS-MS are inherently less sensitive because of poor ionisation, both

techniques gain significantly because of on-line sample introduction and skipping the need for an obligatory derivatisation step. The result is that LC-MS-MS techniques can be routinely carried out on peaks containing as little as 10–25 ng and without the lengthy delays associated with GC-MS. On the other hand, the superior ionisation and fragmentation patterns achieved with GC-MS combined with its excellent ability to resolve isomers are still unsurpassed by LC-MS techniques.

The mainstay of most quantitative LC-MS-MS protocols is the technique referred to as multiple (or single) reaction monitoring (SRM or MRM), which requires MS-MS and involves measuring the transition in the collision cell between a specific parent ion and its chosen fragment ion, that is pre-selected for each analyte under investigation (see Fig. 11.27, right). The data are expressed as a specific mass chromatogram for each parent/fragment pair, typically containing a single integratable peak (Fig. 11.26, left bottom). The peak can be quantitated when compared to the recovery of a suitably labelled (^{2}H-or ^{13}C-) internal standard. The improved sensitivity of MRM arises from the elimination of all background ions except the selected parent ions and the subsequent elimination of all contaminant signals that do not give rise to their specific, pre-selected fragment ions. Even co-eluting analytes can be resolved, provided they differ in molecular weight and/or fragmentation pattern. Conversely, chromatographic resolution is essential for positional or configurational isomers that give rise to identical parent and fragment ions, and therefore cannot be resolved by mass spectrometry alone (e.g. 1α,23(S),25-$(OH)_3D_3$ and 1α,24(*R*),25-$(OH)_3D_3$). The recent report (Singh et al., 2006) of the failure of some LC-MS-based assays of 25-OH-D (25-OH-D_2 and 25-OH-D_3) to resolve the presumed inactive 3-epimer of 25-OH-D_3 in neonatal plasma samples and therefore give falsely high values for blood 25-OH-D illustrates the limitations of LC-MS. In summary, current LC-MS (reviewed by Higashi et al., 2009) offers great promise as a rapid, fairly accurate routine tool to provide quantification of vitamin D metabolites but has not yet become the 'gold' standard which many advocates would like it to be.

Several groups have reported sensitive LC-MS methods to quantitate metabolites of vitamins D and analogues in biological fluids (Higashi et al., 2001a, b, 2002c, 2003; Kissmeyer et al., 2001; Maunsell et al., 2005; Tsugawa et al., 2005; Saenger et al., 2006). Limits of detection range from 200 pg (Higashi et al., 2002a) to 20 pg (Kissmeyer and Sonne, 2001) based upon extractions from plasma samples. Despite this seemingly high level of sensitivity, ionisation efficiencies for non-polar steroids using ESI and APCI are relatively low, in comparison to more polar compounds (Higashi et al., 2004), a situation which has prompted investigators to explore new derivatisation techniques to enhance ionisation efficiencies (Vreeken et al., 1993; Higashi et al., 1999d, 2001a, b; Weiskopf et al., 2001; Higashi and Shimada, 2004; Higashi, 2006). Higashi, Awada and Shimada (2002c) demonstrated that derivatisation of 1α-OH-D_3 with the reagent 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydro-quinoxalyl)-ethyl]-1,2,4-triazoline-3,5-dione (DMEQTAD) and by acetylation, dramatically enhanced the limit of detection from 200 pg to 2.5 pg using APCI in the positive ion mode. Cookson-type reagents (4-substituted 1,2,4-triazoline-3,5-diones or TAD) react with the vitamins D to form a Diels-Alder adduct containing proton-affinitive

atoms of nitrogen and oxygen. Derivatisation, apart from anything else, also increases the mass of the analyte and is thus likely to increase the signal-to-noise ratio by reducing the interference with background ions at a lower *m/z* values (Higashi et al., 2002a; Higashi and Shimada, 2004). Improved ionisation in the LC–ESI(+) mode by derivitisation with PTAD has been used (Higashi et al., 2008) to develop an LC-MS/MS method for 25-OH-D_3 in human saliva with an LOQ of 2 pg/mL – this method uses 1 mL of saliva, deproteinised with acetonitrile and purified using a Strata-X cartridge before the formation of the PTD adduct. Incorporation of 5 mM methylamine in the LC mobile phase further improved sensitivity, forming an adduct ion $[M + CH_3NH_3]^+$ (*m/z* 607), which was easily transformed into *m/z* 298 (an A ring fragment) by CID. This method gave results ranging between 4 and 15 pg/mL (7–37 pmol/L) which are remarkably close to the estimated value of 8 pmol/L reported 27 years ago (Trafford and Makin, 1983) using GC-MS.

The formation of these off-line derivatives such as TAD adducts is reminiscent of the earlier work of the Vouros group which used continuous flow FAB of 4-phenyl-TAD adducts of vitamin D and its metabolites in a tandem mass spectrometer (Yeung et al., 1992, 1993). The FAB mass spectra produced in M1 showed an intense peak at *m/z* 298 and for 1α-hydroxylated vitamin D metabolites this peak shifted to *m/z* 314. By monitoring these fragments in M2 after collision-induced dissociation of MH^+, selected by M1, sensitive and specific measurements of vitamin D and its metabolites could be affected detecting 100–300 pg of all metabolites examined. In this system, the detection limit for 1α,25$(OH)_2D_3$ was 150 pg (with a signal-to-noise ratio of 2.3:1). Early on, Vreeken et al. (1993) also tried LC-MS using thermospray of phenyl-4-TAD adducts of vitamin D_3, 25-OH-D_3, 1α,25-$(OH)_2D_3$, 24,25-$(OH)_2D_3$ and 1α-OH-D_3 and reported a sensitivity of 1–7 nmol/L, which was found to be insufficient for use in the analysis of serum or plasma. Thus, the use of derivatisation in LC-MS is not new but the evidence documents that it does increase sensitivity. Nevertheless, it must be said that enhancing sensitivity by increasing ionisation is inconvenient (cf. need for derivatisation for GC-MS) and (unlike derivatives for GC-MS) does not provide further structural information as these TAD adducts are formed across the 5,7-diene structure common to vitamins D and their metabolites. Use of DMEQ-TAD adducts allowed the development of an LC-APCI(+)-MS/MS method for vitamins D_2 and D_3, and 25-OH-D_2 and 25-OH-D_3 in 6 mL samples of human breast milk (Kamao et al., 2007) and, using 4-phenyl-TAD adducts, the development of a metabolic profile of vitamin D metabolites (25-OH-D_2, 25-OH-D_3, 1α,25-$(OH)_2D_2$, 1α,25-$(OH)_2D_3$ and 24*R*,25-$(OH)_2D_3$) in 0.5 mL serum with a lower limit of quantitation of 25 pg/mL by using UPLC-ESI(+)-MS/MS and MRM (Aronov et al., 2008).

11.4.3.3 LC-MS Using Atmospheric Pressure Photoionisation (APPI)

A relatively new ionisation strategy, termed atmospheric pressure photo-ionisation, is being applied to LC-MS instrumentation. ESI and APCI are based upon the

voltage-induced addition of a charge (typically, in the form of $[M + H]^+$); however, the efficiency of ionisation varies greatly based on the charge affinity of the analytes (Syage and Evans, 2001). The APPI apparatus contains a photon-emitting gas discharge lamp, in place of the corona discharge pin in APCI (Hayen and Karst, 2003). Upon photo-excitation of the analyte, an electron is ejected to form $[M]^+$, provided the photon energy is greater than the ionisation potential of the analyte. The $[M]^+$ ion subsequently forms $[M + H]^+$ in the presence of a protic solvent. APPI mass spectrometry purports to provide relatively uniform ionisation efficiencies for a broad range of compounds and does not discriminate against non-polar compounds (Syage and Evans, 2001). In some preliminary reports, APPI analysis of certain compounds, including progesterone, exhibit an order of magnitude increase in signal if toluene or acetone dopants are added to the mobile phase post-column (Hanold et al., 2004). The dopants act by first becoming ionised themselves, leading to the enhanced ionisation of the surrounding analyte molecules (Hayen and Karst, 2003). Although no exhaustive evaluation of APPI in vitamin D analysis has been reported to date, initial results reveal excellent signal linearity (correlation: 0.999) from 1 to 1,000 ppb for vitamin D_3 (Hanold et al., 2004). Interestingly, the addition of toluene or acetone increased background noise or decreased ionisation efficiencies for vitamin D_2, respectively, as well as for other lipid-soluble vitamins including A, E and K (Hanold et al., 2004). If the advantages of APPI hold true for vitamin D analysis, limits of detection well into the physiological range could be possible, without the need for derivatisation. There is a recent publication of the use of LC-APPI-MS/MS for the measurement of 12 steroids, including 25-OH-D_3, in 200 μL of human serum taking a total of 11 min – this method claiming 10–12% within and between day (batch) precision with a lower limit of detection of 1.5 pg/mL for 25-OH-D_3 (Guo et al., 2006). Quantitative ESI- and APCI-based studies of vitamin D metabolism require deuteriated internal standards for each possible metabolite because of differences in ionisation efficiencies of the variably polar products. This is not economically feasible, and possibly chemically unfeasible for side-chain cleaved products; however, it is possible that only a single internal standard would suffice if APPI could provide similar ionisation efficiencies among the metabolites.

11.5 Nuclear Magnetic Resonance Spectroscopy (NMR)

11.5.1 Introduction

In combination with mass spectrometry, which provides the molecular formula, a structure for almost any chemical entity under investigation (usually as a relatively pure compound) can be deduced using modern NMR techniques (see Chapter 2 for a general discussion of the concepts and methods involved). NMR is by far the most informative spectroscopic technique for the elucidation of molecular structure,

providing not only information on the connectivity of atoms (leading to the gross constitutional formula) but also conformational and configurational information, sometimes on a par with the definitive tool, X-ray crystallography.

NMR in the realm of vitamins D analysis (as in other fields) is employed in routine confirmation of the expected structure of targeted synthetic products and in estimation of purity, possibly including assessment of the degree of isotopic enrichment. It is used in following reaction progress, including stability testing in quality control. Structural identification of degradation products and unexpected products in laboratory synthesis and manufacture is also of great importance, as is that of metabolites isolated from *in vitro* or *in vivo* studies. As regards structural information, emphasis is placed on the distinction of isomers (position isomers, establishment of configuration at one or more stereogenic centres, or of double bond geometry) and conformational analysis with a view to structure–function correlation. NMR is also used to investigate reaction mechanisms and the nature of protein–ligand interactions. In the remainder of this Section, some of these broad (and not mutually exclusive) aspects are illustrated by selected examples (without attempting to be comprehensive). Note that the emphasis is on structural elucidation, i.e. qualitative analysis. The technique is not normally optimal for quantification, except in relative terms as regards mixtures of related compounds.

11.5.2 Routine Confirmation of Expected Structure of Targeted Synthetic Products. Conformational Analysis with a View to Structure–Function Correlation

For compounds (including synthetic intermediates) to be classified as belonging to the vitamins D series, it is assumed that the skeletal structure is basically that of the calciferol secosteroidal nucleus (i.e. C-1 through C-17) (Fig. 11.28). Although seco-calciferol compounds, lacking one or more of the ring bonds (e.g. Reddy et al., 2007), and synthetic intermediates that are truncated between C-6 and C-8 also qualify for inclusion, context permitting ("A-ring building blocks," "C/D-ring building blocks"), these will not be discussed here.

Routine assignments of the proton (^{1}H) and carbon-13 NMR spectra of new calciferol D compounds abound in the synthetic chemistry literature. (For the prototype vitamins D ^{13}C NMR signal assignments, (Tsukida et al., 1975; Berman et al., 1977 and Kruk et al., 1985) and for some full proton and carbon chemical shift assignments, (Dauben and Funhoff, 1988 and Mizhiritskii et al., 1996). Any published errors are, as far as possible, corrected in the present account.) Collected in Table 11.7 are ^{13}C-NMR data for selected standard vitamins D compounds displaying some commonly encountered structural features (full structures can be reconstructed from the partial structures/fragments drawn in Figs. 11.28 and 11.29). These data provide starting points for checking the assignment of signals in the spectrum of a new compound in the appropriate series, and expedite complete assignment. It is valuable to notice that only small changes in the partial spectrum (run under similar

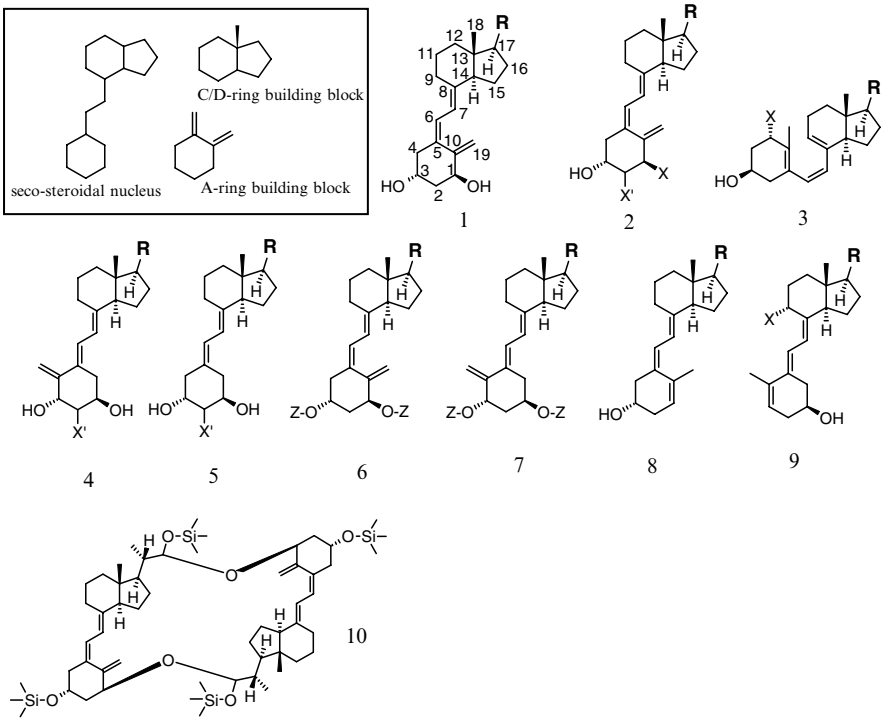


Fig. 11.28 Seco-steroidal structures. **1**: 1α-OH-D (with atom numbering), **2**: D (when X = X′ = H); generally 1α-X-2X′-D, **3**: pre-D (X = H); 1α-X-pre-D, **4**: 5*E*-1α-OH-D (X′ = H); 2-X′-5*E*-1α-OH-D, **5**: 19-nor-1α-OH-D (X′ = H); 2-X′-19-nor-1α-OH-D, **6**: 1α-OH-D-1,3-*bis*-O-[trimethylsilyl (TMSi) ether] (Z = $SiMe_3$), **7**: 5*E*-1α-OH-D-1,3-*bis*-O-[*t*-butyldimethylsilyl (TBDMSi) ether] (Z = $SiMe_2Bu^t$), **8**: 5Z-iso-D, **9**: iso-D (X = H); 9α-X-iso-D, **10**: 1α,21′-epoxy-20S-methylpregnacalciferol-3,21-bis-O-TMSi ether dimer (see text)

conditions) of a compound are attendant on relatively remote structural modification – compare, for example, values for the corresponding side-chain signals (C-20 to C-27) in the ^{13}C-NMR spectra (in $CDCl_3$ solution) of various derivatives possessing the unmodified D_3 side chain (see also cholesterol (compound 9) in Chapter 2, Table 2.15 and Fig. 2.56). The correspondence is within 0.2 ppm. (Variations of this order in reported δ-values even for the same compound are common and can be attributed to experimental factors and are not significant (which is why ^{13}C chemical shifts are quoted here with only one decimal place). Similarly, the respective ^{13}C signals due to the seco-steroidal nucleus (C-1 to C-19) (e.g. vitamin D or 1α-OH-vitamin D) correspond closely within a series of side-chain-modified compounds (see D_3, D_2, or calcipotriol, in Table 11.7).

Even for the case of a relatively pure single compound, it should be noted that intermolecular interactions are still manifested in the NMR spectrum, which is, for example, dependent on the solvent. The standard solvent deuteriochloroform is

Table 11.7 ^{13}C-NMR data for some selected standard vitamins D compounds

Compound	**3a**[a]	**2a**[b]	**2a**[c,f]	**2a**[c,f]	**1a**[d]	**1a**[d,f]	**7a**[e]	**2b**[b]	**1b**[d]	**1c**[e]	**1d**[e,g]	**6e**[e]	**10**[e]
Nucleus (C-1 to C-19)	preD	D	D (alpha)	D (beta)	1α-OH-D	1α-OH-D beta	1α-OH-5*E*-D bisTBDMSi ether	D	1α-OH-D	1α-OH-D	1α-OH-D	1α-OH-D bisTMSi-ether	1α-OR-TMSi-ether
C													
1	29.8	32.1	33.7	31.6	71.0	67.3	70.3	32.0	71.0	70.8	71.5	71.6	77.1
2	30.9	35.3	38.9	35.3	43.0	43.0	44.0	35.3	43.0	42.9	43.8	44.8	42.5
3	67.5	69.2	70.2	66.8	67.0	66.9	67.3	69.2	67.1	66.9	67.4	67.3	66.5
4	37.4	46.0	44.6	44.0	45.4	45.9	36.6	46.2	45.5	45.3	45.3	45.9	46.7
5	125.6	135.3	137.6	135.6	133.1	132.4	135.3	135.4	133.1	133.0	135.7	135.4	133.6
6	128.5	122.5	119.7	121.6	125.2	126.2	121.8	122.4	125.2	125.0	124.9	123.1	124.6
7	128.8	117.8	120.3	117.1	117.2	117.0	116.4	117.8	117.2	117.1	119.0	118.3	117.6
8	136.4	142.2	139.2	140.1	143.5	143.6	143.5	142.2	143.4	143.0	142.6	140.3	142.6
9	124.6	29.1	28.5	31.7	29.3	30.6	29.0	29.1	29.3	29.1	30.0	28.9	29.0
10	129.8	145.3	145.8	147.6	147.8	148.4	153.7	145.3	147.9	147.7	149.9	148.0	143.9
11	24.8	23.7[i]	24.6	25.1	23.8	25.3	23.6	23.7[i]	23.8	23.6	23.3	23.4	23.3
12	39.9	40.6	41.3	41.9	40.7	41.2	40.6	40.4	40.6	40.4	42.0	40.3	40.7
13	41.9	46.0	45.6	46.6	46.1	47.4	46.0	46.0	46.1	45.9	46.2	46.2	45.9
14	50.6	56.5	57.0	57.4	56.6	57.0	56.5	56.5	56.7	56.4	57.6	55.6	56.1
15	23.3	22.4[i]	23.9	23.3	22.5	22.1	22.3	22.4[i]	22.5	22.3	22.0	22.6	22.9
16	28.3	27.8	29.0	28.5	27.9	30.2	27.7	27.8	28.0	27.7	28.8	26.5	28.1
17	54.2	56.7	58.2	53.8	56.8	57.7	56.7	56.5	56.7	56.1	58.0	51.5	52.2
18	11.1	11.9	13.2	12.1	12.2	14.1	12.1	12.3	12.5	12.3	12.4	13.6	13.3
19	19.7	112.5	111.6	111.8	112.0	109.0	106.6	112.5	112.0	111.8	112.0	111.6	116.9
20	36.1	36.2	38.8	35.7	36.3	38.9	36.2	40.4	40.6	39.9	37.5	49.8	44.2
21	18.7	18.9	19.1	19.9	19.1	18.4	18.9	21.1[i]	21.3[i]	20.5	19.4	12.4	11.7
22	36.0	36.2	37.5	33.8	36.3	37.1	36.2	135.7[i]	135.8	138.0	37.8	204.9	96.4
23	23.7	24.0	26.0	19.1	24.1	26.0	23.9	132.0[i]	132.2	129.0	24.7		

	24	39.3	39.6	40.1	41.1	39.7	37.9	39.6	42.8	43.0	77.3	47.0		
	25	28.0	28.1	26.0	26.6	28.2	29.0	28.1	33.2	33.3	17.7	71.5		
	26[h]	22.5	22.7	22.9	21.7	22.8	22.1	22.6	19.7[i]	19.9	3.1	29.2		
	27[h]	22.8	22.9	23.1	23.8	23.1	22.8	22.9	20.0	20.2	1.9	29.3		
	28								17.7[i]	17.8[i]				
Side chain (C-20 to C-28)		D3	D3	D3	D3	D3	D3	D3	D2	D2	Calcipotriol	25-OH-D3	20(*S*)-formyl-pregnane	(bis-oxy-methyl)-pregnane (see text)

Structures are derived from Figs. 11.28 and 11.29 with X = X' = H.
Solution spectra, solvent $CDCl_3$, except where indicated thus:
Sources: Refs. [a]Dauben and Funhoff (1988);
[b]Tsukida et al., (1975);
[c]Olsen et al. (2003);
[d]Kolodziejski et al., (2005)
[e]Calverley MJ, unpublished;
[f]solid state spectra
[g]solvent CD_3OD;
[h]Interchangeable within column.
[i]Incorrectly assigned in source data.

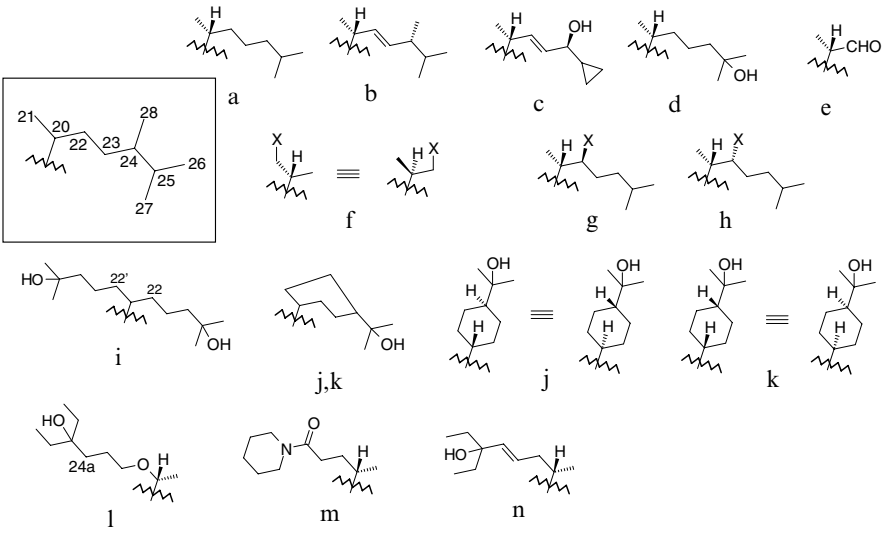


Fig. 11.29 Steroidal side chains (substituent R in Fig. 11.28). R= (*inter alia*) a: as in D_3, b: as in D_2, c: as in calcipotriol, 1: as in KH1060 (lexacalcitol), m: as in ecalcidene, n: as in Ro-26-9228/BXL628 (elocalcitol)

routinely employed as a relatively non-polar, good solvent, and supplementary information can be provided by substitution (or titration) with deuteriobenzene (which produces strong solvent-induced shifts), or with a polar solvent (e.g. deuteriomethanol), when hydrogen bonding, for example, causes a hydroxyl group in effect to become more bulky. It is important to bear in mind that whereas the X-ray structure, like a 3D drawing, of the molecule, provides a "snapshot" view of the relative arrangement (conformation, as well as configuration) of the constituent (non-hydrogen) atoms, the NMR spectrum under normal conditions in solution gives signals for the magnetic nucleus under scrutiny (usually ^{1}H or natural abundance ^{13}C) representing the weighted average of all participating conformations in a dynamic equilibrium, the signals from which can have significantly different chemical shifts and coupling patterns. Measurement of two (or more) low-energy conformations that contribute significantly to the (room temperature) equilibrium can in favourable cases (viz. when the interconversion energy barrier is sufficiently high) be achieved by the so-called dynamic NMR; cooling the solution below the temperature at which coalescence occurs on the NMR time-scale, thus "freezing out" the participants. In the solid-state MAS (magic angle spinning) NMR, again single conformers are analysed, except in regions of the molecule that are very flexible (very low energy barriers to interchange), such as the terminal region of an aliphatic C-17 side chain.

A particularly well-investigated conformational aspect of vitamin D is the existence of two contributing A-ring conformations of similar stability and with a relatively high activation energy for interconversion (ca. 9.5 kcal/mol for the

1α-hydroxylated compounds), a fascinating consequence of the fact that a planar vitamin D triene system is not a stable conformation (in a molecular model, the 7- and the proximal 19-protons would occupy the same space). The alpha-conformation then is defined as having the twist in the triene system (about the 5,10-bond) that places the exocyclic C-10(19) below the plane of the 5,7-diene in the conventional representation. In both the α and β (in which the C-10/C-19 axis correspondingly points above the plane of the 5,7-diene) forms of vitamin D, the A-ring cyclohexane is assumed to adopt the familiar chair conformation, thereby fixing each ring substituent (or proton) in the axial or equatorial position. Thus, for 1α-hydroxyvitamin D the hydroxyl groups on carbons 1 and 3 are axial and equatorial, respectively, in the α-chair form; and equatorial and axial, respectively, in the β-chair form (cf. Figure 11.30).

An early proton-NMR study on vitamin D_2 (La Mar and Budd, 1974) analysing the observed geminal proton coupling constants revealed that these alpha and beta chair forms were represented in about equal amounts (CCl_4 solution). This study is of additional interest in the utilisation of a lanthanide shift reagent to resolve overlapping signals to make the analysis as comprehensive as possible. Further refine-

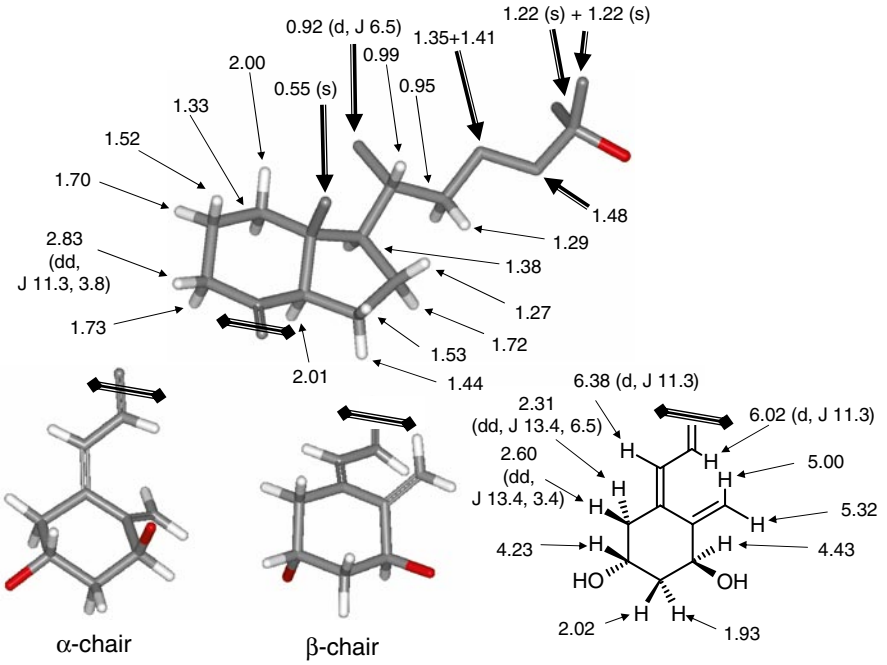


Fig. 11.30 3-Dimensional representation of 1α,25-$(OH)_2D_3$. Illustrates extended side chain conformation and C/D ring system as seen in the X-ray structure, combined with approximations of the two chair forms of the A-ring. Chemical shifts (δ ppm, solvent: $CDCl_3$) of carbon-bonded proton signals (multiplicity (complex multiplets unless indicated otherwise: s-singlet, d-doublet, etc.), coupling constant, *J* in Hz) are indicated (the heavy arrows indicate multiple protons)

ments showed a slight predominance of the alpha-form (57:43) in $CDCl_3$ (or 52:48 in cyclohexane) which increases with the equatorial preference of the 3-hydroxyl group (hydrogen bonding in polar solvents, e.g. CD_3OD, or derivatisation with a bulky group) (Wing et al., 1975; Helmer et al., 1985). For 1,25-dihydroxyvitamin D_3, the situation is similar, and a 45:55 alpha/beta mixture was estimated. In a subsequent analysis, the situation in a polar solvent (CD_3OD) prompted Eguchi and Ikekawa (1990) to propose an alternative, beta-half-chair (also 55%), conformation as the likely contributing beta species. "Freezing out" the two conformers has been achieved in the 1α-hydroxylated compound series for 1α,25-dihydroxyvitamin D_3 (Eguchi and Ikekawa, 1990). Although the resolution was low because of viscosity of the solvent (CD_3OD, with or without $CFCl_3$), approximately equal division of several (broad) signals (3α-, 4α- and 6-Hs) in the ^{1}H-NMR at −100°C was unequivocal. After an initial foray reported in the same paper into dynamic ^{13}C-NMR analysis at natural abundance, the work was crowned by the publication 1 year later of the results obtained with the specifically labelled 19-[^{13}C]-isotopomer of 1α-hydroxyvitamin D_3 (Eguchi et al., 1991). At −100°C (CD_3OD) two completely resolved signals (48:52) for C-19 appeared (ca. 115 and 108.5 ppm) coalescing around −85°C, and eventually averaging to the sharp averaged signal at 112 ppm (cf. value for 1α,25-dihydroxyvitamin D_3 in Table 11.7) at ambient temperature. Thermodynamic parameters for the energy of interconversion of the α and β A-ring conformations were estimated. (In a footnote, the authors recorded the failure of a similar analysis of 19-[^{13}C]-vitamin D_3 itself: a sufficiently low temperature to observe coalescence could not be approached.) A natural abundance dynamic NMR study of 4,4-dimethyl derivatives had appeared already (Berman et al., 1978).

Both 1α-hydroxyvitamin-D_2 and -D_3 crystallise with the A-ring exclusively in the beta conformation, and the X-ray structures have been published (Kolodziejski et al., 2005). The same publication also documents the solid-state (MAS) ^{13}C-NMR spectra of these important compounds including a careful comparison with the solution spectra ($CDCl_3$). Of particular interest are the large differences in the chemical shifts (Δδs) for C-1 and C-19 (see Table 11.7). Indeed, the value of 109 ppm for C-19 in the crystalline beta form is strikingly similar to the solution value for the "frozen out" β-form discussed above. The cyclic "head-to-tail" dimer (compound **10**, Fig. 11.28) furnishes an ideal model compound for conformational analysis of the A-ring, which here is geometrically "locked" in the α-chair conformation (sometimes, the term "locked" is used erroneously to describe a situation where one conformation is only "biased"). This compound was isolated as a silyl ether-trapped hemi-acetal by-product from the transformation of compound **1e** to compound **6e** (Calverley, 2003). The A-ring detail from the modelled structure is illustrated in Fig. 11.31, which includes the measured ^{1}H-NMR data (note the W-coupling observed between the C-2 and C-4 α-protons typical of a rigid system). Examining the ^{13}C-NMR (Table 11.7), noteworthy is the δ_C value observed for C-19, namely 117 ppm, which is close to (albeit even higher than) the "frozen out" value (Eguchi et al., 1991) for the parent system (115 ppm). Furthemore, the value for C-1 in compound **10** (77 ppm) approaches the value required of the alpha-form

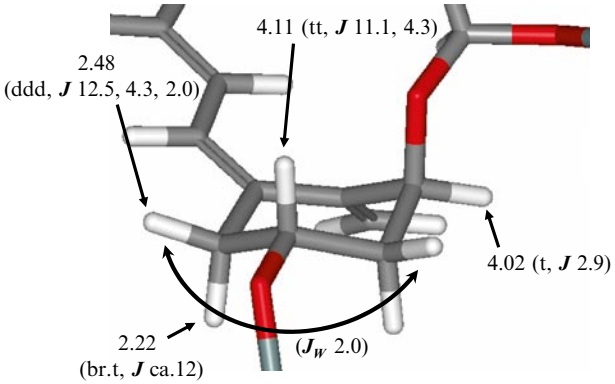


Fig. 11.31 Clip of modelled structure of the head-to-tail dimeric compound 10. Illustrates the locked A-ring system imposed by the bridge feature (not shown). Germane proton chemical shifts (δ ppm, solvent: $CDCl_3$) and couplings (*J* in Hz) are indicated. The configuration at C-22 is arbitrary

to calculate a weighted average (45:55) of around 71 ppm, taking the beta-form value from the solid-state NMR in compound **1a** (67.3 ppm).

For the α and β conformers of vitamin D_3, the Δδ values obtained from solid-state ^{13}C-NMR (Olsen et al., 2003) of the co-crystalline 1:1 mixture are also considerable, though difficult to correlate with the 1α-hydroxylated data (Kolodziejski et al., 2005), and the individual mean values need adjusting systematically (subtracting about 1 ppm) to approach the literature $CDCl_3$ solution values. This Mueller group paper (Olsen et al., 2003) is important in that it shows for the first time that 2D ^{13}C natural abundance correlation in solids can be extended to moderately sized molecules. A uniform-sign cross-peak double-quantum-filtered correlation experiment (UC2QF COSY) was introduced to completely assign the 2 × 27 peaks of the solid-state NMR spectrum of microcrystalline vitamin D_3. A comparison with *ab initio* calculations of the chemical shifts of the two conformers based on the known crystal coordinates was also made as a cross-check.

The question as to which of the two ring A forms constitutes the receptor-active form of the hormone, 1α,25-dihydroxyvitamin D_3, has been frequently addressed. The answer was finally revealed (it is the beta-form) following on the X-ray studies of a VDR-construct complex (Rochel et al., 2000). Although not as yet with the VDR as host, several NMR studies on vitamins D guest/host complexes have been reported. An early study (Szejtli et al., 1980) demonstrated water-soluble complex formation of the parent vitamin D_3 with a β-cyclodextrin derivative (a 1% solution of vitamin D_3 in D_2O was achieved), and the ^{13}C-NMR showed shifted δ values for C-5 and C-18 (relative to $CDCl_3$) that can now be seen to agree with expectations from the recent solid-state data, if the complexed form has the alpha-chair. In addition to extending the range of solubilisation procedures, a study by the Okamura–Mueller group (Okamura et al., 2002) employed specifically triply [7,9,19]-^{13}C-labelled vitamin D_3, together with its hydroxylated metabolites. Analysis of the ring A proton coupling

constants ($J3\alpha,4\beta$ = 11 Hz; cf. Figure 11.31 for the "head-to-tail" dimer compound **10**) indicates the exclusivity of the α-chair conformations in micellar solution. Measurement of distances between protons on the labelled atoms in micellar solution by a NOESY experiment revealed that the distance between the 19- and 7-protons in the 25-hydroxyvitamin D_3 complex is shorter than that in the X-ray structure of the free ligand, which for this compound crystallises in the α-conformation.

Some structurally modified analogues of calcitriol have been designed to probe the active A-ring conformation using NMR as a tool. Typically, analogues where one or more protons or a hydroxyl group have been replaced by fluorine are biologically competent mimics, and this assumption has been elegantly exploited in designing candidates for analysis by ^{19}F-NMR spectroscopy. Thus, Ohno et al. (2002) examined in detail static and dynamic ^{19}F-NMR of the 4,4-difluoro and the both of the 19-monofluoro (*E* and *Z*) derivatives of vitamin D_3 and 1α,25-dihydroxyvitamin D_3, and were able to "freeze out" the α and β A-ring conformers (e.g. for 4,4-difluoro-vitamin D_3, a 79:21 alpha/beta ratio was deduced). The NMR characteristics gleaned for the various species were put to use in conformational studies with solvents of varying polarity, with a view towards future studies with the VDR, for which the hydroxylated analogues were indeed shown to be significant agonists as regards both binding and transactivation.

The biological consequences of introduction of an alpha or beta substituent at the 2-position of 1α-hydroxy calciferols (to bias one A-ring chair conformation over the other) has been explored extensively (2d, X′ = Me: Konno et al., 2000), also in the 5*E*-isomer series (4, X′ = alkyl; all possible 1,2,3-configurations: Fujishima et al., 2001) and especially in the 19-nor series (5, X′ = alkoxy, alkyl or ethylidene (Sicinski et al., 1994; Sicinski et al., 1998 , 2002; Arai and Kittaka,

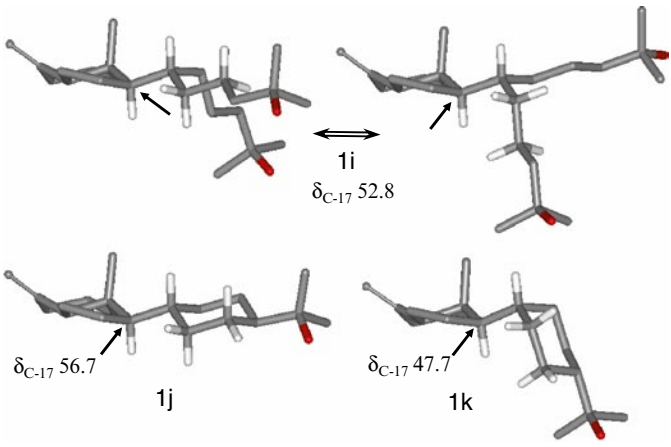


Fig. 11.32 Partial structures of compounds **1i**, **1j** and **1k**, illustrating suggested low-energy conformations to rationalise the chemical shift disparities for C-17. 1i has two similar forms in dynamic equilibrium, while 1j and 1k each have one preferred form. The formal distinction of the oxygen atom (red) from the geminal methyls is arbitrary

2006)). The synthetic studies include routinely a conformational analysis by ^{1}H-HMR (often including NOE measurements).

Delaroff et al. (1963) first demonstrated, by analysing the diene proton coupling constants, that the predominant solution conformation of vitamin D_3 has the transoid (6-*s-trans*) form. The 6-*s-cis* conformer must, however, be invoked as an intermediate in calciferol-precalciferol equilibrium (see Section 11.5.6), and has even been proposed as a candidate for the receptor-active species. An 11-Fluoro-vitamin D_3 derivative was designed as a probe for an ^{1}H-NMR study (Zhu et al., 1994) to detect any significant participation of the 6-*s-cis* conformer but the result was negative.

NMR spectroscopy in the analysis of vitamin D side-chain conformations has been the subject of many studies, again in the pursuit of the "active" conformation associated with receptor-mediated biological effects, or just optimal receptor binding. In this regard, attention is drawn to the discovery of biologically super-agonistic analogues in the (unnatural, synthetic) 20-epi-calciferol (Calverley et al., 1991) series. Conformational analysis confirmed by molecular modelling shows that because of steric crowding, the proton on C-20 prefers to be essentially anti to that on C-17 in the stable conformations in both series, thus placing carbon 22 at opposite positions between normal and epi side chains (Midland et al., 1993). Compounds that are 20-epimers of the same side chain are distinguishable NMR spectroscopically by a rule based on the experimentally observed chemical shifts; the 21-proton signals resonate consistently at higher field in the epi series (by ca. 0.1 ppm), and in the ^{13}C-NMR C-22 resonates higher (by ca. 0.4 ppm, variable). As would be expected, these $\Delta\delta'$s are found to obtain internally in the simplest model compound 1f, and the rule also appears to hold for the interesting situation where TWO side chains diverge from C-20 (20-double, also nicknamed "Gemini", **b**oth the **e**pi and the **n**ormal side chains (incipient in **1f**) can be traced out in these "*bEaN*" compounds) and where similar $\Delta\delta$ tendencies obtain (at least for δ_H; for **1i** the carbon signals for C-22 are coincident, though this is not the case for its 23,23′-dioxa derivative ($\Delta\delta$ 0.5 ppm), nor for the cyclic "bEaN" compounds **1j** and **1k** (0.2 and 0.7 ppm respectively)). (A special numbering system for the epi side chain in "double" side-chain compounds is recommended here: C-21 is considered to be absent and positions after C-20 continue thus: C-22′, -23′, etc. to distinguish those carbons from the corresponding ones in the normal side chain (e.g. 25,25′-dihydroxy-20-double-21-nor-D_3,D_3′ in Fig. 11.29: **1i**).) It has been suggested that the two side chains in the double side-chain compound (**4h**) prefer to adopt respectively conformations about the 20,22-bond where one is extended and the other gauche (see Fig. 11.32) (Calverley, 2001). This hypothesis followed from the observation of a striking difference in the ^{13}C NMR chemical shift values for C-17 between the two cyclohexane compounds wherein these types of conformations are enforced (*trans* compound **1j**: 56.7 ppm; cis compound **1k**: 47.7 ppm). For comparison, the values for calcitriol (**1d**) and its 20-epimer are 56.4 and 56.0 ppm, similar to 1j, while for the double side-chain compound (**1i**) the value observed is 52.8 ppm, almost an average of **1j** and **1k**. In this connection, it may be noted that the X-ray structure of a double side-chain compound (albeit the 23′-oxa version of the double 25-hydroxy D_3 side chain (i) (on a cholesterol nucleus) (Galdecka et al., 1999)) reveals precisely this distribution of the side chains.

The effect of a substituent on the side-chain carbon signals of the D_3 side chain is configuration-dependent and has been studied for 22 and 24 substituents in the cholesterol series (Letourneux et al., 1975; Koizumi et al., 1979). Also, for the 1α-hydroxy vitamin D_3 series the dramatic Δδ for C-23 (around 6 ppm) obtains between 22*R*- and 22*S*-isomers, and is explained in terms of the different preferred conformations. The extended D_3 side-chain conformation as seen in the X-ray structures is still energetically favourable in the 22*S*-hydroxy or methoxy compounds, while a gauche conformation is preferred in the 22*R* compounds, to avoid steric crowding of the C-16 protons (Eguchi et al., 1989). A similar effect (opposite configurations, like a mirror image situation) obtains in the 20-epi-series of 22-oxy compounds (Calverley et al., 1994), evidently to avoid crowding the 12β-H, now by a 22′*S* substituent (the proximity of position 22 is also apparent from the ^{1}H-NMR of 22-oxa-20-epi compounds, where a significant deshielding of H-12β (e.g. 2.15 ppm in lexacalcitol KH1060, compound 1l) relative to the 20-normal isomer (1.98 ppm, which is the same value as in the parent vitamin D (see Fig. 11.30); for H-12α, the values are 1.35 and 1.30, respectively, in the oxa-compounds). The lower biological activities associated with the 22*R* (20-normal) oxy series was an early milestone in probing the active side-chain conformation.

The proton (^{1}H) NMR spectra assignments for 1α,25-dihydroxyvitamin D_3 are indicated in Fig. 11.30, including distinction of the geminal protons where established (α and β twins are often distinguished in absolute terms by an analysis of the geminal coupling constants or chemical shifts. Nuclear Overhauser effect (NOE) studies have also been extensively employed.). Notwithstanding the definitive assignments of the two signals for the (*E* and *Z*) protons on carbon 19 following on a conclusive nuclear NOE study for vitamins D_3 and D_2 in which the C-7 and each of the C-19 protons were separately irradiated (Kotovych et al., 1980), an erroneous earlier tentative assignments has unfortunately been perpetuated in the literature. The confusion is compounded by the fact that the formal interchange of *E*/*Z* descriptors between vitamin D and 1α-hydroxyvitamin D is frequently overlooked. A further NOE study for the 1α-hydroxylated system (Sato et al., 1990) confirms that only the higher field C-19 proton shows a through-space interaction with the proton on C-7. Thus, for vitamin D either with or without the 1α-hydroxyl group it is the 19-proton nearest to C-7 that appears at higher field (lower δ), and this rule should be borne in mind when consulting the references (in the 5*E* series, the order is reversed). The use of specific deuteriation to assist in NMR signal assignment should also be mentioned. Takahashi and Sakakibara (1995) synthesised 28-deuteriated 1α-hydroxyvitamin D_2 in order to confirm the assignment (Koszewski et al.,1987) of the 21- and 28-H_3 (20- and 24-methyl groups) in the ^{1}H-NMR spectra (the methyl doublet from the parent undeuteriated compound at δ_H = 1.01 ppm was retained, while that at δ 0.91 ppm was not represented). For the model compound 1f mentioned above, it was a study of the specifically monodeuteriated (in the incipient "epi" side chain) derivative (1f, X = ^{2}H) that furnished the basis for the rule distinguishing 20-normal from 20-epi side chains in symmetrical cases (the undeuteriated methyl carbon (C-22) resonates at 22.9 ppm, cf. the signals at 22.4 (therefore C-22′) and 22.9 for the parent compound) (Calverley MJ, unpublished).

Geminal protons on C-22 may be distinguishable in absolute terms by NMR. Often conspicuous as an isolated multiplet to the right of the complex "methylene envelope," one of the 22-Hs is the highest-field methylene proton in vitamin D_3 itself and related compounds (δ 0.95 ppm, its twin resonating at 1.29 ppm) (see e.g. the spectra reproduced for previtamin D_3 in Dauben and Funhoff (1988) and 3-epi-calcitriol in Masuda et al. (2000)). The unique assignment of the configuration of the C-22 protons followed on an ^{1}H-NMR experiment with the 22-deuterio-D_3 derivative prepared by reduction (nucleophilic substitution with inversion) of the 22alpha-mesylate (7 g, X = mesyloxy) with the reagent "super-deuteride" (Calverley MJ, unpublished). The product (necessarily having the structure 7 h, X = ^{2}H) was found to retain the 0.95 ppm signal (and lose the signal around 1.3 ppm) in the spectrum, implying that the high-field signal is in fact due to the 22-proS-H. The difference in chemical shifts for these geminal side-chain protons is reminiscent of those on a cyclohexane ring, where the equatorial proton resonates at lower field than its axial twin (cf. Figure 11.31) (suggested to be because of deshielding by the anti-periplanar C–C sigma bond). Indeed, the corresponding proton signals when C-22 (and C-22′) is constrained in the side-chain *trans*-cyclohexane compound (4i) (Calverley, 2001) are close multiplets (at 500 MHz) centred at δ 0.87 and 0.95 ppm (axial protons: 22-proS-H and 22′-HproR-H (sic)) and resolved multiplets (in the two-dimensional spectrum) at 1.83 and 2.05 ppm (equatorial 22- and 22′-protons). (Note the average Δδ between the 22′- and 22-protons is 0.15 ppm, consistent with the "epi/normal" rule, see above. The difference in the carbon signals, 22–22, ′ is 0.2 ppm.) Thus, even in the conformationally flexible open D_3-type side chain situation, the pseudo-axial or -equatorial character of geminal protons from preferred conformations is manifested in the NMR.

11.5.3 Distinction of Position Isomers. Establishment of Configuration at One or More Stereogenic Centres. Double Bond Geometry

It is occasionally necessary to distinguish in absolute terms between possible position isomers encountered in synthesis. As an example, we cite a study of the mono-trialkylsilylated derivatives of the important ring A diols in the 1-hydroxyvitamin D (compounds of types 6 and 7) series where the problem was elegantly solved by using proton-detected one-dimensional ^{1}H-^{29}Si correlation (Aagaard et al., 1996). The *E*/*Z* configuration of double bonds is most easily confirmed by ^{1}H-NMR, e.g. in the D_2-type side chains (Chodynski and Kutner, 1991). The characteristic ^{1}H-NMR "fingerprints" of the 5*E* and 5*Z* systems is useful the routine monitoring of the progress (by sampling over time) of the important compound 7 => compound 6 (in which *Z* is the same group, especially TBS) photoisomerisation, the penultimate step in synthesis of various side-chain modified analogues (Calverley, 1987; Calverley and Jones, 1992), these isomers often being difficult to distinguish chromatographically (Calverley MJ, unpublished).

11.5.4 Structural Identification of Degradation Products

The structures of most vitamin D isomers associated with the triene system were classically established before the advent of NMR, and standard compounds were available for NMR comparison (cf. Tsukida et al., 1976). One latecomer was the rare 5Z-isovitamin (D_2), compound (**8b**), identified in acidic powder formulations of D_2, by Takahashi and Yamamoto (1969) on the basis of the ^{1}H-NMR spectrum. Recently, an acid-catalysed transformation of a 1-hydroxyvitamin D analogue into an isovitamin (compound **9**: note the steromutation into the 5*E* series, a well known isomerisation for vitamins D lacking the 1-hydroxyl) was reported. A solvolytic formal rearrangement of the 1α-hydroxyvitamin D system in ecalcidene (compound 1m) (occurring necessarily via the corresponding previtamin D isomer, 3) to give the 9-hydroxy-isovitamin D (compound 9l, X = OH) occurred (Zhang et al., 2006). In the analogous case of a 1α-fluoro-16-ene-vitamin D compound Ro-26-9228 (1n, the structure contains an additional 16-ene modification, not illustrated), a formal dihomo-allylic substitution is the result in a participating alcohol solvent (X = methoxy in the corresponding compound 9) (Brandl et al., 2003). The configuration of the 9-oxy group in the latter case was deduced from the coupling patterns/NOE experiments and is in agreement with expectations of nucleophile capture from the alpha face of the C/D ring system. These two related examples concern pharmaceutical development of clinical candidates.

11.5.5 Structural Identification of Metabolites Formed In Vitro and In Vivo

Early examples of the use of NMR giving useful structural information even at 100 MHz and using sub-milligram amounts of compound are to be found in the classical reports on the identification of the 25-hydroxy metabolites of vitamins D_3 and D_2 (Blunt et al., 1968; Suda et al., 1969). Although the level of sensitivity at that time only allowed the methyl proton signals to be discerned, this was particularly appropriate in these cases for locating a hydroxyl on the carbon bearing geminal methyl groups. Fourier transform proton NMR at 300 MHz allowed the identification of dihydroxylated vitamin D_2 metabolites with spectra run from 40 μg to as little as 5 μg of material, in which even methine protons are distinct (Koszewski et al., 1987, 1988). The initial identifications of metabolites of calcipotriol and KH 1060 were also based on spectra from samples of less than 10 μg (Soerensen et al., 1990; Andersen et al., 1992). In all these examples, specific side-chain metabolic modifications have left the vitamin D or 1α-hydroxyvitamin D nucleus unchanged. Thus, for vitamin D_2 hydroxylations at C-24(*R*) and either C-26 (configurations still unknown) or C-25 were identified, while for KH 1060 it was the 24α(*S*)-hydroxy derivative (by comparison with the two synthetic epimers). In the case of calcipotriol, the original hydroxyl group present at carbon 24 was found to be oxidised to a ketone, with additional saturation of the 22,23-double bond. Other isolated metabolites identified by NMR include: in the D_2 series: both *R* and *S* isomers of

24-hydroxy (from vitamin D_2) (Engstrom and Koszewski, 1989) and 1α,24,26-trihydroxy (from 1α,24*S*-dihydroxyvitamin D_2) (Jones et al., 1996: the 600 MHz ^{1}H-NMR spectrum of the metabolite is reproduced in the paper); with 1α,25-dihydroxyvitamin D_4, 2D NMR was used to identify the 24*R*-hydroxy-derivatives of both the 26- and the 28-carboxylic acids (Tachibana et al., 1999; Tachibana and Tsuji, 2001); from vitamin D_2 or D_3, the 17,20-dihydroxy and/or 20-hydroxy derivatives (Slominski et al., 2005, 2006); from vitamin D_3, additionally the 20,22-dihydroxylated compound (Guryev et al., 2003). The olefinic mixtures resulting from the elimination of the 25-hydroxyl are also reported as metabolites (for OCT: Kamao et al., 2003) (this dehydration is also observed to occur in acidic solution, as examined by Brandl et al. (2003) for Ro-26-9228 (16-ene-**1n**)).

A few metabolic transformations of the A-ring (apart from 1α-hydroxylation) are known, and NMR has been paramount in structure clarification. An early case in point was the discovery of 19-nor-10-keto compounds as products of microbial oxidation (Napoli et al., 1983). The continuing identification of new metabolites on the 3-epimerisation pathway (Reddy et al., 2000; Brown et al., 2005) relies on NMR comparison with synthetic standards (e.g. Higashi et al., 1999c; Kamao et al., 2003, 2004). A proton spectrum of metabolically generated 3-epi-calcitriol is reproduced in Masuda et al. (2000) (also referred to above). Higashi et al. (2004) isolated a 3-keto derivative of 24,25-$(OH)_2D_3$, wherein the 5,6-double bond has migrated into the A-ring to attain conjugation, reminiscent of another oxidative metabolite of 25-hydroxyvitamin D_3 (Yamada et al., 1987). The ^{1}H-NMR of putative 2α,25-dihydroxyvitamin D_3 from a microbial oxidation of vitamin D_3 has been reported (Takeda et al., 2006).

11.5.6 Investigation of Reaction Mechanisms and Protein–Ligand Interactions

Sheves et al. (1979) studied the mechanistic aspects of the calciferol-procalciferol equilibrium using deuteron (^{2}H) NMR spectroscopy. Vitamin D_3, specifically dideuteriated at C-19, was subjected to conditions of heating under which it equilibrated partially to its previtamin. The ^{2}H-NMR spectrum of the recovered vitamin D_3 showed, in addition to residual deuterium on C-19 (two signals, *E* and *Z*, of equal integration), two new signals (at 1.68 and 2.70 ppm) corresponding respectively to the 9α and 9β protons in the proton NMR spectrum (see Fig. 11.30). The integration ratio of these was found to be ca. 2:1, indicating a preference, but not an exclusivity, for migration from C-19 to the 9α position. Using ^{19}F-NMR, the shift of the fluorine signal as a marker was used to study the rate of previtamin equilibration for the 1α-fluoro analogue Ro-26-9228 (Brandl et al., 2003).

Binding sites in the vitamin D receptor both unoccupied (apo) and occupied (holo) with calcitriol or three 2-substituted analogues were compared by NMR spectroscopy (Sicinska et al., 2005). Comparison of ^{1}H[^{15}N] heteronuclear single quantum correlation (HSQC) spectra revealed that the binding of the three ligands resulted in the same diamagnetic shifts of both a H and a N resonance in the specifically ^{15}*N*-labelled Trp282, an amino acid which occupies the centre of the ligand-binding pocket in the

holo receptor. Thus, the interaction between the ligands and Trp282 could not be invoked as a marker for the known differences in calcemic activity of the various ligands. DFT calculations (see Chapter 2) of the nitrogen chemical shifts were in agreement and confirmed the orientation of the calciferol 7,8-double bond under the tryptophan ring (Sicinska, 2006). It seems that interactions with water molecules in the apo receptor replacing the ligand are responsible for the $\Delta\delta'$s observed.

11.6 Applications

11.6.1 Metabolite Analyses in Clinical Biochemistry

25-OH-D_3 is the metabolite that circulates in human plasma in the highest concentration and it is measured in many laboratories as a means of assessing 'vitamins D status' (Haddad and Stamp, 1974; Mawer, 1980; Hollis, 2000, 2005b, 2008; Heaney, 2004; Holick, 2008; Zerwekh, 2008). Even though this metabolite circulates at relatively high concentrations, its accurate and precise measurement seems to pose difficulties in many laboratories (Binkley et al., 2004, 2006; Hollis, 2004, 2008). It is usual to measure this analyte in serum/plasma from venous blood obtained by standard venepuncture techniques but it also possible to use serum from capillary blood obtained from finger pricks, which is the usual source of blood spots on Guthrie cards. It has recently been shown that finger-prick serum gives 20% higher 25-OH-D results than venous serum (Dayre McNally et al., 2008). A recent LC-MS/MS method has been described for the measurement of 25-OH-D_2 and 25-OH-D_3 in dried blood spots (Eyles et al., 2009). It will be appreciated that vitamin D_2, if present in human plasma, will also be 25-hydroxylated, presumably by similar enzymes which metabolise vitamin D_3. There has been some controversy as to precisely what the desirable levels of 25-OH-D in serum in a healthy individual should be (Lips, 2004) but a consensus has emerged over the last few years suggesting that a minimum concentration of around 70–80 nmol/L correlates with optimal health outcomes (Dawson-Hughes et al., 2005; Bischoff-Ferrari et al., 2006; Vieth, 2006; Bischoff-Ferrari, 2007; Bischoff-Ferrari and Dawson Hughes, 2007) and possibly even higher (90–120 nmol/L) for cancer prevention (Bischoff-Ferrari, 2008), although the consensus view at the 13th Vitamin D Conference was below this concentration (Norman et al., 2007). There is also some debate as to whether a 25-OH-D level of 70–80 nmol/L can be achieved with an oral dose of around 800 IU[1]/day (20 μg/day) of vitamin D (Bischoff-Ferrari and Dawson-Hughes, 2007) and doses of 1,000 IU/day and even higher have been suggested

[1] One International Unit (IU) is defined as being 0.025 μg of vitamin D_3. Thus 400IU is 10 μg. The recommended daily allowance (RDA) in the UK is at present 200IU (5 μg). It is not clear whether this IU definition extends to vitamin D_2 (Norman, 1972).

(Aloia et al., 2008; Bischoff-Ferrari, 2008; Cannell et al., 2008; Grant and Garland, 2008). The relationship between administration of vitamin D_3 and the resultant increase in concentration of serum 25-OH-D_3 is not straightforward (Heaney et al., 2008). Evidence suggests that many populations have 25-OH-D concentrations well below the suggested 70–80 nmol/L minimum concentration (Guillemant et al., 1999; Grant and Holick, 2005; Heaney, 2005; Holick, 2006, 2007; Hypponen and Power, 2007; Hypponen et al., 2007; Vieth et al., 2007), especially in the elderly prone to hip fractures (Dixon et al., 2006) and that this 'deficiency' may be widespread with serious health consequences (Holick and Chen, 2008). A recent review of vitamin D status in 'healthy' children in the US concluded that while overt deficiency is no longer common, serum 25-OH-D measurements suggest that vitamin D insufficiency is widespread (Rovner and O'Brien, 2008). Severe vitamin D deficiency in adults can go unrecognised as clinicians are often unfamiliar with the clinical signs (Sievenpiper et al., 2008). For 3 decades, it has been known that patients with chronic kidney disease (CKD) gradually lose the renal enzyme, 1α-hydroxylase (CYP27B1), during the course of their disease and hence develop reduced plasma 1α,25-$(OH)_2$D levels which if unchecked lead to secondary hyperparathyroidism (Gonzalez and Martin, 1995). It has been shown that these CKD patients also exhibit low 25-OH-D_3 levels which contribute to the myriad of symptoms of vitamin D insufficiency and deficiency seen in these patients (Lambert et al., 1982; Jongen et al., 1984b; Gonzalez et al., 2004; Jones, 2007). While exposure to UVB is a means of increasing vitamin D_3 sufficiency (Sorensen et al., 1986), it is not without risk of skin damage and as oral Vitamin D supplementation is cheap and efficacious, this appears to be the recommended route in Canada and the USA for those at risk of insufficiency to restore adequate serum concentrations of 25-OH-D_3 (Lim et al., 2005; Canadian Dermatology Association, 2006). A recent study (Binkley et al., 2007) has reported low serum 25-OH-D concentrations in adults in Honolulu despite seemingly adequate sunlight exposure – highest 25-OH-D levels recorded being around 150 nmol/L, suggesting that it might be prudent to accept this as the upper limit to aim for.

While this chapter has reviewed assays of vitamins D, analogues and metabolites, it must be recognised that, of these, the measurement of serum 25-OH-D concentration is by far the most important assay carried out in the hospital or clinical laboratory. The serum/plasma concentration of 25-OH-D has been shown to have a strong positive correlation with bone mineral density (BMD) (Bischoff-Ferrari et al., 2004a), improved muscle function (Bischoff-Ferrari et al., 2004b), cognitive function (Przybelski and Binkley, 2007) and optimal antimicrobial peptide synthesis to defend against *Mycobacterium tuberculosis* (Liu et al., 2006), and there is a suggestion that lack of vitamin D in winter months may *inter alia* be the trigger for influenza epidemics (Cannell et al., 2006; Cannell and Hollis, 2008). Routine assays of 1α,25-$(OH)_2$D are useful in only a very few clinical situations (Zerwekh, 2004). Because of the demand for large numbers of assays for 25-OH-D, which can be automated for use in clinical laboratories, there has been considerable commercial interest in developing rapid and simple methodologies, all of which are at present based on competitive binding assays using either human DBP or antibodies as the binding protein.

None of these commercial analyses seem to use any internal standard. These assays are often designed for use in non-specialist laboratories, without particular experience in vitamins D analysis, though even laboratories highly experienced in 25-OH-D assay appear to find difficulties. After a comparison of performance in five such laboratories using both commercial and in-house assays, Lips et al. (1999) concluded that results from different laboratories cannot be assumed to be comparable unless careful cross-calibration has been carried out. In effect, this means that while intra-laboratory precision may be satisfactory, inter-laboratory precision and/or accuracy need considerable improvement – a task which requires a rigorous external QA scheme such as the international DEQAS run in the UK (Carter et al., 2004b). Three automated systems for 25-OH-D and 1,25-$(OH)_2$D (NEXgen Four, Triturus ELISA & Diasorin Liaison) have recently been compared (Kimball and Vieth, 2007), but in the absence of a reference method of known accuracy it is difficult to know how to assess the results. The recent Elecsys fully automated method (for 25-OH-D_3) has been studied by Leino et al. (2008) and Roth et al. (2008), both studies comparing results obtained against LC-MS/MS methodology. The Product Information sheet for this assay (Vitamin D3 (25-OH) – code 03314847 – www.roche.com) gives more data on comparisons with LC-MS/MS.

Table 11.8 lists some of the commercial assays for 25-OH-D which are available in 2009 – see also Binkley, Krueger and Lensmeyer (2009).

Application of these assays to produce accurate and reliable assays for 25-OH-D requires a full evaluation of the assay prior to use and the application of rigorous internal and external quality assurance (Carter et al., 2004b; Wootton, 2005; Jones et al., 2007) to maintain satisfactory performance. Equally, an appreciation by both scientists and clinicians of what the term '25-OH-D' means in this context is absolutely essential – namely, the '25-OH-D' concentration is method-sensitive and depends *inter alia* upon the efficacy of measurement of 25-OH-D_2 and 25-OH-D_3, which must be evaluated in advance of use. Proper independent validation of these commercial assays are not often published in the scientific literature, although some have been, comparing the results from the Diasorin Liaison automated system with those using the Diasorin RIA (Diasorin Inc, Stillwater, MN) (Ersfeld et al., 2004) and Roth et al. (2001) have compared the Nichols Advantage system against an in-house RIA, although this Nichols assay has been withdrawn and is no longer sold. Hollis (2000) has published a comparison of two commercial RIA methods (Diasorin RIA and IDS Gamma-B) for 25-OH-D, suggesting that the IDS kit underestimated circulating 25-OH-D_2. A more recent study using tandem MS-MS as a reference method (Terry et al., 2005), comparing the Diasorin Liaison, Nichols Advantage and Diasorin RIA kits, suggested that the Advantage system also underestimated 25-OH-D_2. Carter et al. (2004b) came to similar conclusions as did Glendenning et al. (2006). As Vitamin D_2 (calciferol BP in the UK, Drisdol in the USA) is widely used for the treatment of vitamin D deficiency both in the UK and USA, the ability to measure 25-OH-D_2 as well as 25-OH-D_3 is very important and it is disconcerting for the clinician, treating patients with large doses of vitamin D_2, to be told by the laboratory that there is no rise in 25-OH-D (Carter et al., 2004a). It is clear that the use of commercial kits produces 25-OH-D concentrations that are method- and/or laboratory-sensitive (Lips et al., 1999; Binkley et al., 2004, 2008)

Table 11.8 Some common commercial 25-OH-D assays available for clinical assessment of vitamin D status

Assay	Details[a]	Supplier/manufacturer	Cross-reactivity[a] (25-OHD_3 as 100%)
OCTEIA	*Enzyme-linked immunoassay*		
	25 μL serum incubated with 25-OHD-biotin complex in microtitre wells coated with sheep anti-25-OHD antibody. Washed and horseradish peroxidase-labelled avidin added which binds to 25-OHD biotin. Wash and colour developed using chromogenic substrate. Colour intensity inversely proportional to 25-OHD concentration	IDS, Ltd., www.idsltd.com [NB IDS demonstrated an automated version of this system using the Grifols TRITURUS (IFCC – May 2005)	25-OHD_2 -75%, 24,25 $(OH)_2D_3 \geq 100\%$
Gamma-B	*Radioimmunoassay, using ^{125}I label*		
	50 μL serum + 50 mL of 1% NaOH, extracted with MeCN. Centrifuge. Use 50 mL extract incubated with ^{125}I-labelled 25-OHD and sheep-anti-25-OHD antibody. After incubation add anti-sheep IgG cellulose, incubate and centrifuge. Decant and count pellet (antibody bound 25-OHD). Count rate inversely proportional to 25-OHD concentration	IDS, Ltd., www.idsltd.com	25-OHD_2 – 75%, 24,25 $(OH)_2D_3$ – >100%
Advantage	*Chemiluminescence assay, using human DBP*		
	20 μL serum + releasing reagent containing 0.5N NaOH. Incubate with human DBP, acridium-ester-labelled-anti-DBP and 25-OHD-coated magnetic particles. Magnetic particles held and remainder washed away. Chemiluminescence developed by addition of hydrogen peroxide and NaOH. Light emitted inversely related to 25-OHD concentration. Automated system	Nichols Institute Diagnostics,[b] www.nicholsdiag.com, 25,26$(OH)_2D_3$ – all 100%	25-OHD_2, 24,25$(OH)2D_3$, 25,26$(OH)2D_3$ – all 100%
Liaison	*Chemiluminesence assay, using antibody*	Diasorin, www.diasorin.com, previously called Incstar	
	Similar to ADVANTAGE but using isoluminol rather than acridium ester. Uses 25-OH-D antibody as binding protein		
RIA	*Radioimmunoassay – using ^{125}I-label*		
	50 μL serum extracted with 500 mL MeCN. Centrifuge and use 2 × 25 mL of extract. Add ^{125}I-labelled 25-OHD and antibody. Incubate, add donkey anti-goat serum which ppts antibody. Centrifuge, decant and count ppt. Count rate. Inversely proportional to 25-OHD concentration	Diasorin/Incstar, www.diasorin.com, www.incstar.com	25-OHD_2, 24,25$(OH)_2D_3$, 25,26$(OH)_2D_3$, 24,25 $(OH)_2D_2$, 25,26$(OH)_2$ D_2 – all 100%

EIA	*Competitive binding assay/enzyme immunoassay* Although described as an EIA, this assay is actually a competitive binding assay using VDBP as the binding protein. 50 μL serum/plasma + precipitation reagent. Centrifuge and use supernatant for tassay, 25-OHD competes with '25-OHD t tracer' coated on well for VDBP. Increasing 25-OHD decreases VDBP immobilised on plate, which is then determined by addition of VDBP-antibody linked to peroxidase with tetramethylbenzidine as substrate. EIA for $1,25(OH)_2D$ is also supplied by this firm	Immundiagnostik AG www.immundiagnostik.com	$25\text{-}OHD_2$, $24,25(OH)_2D_3$, both 100% D_2 and D_3 $1,25(OH)_2D_3$ both <1%
EPBA	*Enzyme-based protein-binding assay* This is a competitive binding assay which appears to be identical in principle and practice to the Immunodiagnostik assay described above. As there are no details as to identity of most reagents, it is not possible to asses just how similar these two assays are	American Laboratory Products Co., www.alpco.com	Identical to Immuno diagnostik assay (*vide supra*)
ELECSYS	*Competitive binding chemiluminescence assay* The binding potein is a preformed complex of poly-clonal $25\text{-}OHD_3$ antibody labelled with ruthenium complexed with biotinylated $25\text{-}OHD_3$, which is displaced by $25\text{-}OHD_3$ in the sample. The complex binds to streptavidin magnetic microparticles via interaction with biotin, which are then magnetically captured on the surface of an electrode. An applied voltage generates chemi-luminescence. The amount of luminescence is inversely related to the amount of $25\text{-}OHD_3$ in the sample	Roche Diagnostics GmbH, Mannheim D-68298, www.roche.com	Specific for $25\text{-}OHD_3$ $25\text{-}OHD_2$ <10%

[a]Method details and cross-reactivity as provided by manufacturers/suppliers.
[b]Nichols Institute Diagnostics has been closed and this kit is no longer available.

and comparisons of results using different assays produces contradictory and disparate results (e.g. Turpeinen et al., 2003; Carter et al., 2004a; Souberbielle et al., 2005; Ibrahim et al., 2007; Chen et al., 2008; Roth et al., 2008). Some form of standardisation is urgently required before these methods can be useful to clinicians seeking a method of determining vitamin D status. The international quality assurance scheme DEQAS (Carter et al., 2004b) operated from the UK offers precisely the forum where such standardisation might be achieved but there is an urgent need for an agreed reference method with high precision against which the accuracy of existing methods can be assessed. While intuitively LC-MS/MS would appear to be a prime candidate, its collective performance at the present time (2009) does not inspire confidence that it can at present fulfil this role (Carter GD, personal communication). While many assays can distinguish between 25-OH-D_2 and 25-OH-D_3, Hollis (2006) has suggested that separate reporting of these two analytes is generally not desirable in order to avoid potential confusion amongst clinicians. The development of certified reference material (Singh, 2008) for 25-OH-Ds will be welcome but will be only a small step towards proper inter-laboratory cooperation and external quality assurance. A standard reference material (SRM972 Vitamin D in Human Serum), although inaccurately named and actually a SRM for 25-OH-D, is apparently under development at the US National Institute of Standards and Technology (Phinney, 2008). – see also Tai, Bedner and Phinney (2010).

There is now an added dimension which must be considered when choosing an assay for 25-OH-D in infant serum and that is the interference in the assay by the presence of the 3-epi isomer of 25-OH-D_3 (Singh et al., 2006). This can be resolved by judicious choice of the LC column system (Lensmeyer et al., 2006a; Singh et al., 2006), which provides the necessary resolution of the 3-epimer of 25-OH-D_3 but not 25-OH-D_2. Estimation of 25-OH-D in saliva may prove to be a useful non-invasive method of assessing vitamin D status (Higashi et al., 2008) but requires a method sensitive to below 8 pmol/L S-MS of OH-D is serum has been revieved by Vogeser (2010) and Singh (2010).

11.6.2 Analysis of Analogues of Vitamins D and Their Metabolites

There have been very few descriptions of the development of methods for the measurement of metabolites of chemically synthesised vitamin D analogues, even though it is clear that many such procedures must have been produced and used in pharmaceutical laboratories throughout the world. However, a GC-MS method for hexafluoro-1α,25-$(OH)_2D_3$ has been described (Komuro et al., 1994) and antisera to 22-oxa-1α,25-$(OH)_2D_3$ (OCT, Chugai Pharmaceutical – Kobayashi et al., 1992) and EB1089 (Leo Pharmaceuticals – Blaehr et al., 2001) have been raised. Interestingly, a similar attempt to produce an specific antibody for 20-epi-1α,25-$(OH)_2D_3$ failed to produce one with sufficient specificity (Blaehr et al., 2003). On the basis of experience in the development of assays for vitamin D metabolites, it is clear that such procedures will utilise either LC with UV detection or some form of saturation analysis,

displacing labelled 1α,25-$(OH)_2D_3$ from a binding protein, such as the procedure used to develop a method for the measurement of calcipotriol in serum/plasma (e.g. Levan et al., 1994). The choice of method will depend upon concentration and affinity for the binding protein. It is likely that *in vivo* concentrations will be so low that methods will usually use saturation analysis. The absence of a suitable naturally occurring binding protein would pose considerable difficulties and would therefore require the generation of a suitable antibody (e.g. Kobayashi et al., 1992) or the use of an alternative methodology, such as mass spectrometry (Schroeder et al., 1993, 1994; Komuro et al., 1994). Where the concentrations of vitamin D analogue are higher than those of endogenous 1α,25-$(OH)_2D_3$ as in pharmacokinetic experiments, microbore-LC can be used with UV detection to monitor the appropriate metabolites directly (Valliere et al., 1994). LC-MS methods have also been described for OCT (Ishigai et al., 1998b) and EB1089 (Kissmeyer et al., 2000).

Analysis in its wider term might be taken to include not only a measure of concentration but also a measure of 'activity'. Here, the analyst is using biological assays (bioassays) to measure the presence and in some cases the amount of the biologically active compound. When the bioassay is used on a crude mixture of active metabolites, the technique cannot resolve the biologically active component. Since most 'active' and 'inactive' vitamin D metabolites retain some, albeit small degree of VDR-binding potential, they will give biological activity in such assays. Thus, in the vitamin D arena, the inherent weakness of such assays is their lack of specificity, as any vitamin D metabolite or vitamin D analogue with significant biological activity will produce a read-out, rendering the assay only useful if there is a single pure test compound or only one bioactive component in a mixture. Today, new metabolites of vitamin D and their chemical analogues are all screened for potential activity in a number of different *in vitro* systems (e.g. VDR or gene transactivation systems), and with an appropriate standard curve such an assay can be transformed into a quantitative tool.

Despite the drawbacks of such methods, researchers over the years in the vitamin D field have attempted to develop bioassays. Perhaps, the earliest was the rat line-test, an *in vivo* semi-quantitative measure of anti-rachitic activity in which a rat was given a measured dose of a vitamin D preparation and the healing of the growth plate of a long bone compared to the healing produced in rats administered measured doses of pure vitamin D. The assay served the vitamin D field for several decades before the advent of *in vitro* assays based upon VDR-dependent activity or gene activation. The cytoreceptor assay of Manolagas (1986) was an ingenious but finicky attempt to bio-assay 1α,25-$(OH)_2D_3$, where [^{3}H]1α,25-$(OH)_2D_3$ was displaced from whole cell-bound VDR and then the intact cell was used to separate bound and free [^{3}H]-labelled ligand. Later attempts at bioassays employed the tools of molecular biology, namely, *in vitro* gene transactivation assays in conjunction with HPLC purification, to measure the activity and concentration of metabolites of DHT. Qaw et al. (1993) measured 'bioactivity' of these metabolites in a more realistic environment, using COS cells into which the VDR/VDRE-linked to a growth hormone gene had been transfected. The system thus contained intact cells which were incubated in a

medium containing the metabolites of interest plus DBP from faetal calf serum and the 'activity' of each metabolite was assessed by measuring the release of growth hormone from these cells into the incubation medium. This experiment measures the whole process of action of a metabolite on a target cell from DBP binding, entry to the cell, interaction of the metabolite with the nuclear receptor protein and gene expression.

Such procedures provide a better measure of 'bioactivity' than simple studies using isolated DBP and/or VDR but do not of course take account of *in vivo* pharmacokinetics which may render a compound with high 'activity' in this *in vitro* cell system 'inactive' *in vivo*. It is possible to envisage utilising such a system for the measurement of concentrations of vitamin D metabolites or analogues in a similar fashion to the cytoreceptor assay of Manolagas (1986), although it would probably be somewhat cumbersome. One problem of assaying the binding of vitamin D metabolites to proteins is the difficulty of ensuring that these relatively hydrophobic molecules are completely solubilised since incomplete solution in the incubation medium will lead to erroneous results. To avoid this problem, Teegarden et al. (1991) used uniform polystyrene beads coated with phosphatidyl choline and the vitamin D metabolite of interest. These beads can then be incubated with varying dilutions of the binding protein of interest and the affinity measured. Bound fraction can be removed by centrifugation. A more traditional approach is the addition of non-specific carrier proteins such as bovine serum albumin or swine skin gelatin at (0.5–1%, v/v) though batches of blood-derived proteins need to be checked for potential DBP contamination.

A more recent example of the use of bioassay of $1\alpha,25\text{-}(OH)_2D_3$ is the work of Kato's group while expression cloning the 1α-hydroxylase (CYP27B1) from a rat kidney library (Takeyama et al., 1997). These researchers used a VDR-driven Lac-Z colorimetric gene expression system to measure $1\alpha,25\text{-}(OH)_2D_3$ production in bacteria expressing the CYP27B1 gene. Bacterial plates were impregnated with $25\text{-OH-}D_3$ and only those colonies incorporating full-length CYP27B1 gene leading to $1\alpha,25\text{-}(OH)_2D_3$ production and in turn operational LacZ gene expression, were able to produce a blue colour. Kato's group mastered this technically demanding approach to detect minute quantities of $1\alpha,25\text{-}(OH)_2D_3$ despite a background of a fairly high concentration of $25\text{-OH-}D_3$. By looking for blue spots Kato's group was able to locate and select the colonies with CYP27B1 and clone the enzyme successfully. The study demonstrates the exquisite sensitivity of bioassays which can be harnessed in times when the physico-chemical approaches, which dominate this chapter, fail the analyst.

11.6.3 In Vitro Metabolic Studies

The metabolism of a large number of vitamin D metabolites and analogues has been studied over the last 20 years. Two topics have been addressed: firstly, identification of the principal metabolites formed *in vitro* using a variety of cell lines.

This application places particular emphasis on the ability of the detection methods to provide data enabling structure to determined, which is best provided by retention time data in both LC and GLC systems and chemical modification in combination with GC-MS. Figure 11.33 illustrates the application of this methodology to the identification of four monohydroxylated metabolites of 1α-OH-D_3 formed using an *in vitro* biological system (Jones and Makin, 2000). From these spectra, together with other data, it is possible to gain a good idea of the structure of these metabolites.

All of the modern studies of *in vitro* metabolism of metabolites and analogues of vitamins D utilise LC separation and many LC-MS-MS spectra are produced. While such LC-MS spectra are extremely useful to demonstrate a major metabolic change (i.e. hydroxylation or side-chain cleavage (Kusudo et al., 2004), more subtle changes (i.e. distinguishing between the four metabolites illustrated in Fig. 11.33) would seldom be possible from LC-MS spectra alone, though of course retention times as compared to authentic standards might well provide significant unequivocal information. On occasions, however, fragmentations during LC-MS-MS do occur which give structural information, as, for example, in the identification of 23,25-$(OH)_2D_3$ (Higashi et al., 2002a). Structural information is, however, still best provided by the use of EI(+) MS spectra either using GC-MS systems or by direct probe insertion into the ion source. Derivitisation prior to GC-MS or direct insertion-MS is still valuable as it provides additional structural information. As an example of this is the EI(+) mass spectrum of underivatised 2α,25-dihroxyvitamin D_3 published by Takeda et al. (2006) where formation of the per-trimethylsilyl ether would likely have given characteristic A-ring fragments, which would have confirmed the 2-OH position The interpretation of EI(+) mass spectra have already been discussed. Table 11.9 gives data from a number of publications which have used GC- and LC-MS for the identification of metabolites formed during *in vitro* (and *in vivo*, as the need for characterisation and methods adopted are identical to those used for *in vitro* studies) studies on vitamins D metabolites and/or analogues or for characterisation of metabolites found in body fluids. It is clear that LC-MS-MS and GC-MS are complementary techniques (Ishigai et al., 1997) and GC-MS still has a valuable contribution to make as a means of structural identification (Araya et al., 2003; Takeda et al., 2006).

Fig. 11.33 (continued) The EI(+) mass spectra of metabolites of 1α-hydroxyvitamin D_3 (1α-OH-D_3). GC–MS was carried out after derivatization to form the per-trimethylsiyl ethers as described by Qaw et al. (1993). Both pyro-and isopyro-isomers of each metabolite were observed but the mass spectrum of the pyro-isomer (the major peak) is shown in each case. The major ions (m/z 632(M^+), m/z 542, 432, and 362 (not highlighted - M^+ losing successive silanols) and m/z 501 (M^+ losing 131 by A-ring cleavage) are the same in all the spectra. M/z 217 is the characteristic ion always seen in these 1,25-dihydroxylated steroids and m/z 251 (not highlighted) arises by side chain cleavage and subsequent loss of three silanols. It is possible, however, to distinguish each isomer from the characteristic fragmentation patterns illustrated for each above the appropriate spectrum. Only important ions have been highlighted

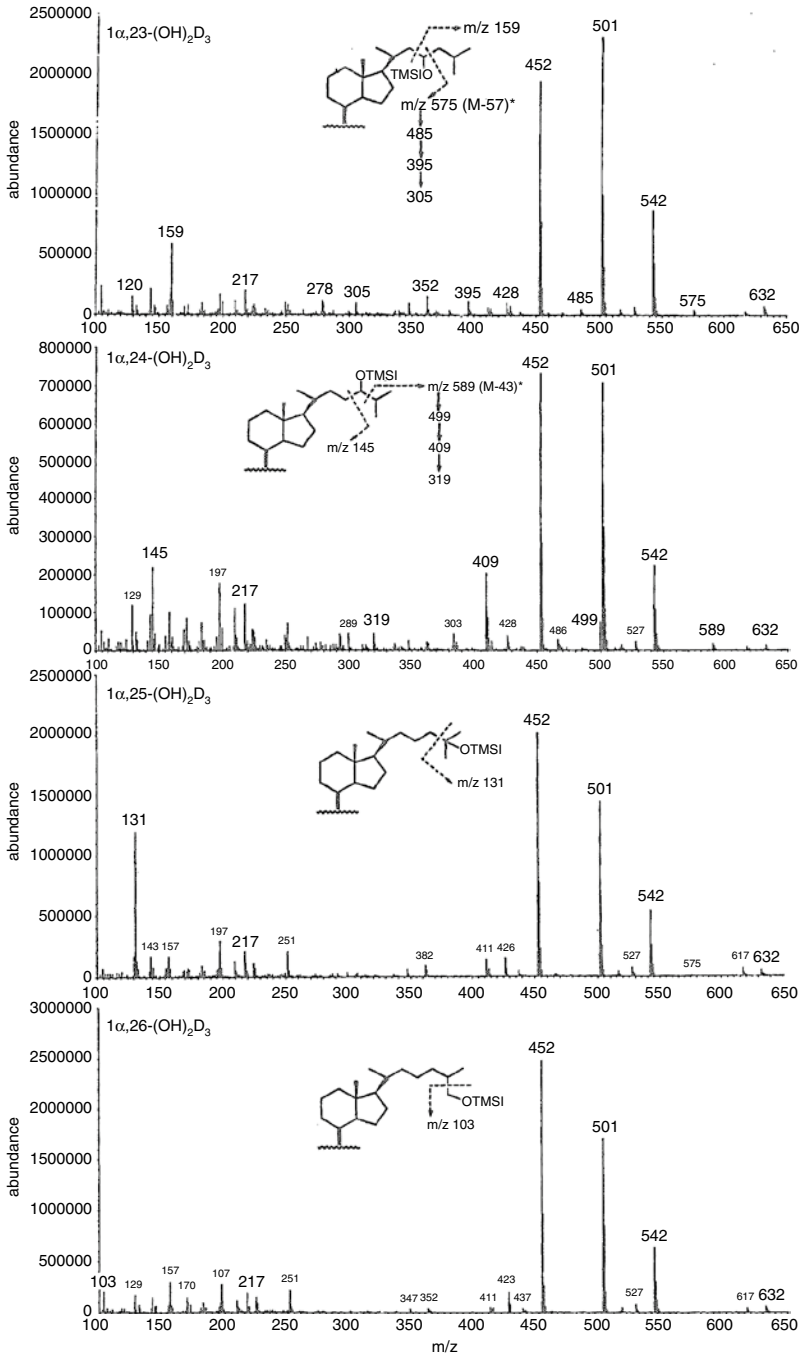


Fig. 11.33 (legend on facing page)

Table 11.9 Characterisation of metabolites of vitamins D and analogues using LC- or GC-MS

Study	Characterisation of products	Reference
Metabolism of 16-ene-1,25$(OH)_2D_3$ in kidney perfusion	LC-MS/MS as PTAD derivatives[a]	Yeung et al. (1995)
Metabolism *in vitro* of 22-oxacalcitriol	GC-MS of TMSi ethers	Masuda et al. (1996)
In vitro and *in vivo* metabolism of EB1089	GC-MS of TMSi ethers	Shankar et al. (1997)
Characterisation of *in vivo* metabolites of OCT	GC-MS of TMSI ethers	Ishigai et al. (1998)
1α-OH- and 1α,23-$(OH)_2$-24-oxo-D_3 in kidney perfusate	GC-MS	Weinstein et al. (1999)
3-epimerisation of 24,25$(OH)_2D_3$ *in vivo*	LC-APCI, ^{1}H-NMR, GC-MS of TMSi eithers	Higashi et al. (1999c)
3-epimerisation of 16-ene-1,25$(OH)_2D_3$ by UMR106 cells	GC-MS and ^{1}H-NMR	Reddy et al. (2000)
In vitro metabolism of D_3 by *E.coli*	LC-APCI(+)	Sawada et al. (2000)
Characterisation of new glucuronides in rat bile after administration of 25-OH-D_3 and 24,25-$(OH)_2D_3$	LC-ESI-MS^n after PTAD[a] and acetate formation and GC-MS of TMSi ethers	Higashi et al. (2000)
1α,25-$(OH)_2D_4$ acidic metabolites from rat bile	GC-MS of Me esters and ^{1}H-NMR	Tachibana and Tsuji (2001)
Calcitroic acid as metabolite of calcitriol in kidney perfusions	GC-MS of Me esters	Zimmermann et al. (2001)
Metabolism of 1α,25-$(OH)_2D_3$ and 3-epimer formation by keratinocytes	LC and GC-MS	Reddy et al. (2001)
Metabolism of 19-nor-1α,25$(OH)_2D_3$	GC-MS of TMSi ethers	Shankar et al.(2001a)
Metabolism of 20-methylvitamin D analogues	GC-MS of TMSi ethers	Shankar et al. (2001b)
Metabolism of D_4 analogues	GC-MS of TMSi ethers	Byford et al. (2002)
Metabolism of D_3 by CYP11A1	GC-MS of TMSi ethers	Guryev et al. (2003)
Metabolism of 25-OHD_3 by CYP2D25 and CYP27A1 – 4,25-$(OH)_2D_3$	GC-MS of TMSi ethers	Araya et al. (2003)
Novel metabolites of OCT	^{1}H-NMR and LC-ESI(+)-MS	Kamao et al. (2003)
Metabolism of [26,27-F_6]-1,25-$(OH)_2D_3$ by CYP24	LC-APCI(+)-MS and GC-MS of TMSi ethers	Sakaki et al. (2003)
Metabolism of D_3 by CYP2R1 in human microsomes	LC-APCI(+)-MS	Shinkyo et al. (2004)
CYP-24 over-expression and knockout systems in 25-OH-D_3 metabolism	LC-MS/MS	Masuda et al. (2004)
3-epimerisation of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ *in vitro*	^{1}H-NMR and LC-ESI(+)-MS	Kamao et al. (2004)
3-epimerisation of 24,25-$(OH)_2D_3$	LC-APCI(+) and ^{1}H-NMR	Higashi et al. (2004)
Metabolism of A-ring isomers of 1,25-$(OH)_2D_3$ by CYP24A1	LC-APCI(+)-MS	Kusudo et al. (2004)
Hydroxylation of 1α,24-$(OH)_2D_3$ by CYP24A1	GC-MS of TMSi ethers	Astecker et al. (2004)
Metabolites of 1α-OH-D_2 and 1α-OH-D_3 by CYP3A4	LC-API-MS/MS and GC-MS of TMSi ethers	Gupta et al. (2005)
Characterisation of D_2 and D_3 metabolites produced by CYP2C11	GC-MS	Rahmaniyan et al. (2005)

(continued)

Table 11.9 (continued)

Study	Characterisation of products	Reference
Metabolism of 1α-OH-D_3 and 3-*epi*-1a-OH-D_3 in ROS 17/2.8 cells	GC-MS as TMSi ethers and ^{1}H-NMR	Brown et al. (2005)
Identification of 2α,25-$(OH)_2D_3$ formed by *P. autotrophica*	LC; GC-MS, ^{1}H- and ^{13}C-NMR	Takeda et al. (2006)
CYP24A1 can activate vitamin D pro-drugs in skin	LC-MS and ^{1}H-NMR	Masuda et al. (2006)
CYP24 expression is increased in lung cancer cells (NSCLC)	LC-MS/MS	Parise et al. (2006)
Metabolism of D_2 by purified $P450_{scc}$ and rat adrenal Mitochondria	MS (EI+) and ^{1}H-NMR, COSY and HSQC	Slominski et al. (2006)
Calcioic and cholacalcioic acids as products of 25-OH-D_3 metabolism	EI(+)-MS and LC-MS – acids as Me esters	Reddy et al. (2006)
Degradation chemistry of ecalcidene	LC-MS, LC- and off-line NMR	Zhang et al. (2006)
Metabolism of nor-C Ring calcitriol analogues	LC-UV and GC-MS (of TMSi ethers)	Reddy et al. (2007)

[a] PTAD = Phenyltriazoline adducts

The second aspect of this application is the need to provide reproducible time course data for metabolites of a number of analogues which can be compared with the necessary degree of confidence. This is easily achieved using LC and requires that one set of data can be overlaid on another set, which may have been obtained at a different time. This can only be achieved if the LC system used has a high degree of precision and retention times remain constant over long periods of time. Major advances have been made in pump, injector and column technology which make this now possible to achieve. Such an example is illustrated in Fig. 11.34 and in Byford et al. (2002) and shows the comparative metabolism of 1α,24*S*-$(OH)_2D_2$ and 1α,25-$(OH)_2D_3$ by HPK1A*ras* cells analysed using a Waters Alliance LC system. These two studies were performed using the same batch of keratinocytes at the same time but each analysis involves separate extraction replicates and different time points which extend the LC analysis time over a period of days. The reproducibility of retention times illustrated here is remarkable.

11.6.4 Analysis of Foodstuffs

The analysis of foodstuffs is not an endeavour widely carried out by biochemists involved in the routine analysis of vitamins D, their metabolites and analogues. It is, however, an area of increasing interest in view of the recognition of the high incidence of hypovitaminosis D amongst the general population in the western world (e.g. Holick, 2007; Hypponen and Power, 2007; Whiting et al., 2007) as well as in the at-risk populations, the elderly and during pregnancy, and the suggestion that

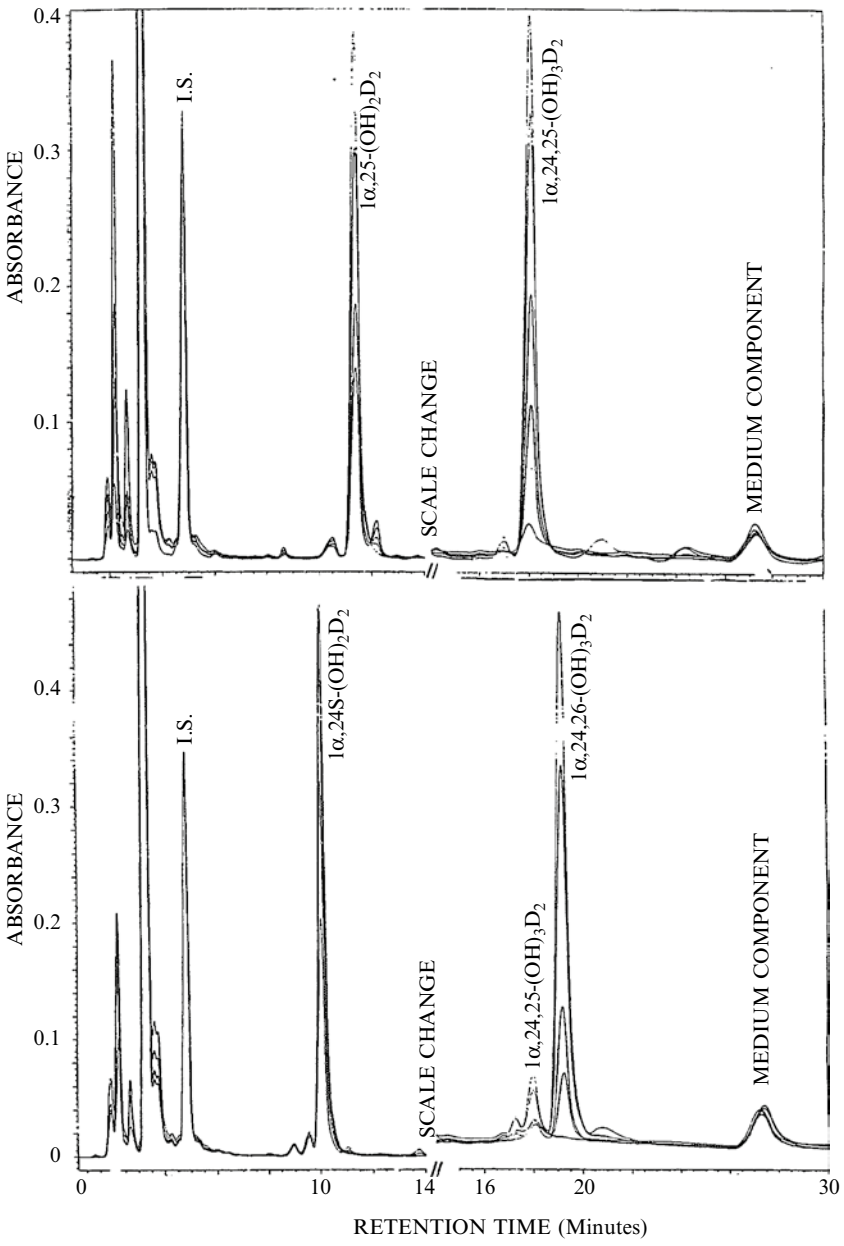


Fig. 11.34 Reproducibility of modern LC instrumentation. Multiple chromatographic profiles in this figure are overlaid to demonstrate the superior reproducibility of modern chromatographs, such as the Waters Alliance 2690 system. The experiment involves lipid extracts from human keratinocytes incubated with synthetic vitamin D analogs 1α,25-$(OH)_2D_2$ and 1α,24S-$(OH)_2D_2$ (10 μM) for various times ranging from 0 to 48 h. Overlays show the remarkably consistent retention times that can be achieved for all components in the extract from the internal standard (labeled I.S., which is

(legend continued on facing page)

rickets may once again become a global health issue for infants and children (Holick, 2006). As already pointed out (*vide supra*), animals obtain their vitamin D from both solar irradiation and from food and there are various factors in humans which affect the proportion of vitamin D derived from these two sources (Chen et al., 2007). One of the major analytical questions which has to be addressed in this area is of course the identification of the analyte – is vitamin D_2 equivalent to vitamin D_3 (Houghton and Vieth, 2006)? Published studies are analysing food for vitamins D only but should we also be measuring the concentrations of 25-OH-Ds, and possibly other metabolites? If hypovitaminosis is the increasing problem which is suggested and restricted solar exposure is the current recommended medical advice for reduction in melanoma incidence, it would seem that food must be the major source of necessary vitamin D. Clearly, a precise and accurate knowledge of vitamin D content of foodstuffs is of considerable importance, together with an agreed quantity and type of vitamin D input required to achieve an agreed vitamin D status (or plasma/serum concentration of 25-OH-D). If food supplementation is being considered, the precise nature of the vitamin D compound used has to be considered as well as the endogenous levels of this compound in the diet (Lamberg-Allardt, 2006). Many research studies concerned with nutrient intake use food frequency questionnaires (FFQs) but these rely on the accuracy of nutrient concentrations in national and international databases, which may have been determined many years ago and may have differing data (Vaask et al., 2004).

The analysis of vitamin D in foodstuffs is a considerable challenge because of the complex and varied matrices in which these analytes are found (Byrdwell et al., 2008; Holden and Lemar, 2008). Detailed consideration has been given in this chapter to the relatively simple task of analysing vitamins D and metabolites in biological fluids, which has advanced considerably over the last decade. Are methods developed for the analysis of foodstuffs satisfactory and are historical values for vitamin D content or 'vitamin D activity' in food reliable in the twenty-first century or do we need to re-analyse using modern methods of analysis such as LC-MS-MS? In general terms, we would suggest that any analysis of 'vitamin D' content of food, which has not been carried out by LC-UV or LC-MS needs to be checked using one of these modern LC methods. Table 11.10 lists some of the LC methods used for the analysis of vitamins D in food since 1990. To the best of our knowledge, no GC-MS procedures have been used in this context.

Fig. 11.34 (continued) 1α-OH-D_3) with a retention time of 4.85 min through to the medium derived; component (retention time 27.2 min). When the LC method is combined with a consistent extraction procedure (giving a remarkably similar I.S. peak heights), the biological time course can be seen to give valuable clues to metabolite identity and origin. In this relatively simple example, 1α,25-$(OH)_2D_2$ (RT = 11.42 min) gives a single product 1α,24,25-$(OH)_3D_2$ (RT=18.05 min), whereas1α,24S-$(OH)_2D_2$ (RT = 10.06 min) gives two products – 1α,24,25-$(OH)_3D_2$ (RT=17.99 min) and 1α,24,26-$(OH)_3D_2$ (RT = 19.18 min). In this case, peak identity was confirmed by off-line GC-MS of derivatized (per-trimethylsiyl ethers) metabolites but currently available LC-MS instrumentation allows for on-line mass spectrometry of un-derivatised peaks, giving immediate information about peak identity

Table 11.10 Some LC methods for the analysis of foods for vitamins D and metabolites (modified from Jones and Makin, 2000)

Matrix	Analyte(s)	Methodology	Reference
Milk powder and gruel, eggs, fish, milk and margarine	D_2 and D_3	Saponification, LC on silica gel, RP-LC-UV	Bognar (1992)
Infant formula	D	LC-UV	Tanner et al. (1993)
Milk	D_2 and D_3	Saponification, LC on Florisil, RP-LC-UV	Hagar et al. (1994)
Milk (liquid and powder)	D_3	SPE on C18 cartridge and LC-UV	Delgado-Zamarreno et al. (1995)
Infant formula and enteral products	D	Liquid extraction, saponification, SPE, RP-LC-UV	Sliva and Sanders (1996)
Animal feeds	D and pro-D_2	Sep-Pak SPE, LC-UV	Qian and Sheng (1998)
Fortified health care foods	D_3	Extraction with $CHCl_3$, RP-LC-UV	Wu and Tang (1998)
Fortified liquid milk	D	Saponification, EtOH + Hx extraction, SPE, RP-LC-UV	Faulkner et al. (2000)
Processed cheese	D_3	Alkaline saponification, extraction with LP:ether SPE, RP-LC-UV	Upreti et al. (2002)
Milk, gruel, cooking oil, margarine, fish oil and infant formula	D	Saponfication, Hpt extraction, SP-LC-UV for D fraction, RP-LC-UV	Staffas and Nyman (2003)
Fortified infant formula	D_3	Saponification, SPE, SP-LC-APCI-MS	Heudi et al. (2004)
Infant formulas and fortified milk	D_3	Saponification, Hx extraction, RP-LC-EC	Perales et al. (2005a)[a]
Variety of fish-edible parts	pro-D_3 and D_3	Saponification, extraction with ChemElut, SP-LC/ RP-LC-EC detection	Ostermeyer and Schmidt (2006)
Oily fish – effect of baking and frying	D_3	Saponification, extract with Hx, 2 x SP-LC-UV, RP-LC-UV or RP-LC-APCI-MRM (MS/MS)	Lu et al. (2007) Chen et al. (2007)
Cheese, milk, cereals, non-carbonated soft drinks and juice	D_3	Saponification, extraction with Hx. RP-LC-APCI(+)-MS	Dimartino (2007)

[a]See Perales et al. (2005b) for a review on LC determination of vitamin D in dairy products. See also reviews by Blake, 2007, Holden and Lemar (2008) and Byrdwell et al. (2008). See also Huang et al. (2009).

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Chapter 12
Industrial and Compendial Steroid Analysis

Sándor Görög

12.1 Introductory Remarks – Similarities and Differences Between Biological–Clinical and Industrial–Compendial Steroid Analysis

The aim of this section is to give an overview of the requirements of steroid analysis in *pharmaceutical industry* and to cover the methodological aspects of these requirements with regard to quality control of bulk steroid drugs and drug formulations. Although this is an important area, the challenges here are not comparable with those in biological–clinical steroid analysis, which was the sole topic of the first edition of this book. This is reflected by the much smaller number of research publications dealing with this pharmaceutical area of steroid analyses. Only a few (more or less obsolete) books are available (Görög and Szász, 1978; Görög, 1983, 1989). The newer developments in this area are summarised in a comprehensive review (Görög, 2004), on which this chapter is based.

Steroid (hormone) drugs and their (semi-)synthetic analogs are among the most important groups in drug therapy. There were about 90 steroid compounds among the top 1,500 drug materials in 2005 representing around 7% of the overall turnover (IMS Chemical Actives Monitor, 2005). In addition, somewhere in the region of one-third of the therapeutic drugs listed in the Monthly Index of Medical Specialities (MIMS – a handbook used in the UK by most clinicians as a reference source for availability and cost of prescription therapeutic drugs) either are steroids or contain, inter alia, steroids. As discussed in detail elsewhere in this book, even the few natural steroid hormones, which are still used for therapy, are semisynthetic materials manufactured from steroids of plant origin. The same applies to an even greater extent to hormone analogs, which are becoming, at an increasing rate, totally synthetic products. The synthesis of a typical steroid drug involves around 10–15 chemical steps. To create the analytical background to this is an important and difficult task. Large numbers of analytical measurements are necessary, not only to increase the safety,

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reproducibility, and economy of production, but also because drug Regulatory Authorities require full documentation of high-level in-process quality control, in which analysis plays a vital role. Very little information is available in the literature on the methods used for this purpose, since the pharmaceutical companies consider this is commercially confidential.

Of course *safety of drug therapy* is an even more important issue than safety of their production. The quality (purity) of bulk drug materials and the careful control of formulations made therefrom are thus of fundamental importance in assuring therapeutic safety in so far as it guarantees that what is prescribed is what is on the label. The possible risk to the patient of a perhaps pure drug with unappreciated side effects, even when acceptable pharmaceutical studies have been satisfactorily completed, is not dealt with here. Although the demands for quality control of drugs in real life (sale and use of drugs) are usually higher than the limits set in the pharmacopoeias, these latter tend to be naturally conservative with regard to the introduction of new analytical techniques, but the state of the art in the analysis of bulk drug materials and formulations can be best characterised by examining the methodologies recommended by the principal pharmacopoeias. This is the subject of Section 12.2.

When the *similarities and differences* between industrial–compendial and biological–clinical steroid analyses are investigated, the following points can be taken into consideration:

a. ***The target compounds*** In compendial analysis, these are restricted to the drug materials themselves, while in industrial analysis, intermediates in their production are also subject to analytical investigation. The determination of the parent drugs as well as the identification and determination of their metabolites in biological samples are parts of the protocol in drug research-oriented analytical laboratories of pharmaceutical companies (or in the case of bioequivalence studies carried out by generic companies in their own or increasingly in contract research organisation laboratories). Of course these pharmaceutical aspects of drug design and *in vitro/in vivo* study are outside the scope of this chapter and are discussed elsewhere in this book. What has been described so far is not a specific steroid-related issue: it applies to all synthetic drugs as well. However, steroids belong to those groups of compounds where the difference between the two main fields is even more pronounced, as among the target compounds of biological–clinical steroid analysis there are many intermediates of the biosynthesis of native steroid hormones and their metabolites. These are intensively investigated in biological–clinical steroid analysis but are outside the field of industrial–compendial steroid analysis.

b. ***Methodological aspects*** Similarities and profound differences are simultaneously present regarding this matter. Although at a decreasing rate, titration and UV-visible spectrophotometry are still fairly important methods in industrial–compendial steroid analysis, but they are seldom used in biological–clinical analysis, where immunoassay is the primary methodology for routine analysis. Liquid chromatography (LC) is widely used in both fields. In industrial–compendial steroid analysis LC with UV detection is almost exclusively employed and

the use of LC-MS is restricted to impurity profiling studies where the mass spectrometer is used for structure elucidation purposes. At the same time, the mass spectrometric detector is becoming the predominantly applied detector in biological–clinical steroid analysis due to its high sensitivity and specifity in quantitative analysis and its capability in elucidation of the structure of metabolites. The importance of NMR spectroscopy attached online to LC is increasing in both fields, and although the high capital cost of this technique has inhibited its adoption, there is no doubt that it will become increasingly useful in the future. Thin-layer chromatography (TLC) is more important in industrial–compendial steroid analysis than in bioanalysis, especially in the (semi-)quantitative determination of related impurities. Gas chromatography (GC) and GC-MS used to be the most widely used chromatographic method in bioanalysis. As a consequence of the introduction of LC and the increasing availability of simple and efficient means of linking LC directly to MS and MS-MS systems, its importance has greatly decreased but GC-MS still retains some of its applications in both fields – primarily for structural analysis of unknown metabolites and as a reference method in quality assurance schemes. Methods related to capillary electrophoresis (CE) but unlike the parent method suitable for the analysis of uncharged steroid molecules (micellar electrokinetic chromatography [MEKC], capillary electrochromatography [CEC]) have proved their suitability in both fields but their future in steroid analysis is not yet predictable, as at present they appear to offer little advantage in the biological–clinical field.

c. ***Matrix effects and selectivity issues*** The issues cause much less serious problems in industrial–compendial than in biological–clinical steroid analysis. Although, even in the former field, it is often necessary to separate six to eight related impurities from each other and from the main components (similarly to the metabolic profiling studies), the matrix effect of pharmaceutical excipients (auxiliary materials used in the production of drug formulations) is much less pronounced than that of non-steroidal endogenous compounds as the excipients can generally easily be separated from steroids by solvent extraction.

d. ***Sample size and sensitivity issues*** The sample size available for analysis is a major difference between the two areas. In contrast to the well-known limitations in the size of the sample and especially its low steroid concentration in bioanalysis, in the case of industrial–compendial steroid analysis, the sample size is practically unlimited. As a consequence of this, in the majority of cases the sensitivity of the applied analytical methods is a far less important issue. Nevertheless, it would be an oversimplification to contrast the methods required by the two areas of steroid analysis as "macro" and "micro" methods, respectively. Fairly high sensitivity is often necessary even in industrial–compendial steroid analysis. For example, the single tablet assay of oestrogens on the order of 10 μg/tablet or identification of impurities or degradation products on the order of 0.01% requires sensitive and specific analytical methods. It should also be mentioned that the demands for accuracy and precision are probably higher in industrial–compendial than in biological–clinical steroid analysis, although stringent QA schemes are in operation in clinical laboratories.

12.2 Steroid Drugs in Pharmacopoeias

In this section the monographs on steroid drugs (hormones, hormone analogs, bile acids and vitamins D) in the main pharmacopoeias, namely, the *5th Edition of European Pharmacopoeia* (Pharm. Eur. 5, 2004), the *United States Pharmacopoeia* (USP 29, 2006) and the *Japanese Pharmacopoeia* (Pharm. Jap. 14, 2001), are summarised in tabulated form. The aim of Table 12.1 is to give an overview on the methods used for the assay and related impurities tests of bulk steroid drug materials. The results of these tests are considered to be the most important data characterising the quality (purity) of the drugs. In addition to these, the monographs contain identification tests (predominantly infrared (IR) spectroscopy), determination of physical constants (melting point, optical rotatory power) and limit tests for volatile impurities, residue of ignition, traces of heavy metals, etc. This information is summarised in Table 12.1.

12.2.1 Assay of Bulk Steroid Drug Materials

As seen in Table 12.1, in the overwhelming majority of cases, non-selective UV spectrophotometric, titrimetric and selective LC methods are used for the assay of bulk steroid drug materials.

UV spectrophotometry at about 240 nm for steroids with strongly absorbing 4-ene-3-oxo- or 1,4-diene-3-oxo groups and at about 280 nm for oestrogens with a phenol-type ring A with its weak, but characteristic, spectra is used mainly by Pharm. Eur. 5. This method is naturally relatively non-specific in that the overwhelming majority of the impurities are measured together with the main component. It is to be noted that UV spectrophotometry around 240 nm with limits for the specific absorbance is often part of the monographs (predominantly in USP 29) even in those cases when the assay is carried out by LC methods. In a few instances, visible spectrophotometric methods based on chemical reactions, such as a measurement around 380 nm after condensation of the unsaturated 3-oxo group with isoniazide or indirect measurement at 525 nm after a reaction with Tetrazolium Blue of corticosteroids with reducing side chain, are also used. The selectivity of these, rather outdated, methods (Görög and Szász, 1978; Görög, 1983, 1989) is not much better than that of the spectrophotometric methods based on UV absorbance measurements on underivatised steroids.

Although the overwhelming majority of steroid drugs do not posses functional groups suitable for *titrimetric determination*, in some cases this method can be used even though it is not specific. Bile acids are titrated directly with sodium hydroxide while ethinyl steroids can be titrated after a reaction with silver nitrate and liberation of nitric acid (Pharm. Jap. 14, 2001; Pharm. Eur. 5, 2004; USP 29, 2006) (see Fig. 12.1). Other titrimetric methods are restricted to the titration of pancuronium bromide (Pharm. Jap. 14, 2001; Pharm. Eur. 5, 2004), dexamethasone isonicotinate (Pharm. Eur. 5, 2004) and stanozolol (Pharm. Eur. 5, 2004; USP 29, 2006) with acetous perchloric acid.

Table 12.1 Assay and related impurities tests in European, USP and Japanese pharmacopoeias (steroid hormones and hormone analogues, bile acids and vitamins D)

	Assay			Related impurities		
	Ph. Eur. 5	USP XXIX	Ph.Jp. XIV	Ph. Eur. 5	USP XXIX	Ph. Jp XIV
Alclometasone dipropionate	–	RP-HPLC 254 nm	–	–	TLC 254 nm	–
Amcinonide	–	RP-HPLC 254 nm	–	–	–	–
Beclomethasone dipropionate	RP-HPLC 254 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	–	TLC – tetrazolium
Betamethasone	UV 238.5 nm	RP-HPLC 240 nm	RP-HPLC 240 nm	RP-HPLC 254 nm	TLC – H_2SO_4	TLC 254 nm
Acetate	UV 240 nm	RP-HPLC 254 nm	–	RP-HPLC 254 nm	TLC – H_2SO_4	–
Benzoate	–	RP-HPLC 254 nm	–	–	TLC 254 nm	–
Dipropionate	UV 240 nm	RP-HPLC 254 nm	UV 239 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	TLC 254 nm
Sodium phosphate	UV 241 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	Betamethasone – extraction 239 nm	TLC 254 nm
Valerate	UV 240 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	TLC – tetrazolium
Budesonide	RP-HPLC 240 nm	–	–	RP-HPLC 240 nm	–	–
Chlormadinone acetete	–	–	UV 285 nm	–	–	RP-HPLC 236 nm
Cholecalciferol	NP-HPLC 254 nm	NP-HPLC 254 nm	NP-HPLC 254 nm	–	–	–
Clobetasol propionate	RP-HPLC 240 nm	RP-HPLC 240 nm	–	RP-HPLC 240 nm	RP-HPLC 240 nm	–
Clobetasone butyrate	UV 235 nm	–	–	RP-HPLC 241 nm	–	–
Clocortolone pivalate	–	VIS–isoniazid	–	–	TLC/elut 238 nm	–
Cortisone acetate	UV 237 nm	RP-HPLC 254 nm	–	RP-HPLC 254 nm	RP-HPLC 254 nm	–
Cyproterone acetate	UV 282 nm	–	–	RP-HPLC 254 nm	–	–
Danazol	–	UV 285 nm	–	–	TLC – iodine	–
Dehydrocholic acid	–	Titration NaOH	Titration NaOH	–	–	–
Desogestrel	RP-HPLC 205 nm	–	–	RP-HPLC 205 nm	–	–
Desoxycort(icoster) one acetate	UV 240 nm	VIS–tetrazolium	–	RP-HPLC 254 nm	–	–
Pivalate	–	RP-HPLC 254 nm	–	–	–	–
Desoximetasone	–	RP-HPLC 254 nm	–	–	–	–

(continued)

Table 12.1 (continued)

	Assay			Related impurities		
	Ph. Eur. 5	USP XXIX	Ph.Jp. XIV	Ph. Eur. 5	USP XXIX	Ph. Jp XIV
Dexamethasone	UV 238.5 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	TLC 254 nm
Acetate	UV 238.5 nm	RP-HPLC 254 nm	–	RP-HPLC 254 nm	RP-HPLC 254 nm	–
Isonicotinate	Titration $HClO_4$	–	–	RP-HPLC 240 nm	–	–
Sodium phosphate	UV 241.5 nm	RP-HPLC 254 nm	–	RP-HPLC 254 nm	RP-HPLC 254 nm	–
Diflorasone diacetate	–	NP-HPLC 254 nm	–	–	NP-HPLC 254 nm	–
Drostanolone propionate	–	–	GC	–	–	TLC – vanillin
Dydrogesterone	–	RP-HPLC 280 nm	–	–	RP-HPLC 280 nm	–
Equilin	–	RP-HPLC 280 nm	–	–	–	–
Ergocalciferol	NP-HPLC 254 nm	NP-HPLC 254 nm	NP-HPLC 254 nm	TLC – $SbCl_3$	–	–
Estradiol	UV 238 nm NaOH	RP-HPLC 205 nm	–	RP-HPLC 280 nm	NP-HPLC 280 nm	–
Benzoate	UV 231 nm	–	RP-HPLC 230 nm	RP-HPLC 230 nm	–	TLC 254 nm
Cypionate	–	RP-HPLC 280 nm	–	–	–	–
Valerate	UV 280 nm	RP-HPLC 280 nm	–	RP-HPLC 220 nm	TLC – H_2SO_4	–
Estriol	UV 281 nm	UV 281 nm	RP-HPLC 280 nm	NP-HPLC 254 nm	TLC – H_2SO_4	TLC–H_2SO_4
Oestrogens, conjugated	GC	GC	–	GC	GC	–
Oestrogens, esterified	–	GC	–	–	GC	–
Estrone	–	RP-HPLC 280 nm	–	–	TLC – H_2SO_4	–
Estropipate	–	RP-HPLC 213 nm	–	–	RP-HPLC 213 nm	–
Ethinylestradiol	Titration ethinyl	RP-HPLC 280 nm	Titration ethinyl	RP-HPLC 280 nm	–	Estrone–colorim.
Ethynodiol diacetate	–	RP-HPLC 200 nm	–	–	RP-HPLC 200 nm	–
Finasteride	RP-HPLC 210 nm	RP-HPLC 210 nm	–	RP-HPLC 210 nm	RP-HPLC 210 nm	–
Fludrocortisone acetate	UV 238 nm	VIS–tetrazolium	–	RP-HPLC 254 nm	TLC 254 nm	–
Flumetasone pivalate	UV 239 nm	VIS–tetrazolium	–	RP-HPLC 254 nm	TLC-H_2SO_4	–
Flunisolide	–	RP-HPLC 254 nm	–	–	TLC 254 nm	–

Fluocinolone acetonide	UV 238 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	RP-HPLC 238 nm	–	RP-HPLC 254 nm
Fluocinonide	–	RP-HPLC 254 nm	RP-HPLC 254 nm	–	RP-HPLC 254 nm	TLC – tetrazolium
Fluocortolone pivalate	UV 242 nm	–	–	RP-HPLC 243 nm	–	–
Fluorometholone	–	RP-HPLC 254 nm	RP-HPLC 254 nm	–	–	TLC 254 nm
Fluoxymesterone	–	RP-HPLC 254 nm	NP-HPLC 254 nm	–	RP-HPLC 254 nm	TLC 254 nm
Flurandrenolide	–	RP-HPLC 240 nm	–	–	TLC 254,366 nm	–
Fluticasone propionate	RP-HPLC 239 nm	RP-HPLC 239 nm	–	RP-HPLC 239 nm	RP-HPLC 239 nm	–
Halcinonide	–	UV 239 nm	–	–	TLC/elut. 239 nm	–
Hydrocortisone	RP-HPLC 239 nm	RP-HPLC 254 nm	NP-HPLC 254 nm	RP-HPLC 239 nm	NP-HPLC 254 nm	TLC 254 nm
Acetate	UV 241.5 nm	NP-HPLC 254 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	TLC – tetrazolium
Butyrate	–	RP-HPLC 254 nm	UV 241 nm	–	RP-HPLC 254 nm	TLC – tetrazolium
Hydrogen (hemi) succinate	UV 241.5 nm	NP-HPLC 254 nm	–	RP-HPLC 254 nm	RP-HPLC 254 nm	–
Sodium phosphate	–	Enzime, extraction 239 nm	RP-HPLC 254 nm	–	Hydrocortisone, extraction 239 nm	RP-HPLC 254 nm
Sodium succinate	–	VIS–tetrazolium	UV 240 nm	–	–	TLC 254 nm
For injection	–	NP-HPLC 254 nm	–	–	NP-HPLC 254 nm	–
Succinate	–	–	RP-HPLC 254 nm	–	–	TLC 254 nm
Valerate	–	RP-HPLC 254 nm	–	–	–	–
Hydroxyprogesterone caproate	–	UV 240 nm	–	–	TLC – H_2SO_4	–
Isofluprednone acetate	–	NP-HPLC 254 nm	–	–	RP-HPLC 254 nm	–
Levonorgestrel	Titration ethinyl	UV 241 nm	–	TLC – phosphomolybdate	TLC – phosphomolybdate	–
Lynestrenol	Titration ethinyl	–	–	TLC – H_2SO_4	–	–
Medroxyprogesterone acetate	UV 241 nm	RP-HPLC 254 nm	–	TLC – *p*-toluene-sulphonic acid	RP-HPLC 254 nm	–
Megestrol acetate	UV 287 nm	RP-HPLC 280 nm	–	RP-HPLC 254 nm	–	–

(continued)

Table 12.1 (continued)

	Assay			Related impurities		
	Ph. Eur. 5	USP XXIX	Ph.Jp. XIV	Ph. Eur. 5	USP XXIX	Ph. Jp XIV
Melengestrol acetate	–	RP-HPLC 287 nm	–	–	RP-HPLC 240, 262 nm	
Mepitiostane	–	–	RP-HPLC 265 nm	–	–	TLC – H_2SO_4
Meprednisone	–	TLC 238 nm extr.	–	–	–	–
Mesterolone	RP-HPLC 254 nm	–	–	RP-HPLC 200 nm TLC – *p*-toluene-sulphonic acid	–	–
Mestranol	Titration ethinyl	VIS –H_2SO_4	UV 279 nm	TLC – H_2SO_4	–	TLC – H_2SO_4
Metenolone acetate	–	–	UV 242 nm	–	–	TLC 254 nm
Metenolone enanthate	–	–	UV 242 nm	–	–	TLC 254 nm
Methylprednisolone	UV 243 nm	NP-HPLC 254 nm	UV 243 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	TLC – tetrazolium
Acetate	UV 243 nm	NP-HPLC 254 nm	–	RP-HPLC 254 nm	RP-HPLC 254 nm	–
Hydrogen succinate	UV 243 nm	NP-HPLC 254 nm	–	RP-HPLC 254 nm	RP-HPLC 254 nm	–
Sodium succinate	–	VIS-tetrazolium	–	–	–	–
Methyltestosterone	UV 241 nm	RP-HPLC 241 nm	UV 241 nm	TLC 254 nm	RP-HPLC 254 nm	TLC 254 nm
Mibolerone	–	RP-HPLC 254 nm	–	–	–	–
Mometasone furoate	UV 249 nm	RP-HPLC 254 nm	–	RP-HPLC 254 nm	TLC 254 nm	–
Nandrolone decanoate	UV 240 nm	RP-HPLC 240 nm	–	TLC – H_2SO_4 RP-HPLC 254 nm	NP-HPLC 238 nm	–
Nandrolone phenylpropionate	–	TLC 239 nm extr.	–	–	–	–
Norethisterone (norethindrone)	Titration ethinyl	UV 240 nm	Titration ethinyl	RP-HPLC 210, 254 nm	TLC – H_2SO_4	–
Acetate	Titration ethinyl	UV 240 nm	Titration ethinyl	RP-HPLC 210, 254 nm	TLC 254 nm RP-HPLC 254 nm	RP-HPLC 254 nm
Norethynodrel	–	UV 240 nm-HCl	–	–	TLC – H_2SO_4	–

Norgestimate	–	RP-HPLC 244 nm	–	–	RP-HPLC 244 nm NP-HPLC 210 nm	–
Norgestrel	Titration ethinyl	UV 241 nm	Titration ethinyl	TLC – phosphomolybdate	TLC – phosphomolybdate	TLC 254 nm
Oxandrolone	–	Titration lactone	–	–	TLC – H_2SO_4	–
Oxymetholone	–	TLC 315 nm extr.	–	–	–	–
Pancuronium bromide	Titration $HClO_4$	–	Titration $HClO_4$	TLC – $NaNO_2$+ iodobismuthate	–	TLC – $NaNO_2$ + iodobismuthate
Paramethasone acetate	–	TLC 242 nm extr.	–	–	–	–
Paricalcitol	–	RP-HPLC 252 nm	–	–	RP-HPLC 252 nm	–
Prasterone sodium sulfate	–	–	Ion-exchange/ Titration NaOH	–	–	TLC – H_2SO_4
Prednicarbate	RP-HPLC 243 nm	–	–	RP-HPLC 243 nm	–	–
Prednisolone	UV 243.5 nm	NP-HPLC 254 nm	RP-HPLC 247 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	TLC – tetrazolium
Acetate	UV 243 nm	NP-HPLC 254 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	NP-HPLC 254 nm	TLC 254 nm
Hemisuccinate	–	TLC 243 nm extr.	–	–	–	–
Pivalate	UV 243 nm	–	–	RP-HPLC 254 nm	–	–
Sodium phosphate	UV 247 nm	UV 241 nm enzime, extract.	–	RP-HPLC 254 nm	Prednisolone extraction 241 nm	–
Sodium succinate	–	VIS–tetrazolium	RP-HPLC 254 nm	–	–	–
Succinate	–	–	RP-HPLC 242 nm	–	–	TLC 254 nm
Ttebutate	–	NP-HPLC 254 nm	–	–	–	–
Prednisone	UV 238 nm	RP-HPLC 254 nm	–	RP-HPLC 254 nm	NP-HPLC 254 nm	–
Progesterone	UV 241 nm	RP-HPLC 254 nm	UV 241 nm	RP-HPLC 241 nm	–	TLC 254 nm
Rimexolone	–	RP-HPLC 242 nm	–	–	RP-HPLC 242 nm	–
Rocuronium bromide	Titration $HClO_4$	–	–	NP-HPLC 210 nm	–	–

(continued)

Table 12.1 (continued)

	Assay			Related impurities		
	Ph. Eur. 5	USP XXIX	Ph.Jp. XIV	Ph. Eur. 5	USP XXIX	Ph. Jp XIV
Spironolactone	UV 238 nm	RP-HPLC 230 nm	UV 238 nm	RP-HPLC 254/283 nm	TLC – H_2SO_4	TLC – H_2SO_4
Stanozolol	Titration $HClO_4$	Titration $HClO_4$	–	TLC – H_2SO_4	TLC – H_2SO_4	–
Testolactone	–	VIS – isoniazid	–	–	TLC 254 nm + acid dichromate	–
Testosterone	UV 241 nm	TLC 239 nm extr.	–	TLC- *p*-toluene-sulphonic acid RP-HPLC 254 nm	–	–
Cypionate	–	GC	–	–	–	–
Enantate	UV 241 nm	VIS – isoniazid	UV 241 nm	TLC – H_2SO_4 RP-HPLC 240 nm	TLC – *p*-toluene sulphonic acid	–
Propionate	UV 241 nm	VIS – isoniazid	UV 241 nm	RP-HPLC 254 nm	–	TLC 254 nm
Trenbolone acetate	–	RP-HPLC 344 nm	–	–	TLC – phosphomolybdate RP-HPLC 344 nm	–
Triamcinolone	UV 238 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	RP-HPLC 238 nm	–	–
Acetonide	UV 238.5 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	RP-HPLC 254 nm	TLC 254 nm
Diacetate	–	RP-HPLC 254 nm	–	–	–	–
Hexacetonide	UV 238 nm	RP-HPLC 254 nm	–	RP-HPLC 254 nm	RP-HPLC 254 nm	–
Ursodeoxycholic acid (ursodiol)	Titration NaOH	RP-HPLC 254 nm RI detector	Titration NaOH	TLC – phosphomolybdate	TLC – phosphomolybdate	TLC –phosphomolybdate
Vecuronium bromide	–	NP-HPLC 215 nm	–	–	RP-HPLC conductivity det.	–

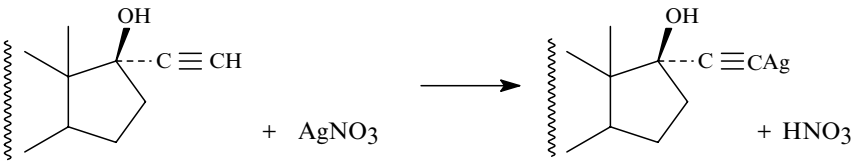

Fig. 12.1 Reaction equation for acidimetric determination of ethinylsteroids

The lactone group in oxandrolone (USP 29, 2006), after opening it with sodium hydroxide, and the –O–SO_2–ONa group in sodium prasterone sulphate (dehydroepiandrosterone sulphate) (Pharm. Jap. 14, 2001) after $Na^+ \rightarrow H^+$ ion exchange are also suitable for acidimetric titration.

The predominant methods in the *United States Pharmacopoeia* (USP 29, 2006) use specific well-optimised and fully validated reversed-phase (RP), and in some cases normal-phase (NP), high-performance liquid chromatography (LC), which is somewhat less widely used in the other two pharmacopoeias. In the overwhelming majority of cases, porous octadecylsilica (C_{18}) support (3–5 μm) is used for the RP and porous silica support (5 μm) for the NP separations. The wavelength of the UV detector is most often 254 nm, which is not exactly the maximum of the spectrum of 4-ene-3-oxo- or 1,4-diene-3-oxo-steroids. This is attributable to the early period in the history of LC when the majority of instruments were equipped with only a mercury lamp, giving out UV light of this wavelength.

As mentioned above, many of the non-chromatographic assay methods used for bulk steroid drugs are non-selective and, for this reason, results obtained by these may not measure, with any degree of confidence, the real active ingredient content. (The prescribed limits are usually 98–102% or 97–103%.) Well-established LC methods are more selective but extremely time-consuming, although the availability of computer-controlled LC systems allows unattended operation throughout the day. Although it is mandatory to establish the reliability of the method, the "system suitability test" prescribed prior to the assay is itself often very time-consuming. To illustrate this, the system suitability test before the LC assay of hydrocortisone (cortisol) bulk material in the *United States Pharmacopoeia* requires that the resolution (*R*) between the peaks of hydrocortisone and propylparabene (internal standard) is not less than 9.0, the column efficiency is not less than 3,000 theoretical plates for hydrocortisone, the tailing factor is not more than 1.2 and the relative standard deviation of at least five replicate injections is not more than 2.0% (USP 29, 2006).

Owing to the limited accuracy and precision of even selective LC methods and the non-selective nature of other methods the results of the assay methods are of limited value in characterising the quality (purity) of bulk drugs, among them steroids. Much more appropriate means for this is the "related impurities test" to be discussed in the subsequent section. In the author's opinion (Görög, 2005, 2008) the importance of assay methods of bulk drug materials, among others of steroid drugs, in modern pharmaceutical analysis is at least questionable, and the real

active ingredient content is better characterised by the formula $(100 - \Sigma_{impurities})\%$. Of course this does not apply to the assay of drug formulations, where the question is whether the drug concentration is as specified.

12.2.2 Related Impurities Test of Bulk Steroid Drug Materials

As already mentioned, the test for "related substances" (Pharm. Eur. 5, 2004), also named "chromatographic purity" (USP 29, 2006), "ordinary impurities" (USP 29, 2006), "related steroids" (USP 29, 2006) or "other steroids" (Pharm. Jap. 14, 2001), is the most important test to characterise the quality of bulk steroid drug materials.

As can be seen in Table 12.1, with two exceptions (conjugated and esterified oestrogens, where gas chromatography is used), this test is carried out by semi-quantitative TLC or quantitative (mainly, but not exclusively, reversed-phase) LC methods. The latter is the predominant method for bulk steroids in Ph. Eur. 5 and is also widely used in USP 29 and to a lesser extent in Pharm. Japan 14. It has to be mentioned that in many cases the USP 29 and Pharm. Japan 14 do not always contain tests for related impurities.

In the case of LC tests, a UV detector is used at the wavelength corresponding to maximum absorbance of the main component or at 254 nm. For the thin layer chromatographic purity test, in the majority of cases, sorbent layers are used that are impregnated with dyes strongly fluorescing when irradiated at 254 nm by a mercury lamp. Since most of the steroid hormone drugs strongly absorb UV light at this wavelength, the drugs and their impurities appear as dark spots in the chromatogram, suitable for their semi-quantitative estimation. These appear as "TLC 254 nm" in Table 12.1. In some cases the use of various visualising reagents is necessary. Reagents containing sulphuric acid are most frequently used. "TLC – H_2SO_4" in Table 12.1 means spraying with this reagent, heating and visual inspection of the plate in daylight and using long-wavelength UV light (366 nm). Other visualising reagents are *p*-toluenesulphonic acid, vanillin/sulphuric acid (TLC – vanillin), potassium dichromate/sulphuric acid (TLC – acid dichromate), phosphomolybdate, iodine vapour, sodium nitrite/iodobismuthate and alkaline blue tetrazolium (TLC – tetrazolium).

The requirements regarding the limit of related impurities vary from steroid to steroid and pharmacopoeia to pharmacopoeia. For example, in the case of hydrocortisone in the *United States Pharmacopoeia* (USP 29, 2006) the level of any individual impurity and the sum of impurities must not exceed 0.5% and 2.0%, respectively. The same limits for triamcinolone acetonide in the *European Pharmacopoeia* (Pharm. Eur. 5, 2004) are 0.25% and 0.5%. It is important to note that with a few exceptions, both the TLC and LC tests express the impurities as the main component, which can be the source of serious errors (under- or overestimation) if the chromophoric system or the colour and/or the intensity of the TLC spot of the main component and the impurity are different. For this reason, requirements of the drug regulatory authorities are much stricter and require identification of the

impurities above a certain level (usually 0.1%) and their quantification by specific tests or at least determination by use of relative response factors in the LC and quantitative TLC-densitometric tests. Examples for this are shown in Section 12.7.1.

12.2.3 Assay of Steroid Drug Formulations

In this section the state of the art in the field of determining the active ingredient content of steroid drug formulations is outlined based on the *United States Pharmacopoeia* (USP 29, 2006), since the *European Pharmacopoeia* (Pharm. Eur. 5) does not contain such methods for formulations and the *Japanese Pharmacopoeia* (Pharm. Jap. 14, 2001) contains only a few. Unlike in the case of bulk steroid drugs, this assay is of fundamental importance, since this is the guarantee that the active ingredient content is in accordance with the label claim. The requirement is usually ±10% (or in real life often ±5%). Another difference is that the use of selective methods (mainly LC) is much more widespread here as these methods are necessary to demonstrate the stability of the active ingredient in the formulations. This is reflected by the much higher proportion of LC use than in the case of bulk steroid drugs. Although – as will be shown – a few non-selective methods are still officially included in the pharmacopoeia, the use of selective methods is required by the drug regulatory authorities.

A great variety of *sample-preparation* methods are used in the assay of formulations. Solid dosage forms (*tablets* etc.) are either dispersed with water, followed by dilution with suitable solvent (methanol, acetonitrile, or the mobile phase of the RP-LC system) or extraction of the aqueous phase with chloroform. More common is direct extraction – mainly with methanol. Direct extraction with methanol, chloroform, acetonitrile, etc. is the most widely used sample-preparation method for *creams*, while in addition to these, extraction with hexane, heptane, isooctane followed by extraction of the apolar phase with mixtures of water and methanol, acetonitrile, etc. A similar approach is adopted for *ointments*. Aqueous *solutions* are usually diluted with water or the mobile phase of the RP-LC method, or extracted with chloroform, ethyl acetate, etc. Aqueous *suspensions* are homogenised by simple dilution with methanol, 2-propanol or the mobile phase of the RP-LC system. Oily *injections* are either simply diluted, mainly with chloroform or diluted with hexane etc., followed by extraction with polar solvents or solvent mixtures.

The predominant method for the assay is *reversed-phase LC* with UV detection, either at 254 nm or at the maxima of the UV spectra of the active ingredients. This method is used among others for the assay of aclometasone dipropionate and amcinonide cream and ointment, betamethasone cream and tablets, betamethasone benzoate gel, betamethasone dipropionate cream, ointment, lotion, and topical aerosol, betamethasone sodium phosphate injection, betamethasone sodium phosphate and acetate injectable suspension, betamethasone valerate cream, ointment and lotion, clobetasol propionate cream, ointment and topical solution, cortisone acetate tablets and injectable suspension, danazol capsules, desoxycorticosterone pivalate

injectable suspension, desoximetasone cream and ointment, dexamethasone injection, ophthalmic suspension, oral solution, elixir and tablets, dexamethasone acetate injectable suspension, dexamethasone sodium phosphate cream, injection, ophthalmic ointment and ophthalmic solution, dydrogesterone tablets, estradiol tablets and vaginal cream, estradiol cypionate and valerate injection, estrone injectable suspension, estropipate tablets and vaginal cream, ethynodiol diacetate and mestranol tablets, ethynodiol diacetate and ethinylestradiol tablets, finasteride tablets, fludrocortisone acetate tablets, flunisolide nasal solution, fluocinolone acetonide cream, ointment and topical solution, fluocinonide cream, ointment, gel and topical solution, fluorometholone ophthalmic suspension, fluoxymesterone tablets, flurandrenolide cream, lotion, tape, halcinonide cream and ointment, hydrocortisone cream, ointment, gel, lotion, rectal suspension and tablets, hydrocortisone butyrate cream, hydrocortisone valerate cream and ointment, levonorgestrel and ethinylestradiol tablets, medroxyprogesterone acetate tablets, megestrol acetate oral suspension and tablets, mometasone furoate cream, ointment and topical solution, nandrolone decanoate injection, norethindrone and ethinylestradiol tablets, norethindrone and mestranol tablets, norgestrel and ethinylestradiol tablets, paricalcitol injection, prednisolone oral solution, prednisolone acetate injectable suspension and ophthalmic suspension, prednisone oral solution, injectable suspension and tablets, progesterone injection, injectable suspension and vaginal suppositories, rimexolone ophthalmic suspension, spironolactone tablets, spironolactone and hydrochlorothiazide tablets, triamcinolone tablets, triamcinolone acetonide cream, ointment, topical aerosol, lotion and injectable suspension, triamcinolone diacetate oral solution and injectable suspension, triamcinolone hexacetonide injectable suspension and ursodiol capsules and tablets.

Normal-phase LC is used for the assay of cholecalciferol (vitamin D_3) solution (in oil), cortisone acetate tablets, diflorasone diacetate cream, ergocalciferol capsules, tablets and oral solution, fluorometholone cream, hydrocortisone tablets, hydrocortisone acetate cream, ointment and lotion, isofluprednone acetate injectable suspension, medroxyprogesterone acetate injectable suspension, methylprednisolone tablets, methylprednisolone acetate injectable suspension, prednisolone tablets and prednisolone tebutate injectable suspension.

Of the other chromatographic methods, *gas chromatography*, using exclusively flame ionisation detection, is used for the assay of esterified and conjugated oestrogen tablets, mibolerone oral solution, oxandrolone tablets and testosterone cypionate injection. *TLC* separation, extraction followed by tetrazolium blue colorimetry is the assay method for betamethasone syrup and methylprednisolone acetate cream, while very outdated column chromatographic separation followed by tetrazolium blue colorimetry or iron-phenol colorimetry is prescribed for the assay of dexamethasone gel and estradiol pellets and injectable suspension, respectively.

The above-discussed chromatographic methods are selective and can thus be used for stability testing. This does not apply to non-chromatographic methods. *UV spectrophotometric* assay based on natural absorption is carried out for dexamethasone sodium phosphate inhalation aerosol, methyltestosterone tablets and capsules, oxymetholone tablets, prednisolone sodium phosphate injection and

ophthalmic solution (after enzymatic hydrolysis and extraction), progesterone in intrauterine contraceptive system, stanozolol tablets and testosterone injectable suspension. UV spectrophotometry is used in some other cases such as in the content uniformity and dissolution tests of tablet formulations, where selectivity is not such an important issue. The selectivity of some colorimetric methods, also used for the assay of steroid hormone formulations, is not better either (Görög and Szász, 1978; Görög, 1983, 1989). For example, reactions based on the condensation of unsaturated 3-oxo group with isoniazid (clocortolone pivalate cream, nandrolone phenylpropionate injection, norethindrone tablets, norgestrel tablets, testolactone tablets) or 4-aminoantipyrine (flumethasone pivalate cream) measure the more stable A ring part of the molecules. The situation is somewhat better with the colour reactions of corticosteroids with sensitive, reducing side chains at position 17. Most of their degradation products do not react with the alkaline tetrazolium blue (desoxycorticosterone acetate injection and pellets, dexamethasone topical aerosol, hydrocortisone injectable suspension and hydrocortisone acetate ophthalmic ointment and suspension, injectable suspension) or phenylhydrazine-sulphuric acid reagents (hydrocortisone sodium phosphate injection and prednisolone cream).

The use of acidimetric titration for the assay of dehydrocholic acid tablets and IR spectroscopy for paramethasone acetate tablets can be regarded as curiosities.

12.3 Spectroscopic and Spectrophotometric Methods in the Analysis of Steroid Drugs

12.3.1 Ultraviolet-Visible Spectrophotometry

As shown in the previous sections, in spite of its poor selectivity, UV spectrophotometry (Görög, 1995) is widely described in the pharmacopoeias for the assay of bulk steroid drugs. The assay is carried out either using a reference standard material or on the basis of specific absorbance values, presented in the pharmacopoeia. The validation of the determination of the latter by interlaboratory study was described on the example of triamcinolone (Rose and Fuchs, 1998).

It was also shown that the non-selective but very rapid assay of single-component drug formulations can also be carried out by UV spectrophotometry. Various possibilities are available for the solution of more delicate problems such as the assay of drug formulations with two or three active ingredients. These methods excel with their speed and simplicity but of course their selectivity is not comparable with chromatographic methods. For example, the classical dual-wavelength spectrophotometry (Vierordt method) was successfully used for the simultaneous determination of spironolactone and hydrochlorothiazide with their overlapping spectra in a tablet formulation (Erk, 1999). The performance of this method can be improved by multiwavelength measurements and *chemometric methods*. Partial least-squares regression analysis was applied to other diuretic preparations, such as spironolactone – althiazide (Linares et al., 1999) and spironolactone – chlorthalidone

(Luis et al., 1999) formulations. The much more delicate problem of the assay of low-dosed levonorgestrel – ethinylestradiol (Berzas et al., 1997a) and gestodene – ethinylestradiol (Berzas et al., 1997b) oral contraceptive tablets was also solved by the partial least-squares regression analysis. Even three-component drug formulations were successfully analysed by this and other chemometric methods, such as the principal component regression method. One example is the mixture of hydrocortisone, nystatin and oxytetracycline (Lemus Gallego and Pérez Arroyo, 2002a). The accuracy and precision of the determination of even as low a quantity as 2 mg/vial dexamethasone in the presence of 250 and 10 mg of vitamins B_6 and B_{12}, respectively, in an injection formulation were good (Nepote et al., 2003). Other 3-component formulations containing low quantities of dexamethasone resolved by partial least-squares regression analysis and other chemometric methods contained Polymyxin B, trimethoprim (Lemus Gallego and Pérez Arroyo, 2001a) and chlorpheniramine, naphazoline (Goicoechea et al. 2002), respectively.

Derivative UV spectrophotometry (Görög, 1995) where the assay is based on the first, second, and sometimes third derivative spectra, easily obtainable by modern recording UV spectrophotometers was also successfully applied to the analysis of steroid formulations. In simple cases (single-component formulations) only the spectral background caused by the excipients should be eliminated. For this purpose first-derivative spectra are usually sufficient. An example for this is the determination of triamcinolone acetonide in an ointment (Kedor-Hackmann et al., 1997). The same technique using the "zero-crossing" method was found to be suitable for the simultaneous determination of the components of dual-component drug formulations. In this technique the steroid is measured at such a wavelength where one of the derivative curves of the other component is zero (it crosses the *X*-axis) and vice versa. Examples for this are creams and tablets containing triamcinolone acetonide and terbinafine hydrochloride (El-Saharty et al., 2002), cyproterone acetate – ethinylestradiol tablets (Souri et al., 2005), medroxyprogesterone acetate - estradiol tablets (Toral et al., 2002), oral contraceptives containing ethinylestradiol and levonorgestrel (Berzas et al., 1997c) as well as ethinylestradiol and gestodene (Berzas et al., 1997). A further possibility to improve the selectivity of the method is the use of "*ratio spectrum* (zero-cross) derivative spectroscopic" method, suitable for the resolution of even ternary mixtures. Examples for this are the determination of spironolactone and hydrochlorothiazide (Erk, 1999) or furosemide (Millership et al., 2005), cyproterone acetate and estradiol valerate (Dinç et al., 2003), hydrocortisone, nystatin and oxytetracycline (Lemus Gallego and Pérez Arroyo, 2002) in pharmaceutical formulations. Figure 12.2a shows the strongly overlapping spectra of the three compounds and their mixture in the latter example. Figure 12.2b is the representation of the first derivative of the ratio spectra while Fig. 12.2c shows the same curve at various concentrations of hydrocortisone. See the figure legend for details. Good assay results were obtained using this method for formulations containing the three active ingredients.

The performance of various multivariate chemometric and derivative spectrophotometric methods is compared, for example, on formulations containing hydrocortisone, nystatin and oxytetracycline (Lemus Gallego and Pérez Arroyo, 2002),

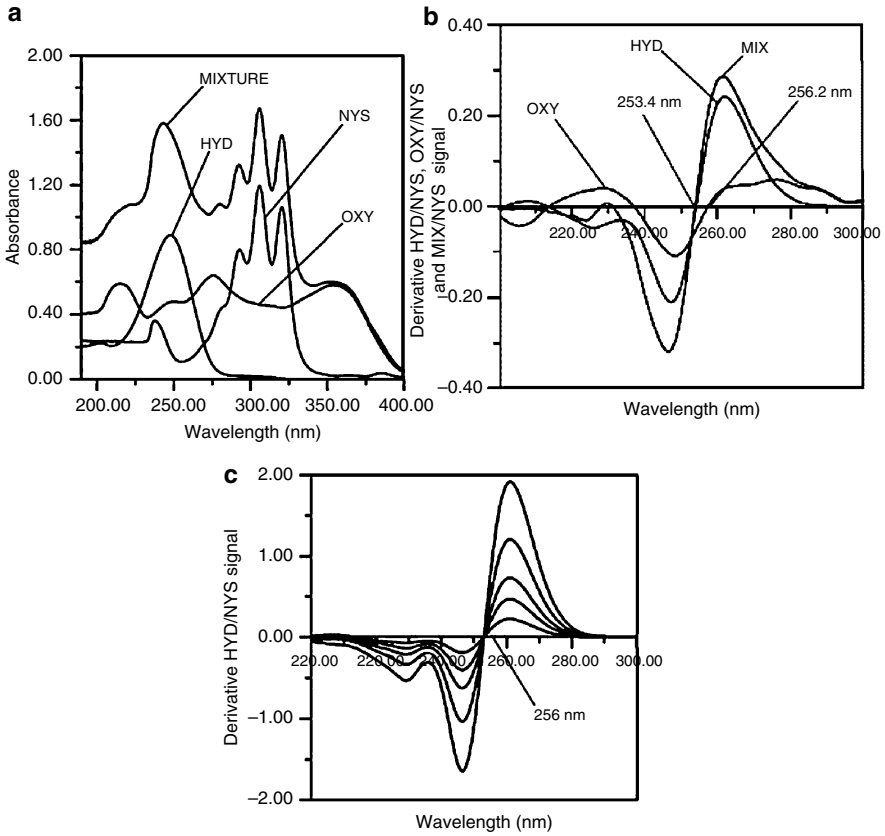

Fig. 12.2 Spectra for the simultaneous determination of hydrocortisone (HYD), oxytetracycline (OXY) and nystatin (NYS). Solvent: acetate buffer (pH 4.5) containing 16% v/v ethanol. (**a**) Absorption spectra of 20 mg/L of the three drugs and their mixture. (**b**) First derivative of the ratio spectra of 4–4 mg/L of HYD and OXY and their mixture obtained using as divisor the spectrum of 6 mL/L of NYS. (**c**) First derivative of the ratio spectra for different concentrations of HYD obtained using as divisor the spectrum of 6 mL/L of NYS (from Lemus Gallego and Pérez Arroyo, 2002, with permission)

hydrocortisone and Zn-Bacitracin (Lemus Gallego and Pérez Arroyo, 2003a) as well as dexamethasone and polymyxin B and trimethoprim (Lemus Gallego and Pérez Arroyo, 2001). Making use of a rapid-scanning diode-array spectrophotometer, the ratio spectrum derivative spectroscopic method can be adapted to flow-injection analysis for the assay of spironolactone and hydrochlorothiazide (Martín et al., 1997).

The importance of spectrophotometric/colorimetric methods based on chemical reactions has dramatically decreased in the last years both in the analysis of bulk steroid drugs and formulations made thereof. As can be seen in Table 12.1 and in Section 12.2.3, some of the classical reactions (e.g. condensation with isoniazid or phenylhydrazine, redox reaction with tetrazolium reagents) are still in use in some instances.

Many other reactions have also been described and reviewed (Görög and Szász 1978; Görög 1983, 1989, 1995). Further research in this field can be considered to be unnecessary. Publications of this kind, although still published from time to time, can in the author's view be considered to be obsolete and therefore are not referred to further in this section.

UV spectroscopy of underivatised steroids is definitely the least informative of the spectroscopic techniques for the identification/structure elucidation of steroid drugs, their impurities and degradation products, etc. However, because UV spectra can easily be obtained by means of the diode-array detector or reflection spectra by TLC-densitometry, such methodology is still widely used and remain valuable, and simple, aids to structural analysis.

12.3.2 Fluorimetry

Of the various groups of steroid drugs only oestrogens possess native fluorescence, suitable for analytical purposes. The main field of application of this technique is for sensitive LC detection. For example, in the case of the analysis of contraceptive pills, the very low amount of ethinylestradiol (20–30 μg/tablet) causes difficulties if UV detection is used. For this reason, in the monograph dealing with the dissolution test of norgestrel – ethinylestradiol tablets, the *United States Pharmacopoeia* (USP 29, 2006) uses UV detection at 247 nm for monitoring norgestrel and fluorimetric detection (excitation at 285 nm, emission at 310 nm) for ethinylestradiol.

Measurements based on fluorescence induced by non-stoichiometric reactions with various strongly acidic reagents (Görög and Szász, 1978; Görög, 1983, 1989) have almost completely lost their importance in modern pharmaceutical analysis. However, methanol-sulphuric acid and other reagents containing sulphuric or phosphoric acids are still important spray reagents in the thin-layer chromatographic analysis of steroid drugs.

Two specific and sensitive flow injection analysis (FIA) methods based on fluorescence after chemical reactions are worth mentioning. As shown in Fig. 12.3 the –CO–S–CH_2F side chain of fluticasone propionate is cleaved with sodium hydroxide to form fluoromethylthiol, which is further reacted with glycine and *o*-phthalaldehyde. The strongly fluorescent isoindole derivative is formed in the reaction coil of the FIA apparatus (Vannecke et al., 2000). Photochemically-induced fluorescence was used for the determination of hydrocortisone acetate and succinate (Cardoso et al., 2004).

12.3.3 Infrared, Near Infrared (NIR) and Raman Spectroscopy

Infrared (IR) spectroscopy is the most generally used method for the identification of bulk steroid drugs in modern pharmacopoeias largely but not completely replacing the classical colour tests (Pharm. Jap. 14, 2001; Pharm. Eur. 5, 2004; USP 29, 2006).

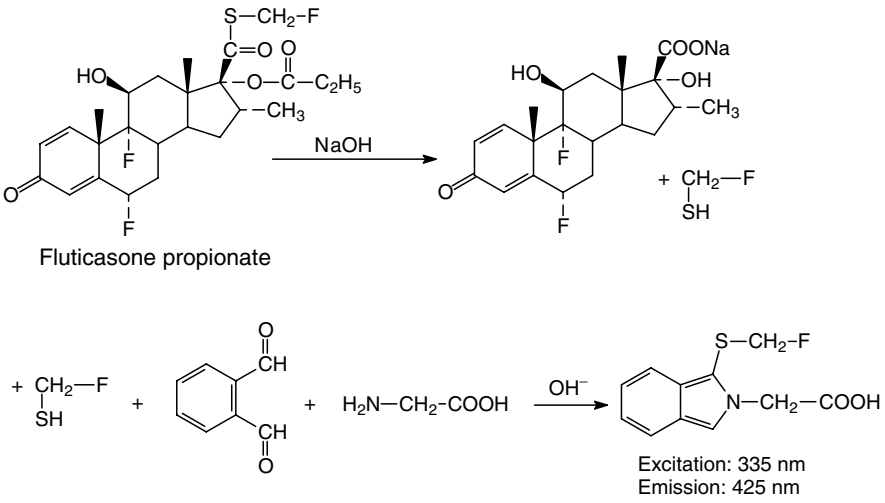

Fig. 12.3 Indirect fluorimetric determination of fluticasone propionate (Vannecke et al., 2000)

In addition to this, IR and Raman spectroscopies, especially their Fourier-transform variants (FT-IR and FT-Raman) are among the most important modern methods, together with x-ray powder diffractometry, solid state NMR spectroscopy and thermal methods (Salole, 1991) such as differential scanning calorimetry (DSC), thermogravimetry (TGA) and thermomicroscopy to characterise the solvates and polymorphic modifications frequently occurring among steroid drugs. One of the most characteristic examples is spironolactone, where, in the early literature, several polymorphs and solvates were described. Conventional IR spectrometry using fused KBr discs is not suitable for their differentiation due to the easy transformation of the polymorphs under pressure. On the basis of diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) and FT-Raman spectroscopic investigations supported by DSC and TGA measurements (Neville et al., 1994), four different polymorphic forms (Neville et al., 1992) and five solvates (Beckstead et al., 1993) were identified and characterised. FT-IR studies combined with all of the above mentioned solid-phase techniques revealed the existence of a three polymorphs of fluocinolone acetonide (Bartolomei et al., 1997) as well as two polymorphs and a hemihydrate of flunisolide (Bartolomei, 2000) and characterised the anhydrous and monohydrate forms of momethasone furoate (Chen et al., 2005).

The DRIFTS method is also suitable for the rapid verification of identity, as well as the content of various drugs (i.e. finasteride) in their solid formulations (Ryan et al., 1991). TG/FT-IR studies supported by x-ray diffraction and DSC revealed the mechanism of the decomposition of prednisolone hemisuccinate in a freeze-dried formulation (Claussen et al., 2001). In addition to its above-mentioned role in characterising polymorphic modifications and solvates of steroid drugs, FT-Raman spectroscopy can be a useful tool for the quantitative analysis of formulations, too. Medroxyprogesterone acetate was determined in a pharmaceutical suspension

(De Beer et al., 2004) and prednisolone in a tablet formulation (Mazurek and Sztosak, 2006).

Near-infrared reflectance spectroscopy is a versatile tool in drug analysis and in-process control of drug production. In addition to its use in the rapid identification of bulk materials and the active ingredients of formulations, it was found to be capable of resolving difficult problems such as the simultaneous determination of norethisterone and ethinylestradiol in oral contraceptive tablets (Corti et al., 1990) and testosterone in mucoadhesive bi-layer thin-film composites (Fountain et al., 2003).

12.3.4 Mass Spectrometry

The fundamentals of mass spectrometry (MS) are summarised in Chapter 2 and general applications to steroid analysis in Chapter 3. As in the case of other groups of drugs, mass spectrometry is mainly applied to steroid analysis associated with various chromatographic techniques. Examples for these are presented in Sections 12.4, 12.5 and 12.7. Here, only one direct application is mentioned. Time-of-flight secondary ion mass spectrometry was successfully applied to the characterisation and imaging of a controlled-release drug delivery system containing prednisolone sodium metasulfobenzoate. Cross sections of the device containing a multi-polymer-layer system were probed for the distribution of the active ingredient (Belu et al., 2000).

12.3.5 NMR Spectroscopy

Chapter 2 contains an introduction to basic NMR spectroscopy of steroids. One of the relatively rare *direct applications of NMR* spectroscopy in pharmaceutical steroid analysis is its use for the rapid screening and compositional analysis of steroid cocktails and veterinary drug formulations illegally administered to livestock. Stanozolol, fluoxymesterone, methylboldenone, norethandrolone, clobestol acetate, testosterone and its cypionate and decanoate and other steroids were identified directly or after LC fractionation with the aid of a ^{1}H-NMR database (Lommen et al., 2002). Another example relating to drug delivery studies is the application of ^{1}H-NMR in skin permeation studies with transdermal patches containing cyproterone acetate (Kaehlig et al., 2005).

As already mentioned in Section 12.3.3 *solid state NMR* is becoming an important tool in characterising bulk steroid drugs and solid formulations made therefrom. For example, the intermolecular hydrogen bonding in cortisone acetate polymorphs and solvates were successfully studied using this technique (Harris, 2004). When using interrupted decoupling, solid state ^{13}C-NMR is suitable not only for detecting low-dose active ingredients (among others prednisolone) in solid dosage

forms, but also for discrimination between the two polymorphic forms of the latter (Saindon et al., 1993).

In addition to analytical applications, NMR spectroscopy is a good tool for studying the mechanism of interactions taking place between the steroid drug and the stationary phase used in their chromatographic analysis as well as excipients used in the preparation of their pharmaceutical formulations. An example for the former is the transferred NOESY study of the interactions between various steroids and calix[8]arene-based stationary phases (Skogsberg et al., 2003). NMR spectroscopy is a useful tool for determining stability constants of complexes of steroids with various cyclodextrin derivatives as demonstrated on the example of betamethasone (Piel et al., 2004). Similar studies related to chiral interactions are shown in Section 12.7.3. Further analytical applications of NMR spectroscopy are discussed in Sections 12.4.4 and 12.7.1.

12.3.6 Chemiluminometry

Although the main application field of chemiluminescence in steroid analysis is chemiluminescence immunoassay in biological–clinical analysis, a few direct applications in pharmaceutical analysis are also described. The sensitising effect of corticosteroids on the chemoluminogenic reaction between sulphite and cerium(IV) was exploited for their flow-injection (FIA) determination in pharmaceutical formulations (Koukli and Calokerinos, 1990; Deftereos and Calokerinos, 1994). A luminol chemiluminescence determination for the same purpose has also been described (Iglesias et al., 2002).

12.3.7 Circular Dichroism Spectropolarimetry

Circular Dichroism (CD) spectroscopy is an important tool in the structural analysis of steroids but this technique can also be successfully used for analytical purposes (Gergely, 1994). The profound difference between the ellipticities of ethisterone and its 5-ene isomer enabled their selective determination: ethisterone at 348 nm at its negative maximum of the CD spectrum and the isomer at 296 nm at the positive maximum of the CD curve. When simultaneous determination was carried out by LC, the spectropolarimeter could act as a CD-detector (Szegvári et al., 2003). The applicability of the CD method can be enhanced by the use of preliminary chemical reactions. A difference CD method was developed for the determination of 4-ene-3-oxo steroids based on oxime formation making use of the large difference between the ellipticities before and after oximation. This method was found to be suitable for the determination, at as low a concentration as 0.02%, of levonorgestrel acetate as an impurity in norgestimate (Szentesi et al., 2000).

12.4 Chromatographic and Hyphenated Methods

12.4.1 Planar Chromatography

As shown in Section 12.2.2, in pharmacopoeias thin-layer chromatography is used only as a semi-quantitative limit test for purity check of bulk steroid drugs. In contrast to this, in the literature of the last 20 years, almost exclusively quantitative *densitometric* methods are described for purity check and also for the assay of steroid drug formulations. Typical papers describing the assay of formulations are determination of betamethasone valerate in various creams together with other active ingredients. Conventional silica gel 60 F254 plates were used. The densitograms were scanned in the reflectance mode at wavelengths which correspond to the isobestic point of the two components: 228 nm for betamethasone – clotrimazole cream (Indrayanto et al., 1998), 233 nm for betamethasone – miconazole nitrate cream (Indrayanto et al., 1999) and 250 nm (betamethasone) and 310 (salicylic acid) in a lotion formulation (Wulandary and Indrayanto, 2003a). The determination of the six components of conjugated oestrogens in raw materials and tablet formulations was carried out after hydrolysis, extraction, followed by TLC separation and detection at 280 nm (Novakovic et al., 1990).

As described in Section 12.2.2, in the overwhelming majority of cases, TLC purity testing in pharmacopoeias is restricted to semi-quantitative estimation of the impurities. However, if carefully validated, the quantitative TLC densitometric method is equivalent to the LC purity test. It was demonstrated on the example of the separation of estradiol, estrone, estriol and estetrol that in particular cases TLC can provide more robust system than LC with regard to separation efficiency and peak distribution (Zarzycki et al., 2005). The principles and practice of validation of quantitative TLC densitometry were described using danazol (Ferenczi-Fodor et al., 1993) and estradiol benzoate (Ferenczi-Fodor et al., 1995) as examples (scanning at 252 and 229 nm, respectively).

The popularity of *high-performance thin-layer chromatography* (*HPTLC*) is continuously increasing. In the majority of papers published in the last 20 years the use of this method is reported. The advantages of this technique are clearly due mainly to the smaller particle size (about 4 μm) and narrow particle-size distribution of the stationary phases. Because of this, the separation efficiency increases, allowing well-separated and well-quantifiable spots to be achieved with smaller plates, shorter running distance and shorter running time. Some of the important parameters (retention behavior, separation efficiency, selectivity) of TLC and HPTLC were compared using steroids as the model compounds (Hauck et al., 1995). In the majority of cases, silica gel 60 F254 plates were used for the assay of steroid preparations. Some of the investigated formulations are as follows (wavelength of the densitometric scan in parentheses): finasteride tablets (228 nm) (Meyyanathan et al., 2001), betamethasone tablets (245 nm) (Wulandari and Indrayanto, 2003), mometasone furoate topical preparations (260 nm) (Wulandari et al., 2003), cyproterone acetate – ethinylestradiol tablets (284 nm) (Novakovic and Vasatová, 1990)

or ethinylestradiol (220 nm) and cyproterone acetate (284 nm) (Pavic et al., 2003), dexamethasone and xylometazoline in nasal drops (240 nm) (Agbaba et al., 2001), dexamethasone sodium phosphate – neomycin sulphate solution (246 nm) (Agbaba et al., 2000), prednisolone acetate in pharmaceutical formulations (243 nm; stepwise gradient) (Matysik et al., 1995), levonorgestrel in a drug delivery system based on poly(DL-lactide-*co*-glycolide) and *N*-methylpyrrolidone. (250 nm) (Khakpour et al., 2005), progesterone in a silicon-based delivery system (Jamshidi, 2004). The basis for the assay of hydrocortisone, hydrocortisone acetate and clioquinol containing ointment and cream preparations was fluorescence quenching at 254 nm (Sherma et al., 1990). In the case of estradiol tablets (284 nm), the HPTLC method was suitable not only for the assay but also for the detection of degradants formed under stress conditions (Kotyiyan and Vavia, 2000).

A reversed-phase (ion pair) TLC system was used for the assay of corticosteroid sodium phosphate salts in parentheral preparations and ear drops (Datta and Das, 1993) while various other steroids were analysed by reversed-phase HPTLC (Lamparczyk et al., 1990).

Overpressured thin-layer chromatography (*OPLC*) is a variant of HPTLC in which the sorbent layer is covered with a pressurised elastic plate thus eliminating the vapour phase. Due to the forced flow of the eluent delivered by an automatic pump, its migration velocity is constant and the migration distance can be increased up to 18 cm thus enabling excellent separations and improved quantification to be achieved. As a result of these factors, OPLC is eminently suitable for the separation of impurities and degradants in bulk drugs and their formulations. Examples of the application of OPLC to steroid hormone drugs are the purity test of ethinylestradiol (visualisation by sulphuric acid) (Ferenczi-Fodor et al., 2006), norgestrel and levonorgestrel (visualisation by fluorescence quenching at 254 nm, phosphomolybdic acid or sulphuric acid) (Ferenczi-Fodor et al., 1997), allylestrenol drug substance and tablet (sulphuric acid) (Wiszkidenszky et al., 1998), nandrolone (sulphuric acid) containing comparison of the impurity profile with those obtained by TLC, LC and GC) (Bagócsi et al., 2002), norethisterone drug substance and tablet (sulphuric acid) (Bagócsi et al., 2003). OPLC was successfully used in in-process monitoring (Kassai et al., 2000) and cleaning validation of equipment (Katona et al., 2000) in the course of the production of bulk steroids and steroid formulations.

Figure 12.4 shows a comparison of OPLC and LC on the example of ethinylestradiol and its potential impurities (Ferenczi-Fodor et al., 2006).

The usefulness of further innovations, such as temperature-controlled programmed TLC (Zarzycki, 2002), programmed multiple development TLC with a new modification of the horizontal sandwich chamber (Matyska et al., 1991) and ultra-thin-layer chromatography (UTLC) where the granular adsorbents are replaced by a monolithic structure based on a silica-gel matrix, have been demonstrated by using, amongst other examples, the separation of steroid mixtures (Hauck et al., 2001). An interesting study dealing with the reasons for and the elimination of the irreversible adsorption of some steroids during multiple development TLC is also worth mentioning (Ferenczi-Fodor et al., 1999).

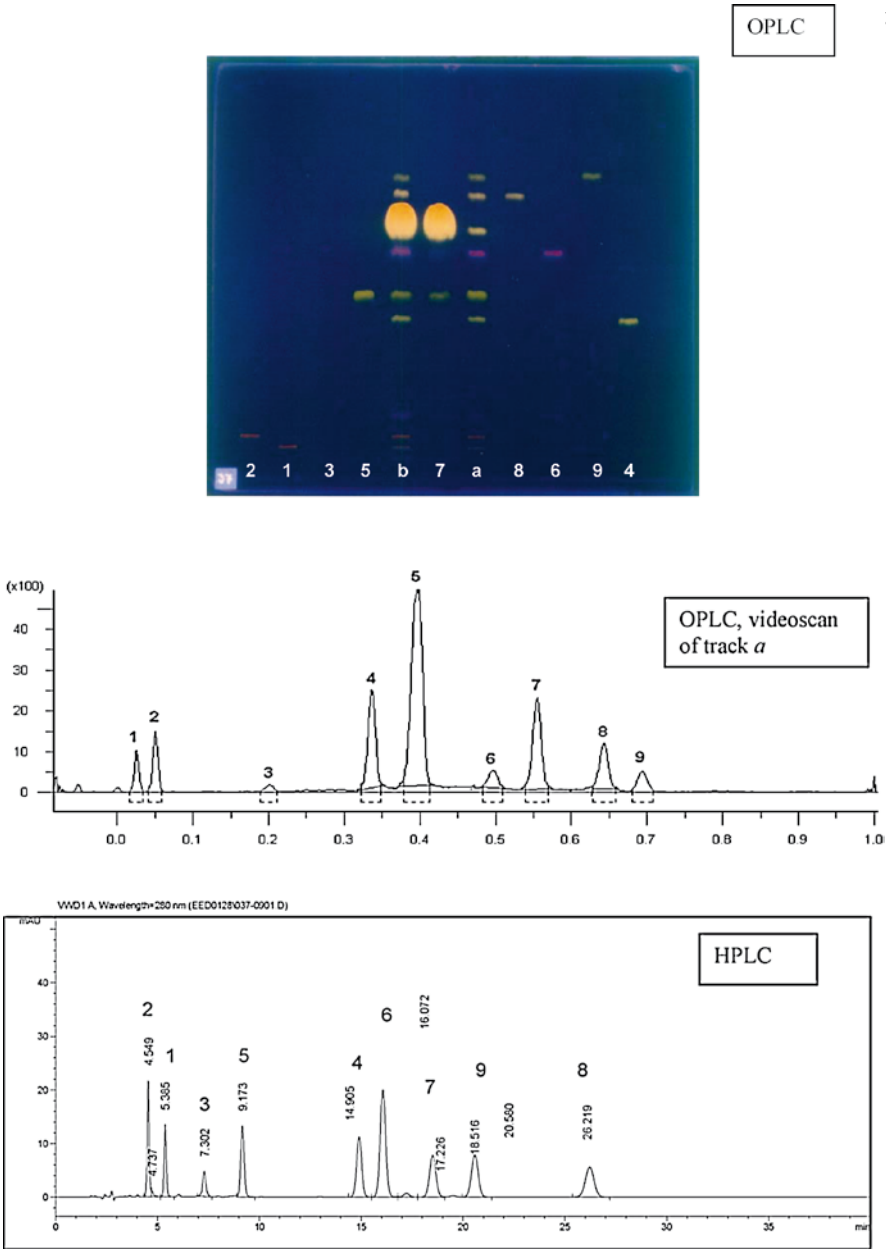

Fig. 12.4 Ethinylestradiol and its related substances, separated by OPLC and LC (from Ferenczi-Fodor et al., 2006, with permission)

Key: 1. 6β-hydroxy-ethinylestradiol; 2. 6α-hydroxy-ethinylestradiol; 3. 6-oxo-ethinylestradiol; 4. estradiol; 5. 16-oxo-ethinylestradiol; 6. 9(11)-dehydro ethinylestradiol; 7. ethinylestradiol; 8. 17-epi-ethinylestradiol; 9. estrone

OPLC. Plate: sealed HPTLC silica gel adsorbent layer (Merck # 5548); mobile phase: cyclohexane/ethyl acetate/chloroform (3:1:1, v/v) at 50 bar external pressure; instrument: *P-OPLC BS-50* overpressured layer chromatograph (OPLC-NIT Ltd., Budapest, Hungary); spray reagent: 10% ethanolic sulphuric acid; videodesitometry (VideoScan, Camag)

A tandem TLC-LC method is described for screening cosmetic products for the presence of undeclared synthetic corticosteroids (Gagliardi et al., 2002). Chiral selectors improved the separation of diastereomeric steroid pairs, e.g. the addition of β-cyclodextrin to the mobile phase in the case of the separation of R(+)- and R(−)-budenoside on cellulose-coated HPTLC plates (Krzek et al., 2002).

12.4.2 Gas Chromatography and GC-MS

As seen in Table 12.1, gas chromatography is used only in a very limited number of cases in the pharmacopeial analysis of steroid drugs. The reasons for this are their low volatility and (especially in the case of corticosteroids) thermal instability requiring derivatisation prior to the analysis. Although since the advent of LC, the importance of GC has decreased considerably in all branches of steroid analysis, in some cases it is still a good method of choice for assay or purity control. Two of the rare publications dealing with the application of GC in the analysis of steroid formulations are the assay of methyltestosterone (after dimethylethyl- or dimethylisopropylsilylation) (Zakhari et al., 1991) and cyproterone acetate – ethinylestradiol (Novakovic et al., 1991) tablets.

GC-MS is a good tool for screening purposes. As many as 134 underivatised drugs (among them 10 steroids) were included in a study to screen ethnic patent medicines available in health-food stores and ethnic markets. It is to be noted that two of the ten (dexamethasone and prednisolone) could not be detected by GC-chemical ionisation MS, probably due to their vulnerable 17-side chain (Hsu and Au, 2001). One possibility to enhance the applicability of GC and GC-MS to some of the thermally labile compounds (in addition to derivatisation) is "supersonic GC-MS" using shorter capillaries with lower film thickness, an increased carrier gas flow rate and a decreased temperature. This enables several steroids (among them corticosterone with a vulnerable side chain) to be determined (Fialkov et al., 2003).

12.4.3 Supercritical Fluid Chromatography

Although the problem of thermal instability in GC can be solved by using supercritical fluid chromatography (SFC), and several steroids were separated by SFC with light-scattering detection (Loran and Cromie, 1990), this technique seems to have attracted little interest in steroid analysis. The sensitivity of the laser light

Fig. 12.4 (continued) **LC**. Column: 250 × 4.6 mm, ODS, 5 μm; eluent: water/acetonitrile/methanol (50:30:20 v/v), 1 mL/min; wavelength: 280 nm.
Application on OPLC plate: 1–6 and 8–9, 0.5 μg of the above listed impurities; 7, 100 μg of ethinylestradiol; a, mixture of 0.5–0.5 μg of tested substances; *b*, 100 μg of ethinylestradiol spiked with 0.5–0.5 μg of the impurities. Peaks of LC chromatogram correspond to 0.5 μg of the substances.

scattering detector is similar to that of the UV detector with the advantage that UV-inactive steroids can also be detected with the same sensitivity.

Supercritical fluid extraction can be a useful alternative to classical extractions as demonstrated on the examples of megestrol acetate (Dean and Lowdon, 1993), medroxyprogesterone acetate, cyproterone acetate (Yamini et al., 2002) and vitamins D (Gamiz-Garcia et al., 2000) from tablet matrices.

12.4.4 High-Performance Liquid Chromatography and LC-MS

LC is the most important and most generally used method in pharmaceutical steroid analysis (see Section 12.2.1). Assay and purity tests recommended by pharmacopeas for 124 bulk steroid drugs by LC are summarised in Table 12.1 and several formulations assayed by LC are also listed. A large number of papers have been published in the past 30 years describing "pharmacopoeial-like" reversed-phase LC methods for the assay of steroid formulations and the analysis of these single-, two or three-component *formulations* by LC has become a routine task. More informative are the publications describing stability-indicating assays, including identification of impurities and degradation products. Some modern publications from the past 5 years are presented below, followed by a few earlier stability-indicating assays. A more comprehensive list is given in Görög (2004).

Fluticasone propionate and salmeterol xinafoate (Murnane et al., 2006) as well as beclomethasone dipropionate (Gupta and Myrdal, 2004) were determined in a pressurised metered dose inhaler, budenoside in loaded microparticles (Gupta and Bhargava, 2006) budesonide and formoterol in a turbohaler (Assi et al., 2006) and finasteride in tablets (Demir et al., 2006). Dexamethasone acetate was assayed in suppositories and ointment in the presence of several drugs, excipients and degradation products (Zivanovic et al., 2005), hydrocortisone and its acetate in a pharmaceutical emulsion in the presence of preservatives (Cauhan and Conway, 2005). The simultaneous determination of triamcinolone acetonide and oxymetazoline hydrochloride in a nasal spray (Sudsakorn et al., 2006), gestodene and ethinylestradiol in contraceptive pills (Laban et al., 2004), vitamins D_2 and D_3 in oil-injectables (Yan et al., 2005) as well as cholic, ursodeoxycholic, hyodeoxycholic acids and other drugs in an injection (Sarioglu et al., 2001) is also described.

Some further stability-indicating LC assays are described for beclomethasone dipropionate formulations (De Orsi et al.,1995), cyproterone acetate tablet (Segall et al., 2000), estradiol – levonorgestrel transdermal drug delivery formulation (Li, 2002), hydrocortisone acetate topical cream (Hájková et al., 2003), hydrocortisone sodium phosphate gel (Galmier et al., 1999), nestorone implants (Ahmed et al., 1995), pancuronium bromide injection (Zecevic et al., 2002), prednisolone sodium phosphate implantable infusion pump (Bachman and Gambertoglio, 1990), triamcinolone, its acetate and acetonide and several other corticosteroids in various formulations (Gonzalo-Lumbreras et al., 1997), and triamcinolone acetonide topical cream (Matysová et al., 2003). Stability studies by LC of budesonide (Hou et al.,

2001), aqueous spironolactone solutions (Kaukonen et al., 1998), and its use for the photodegradation kinetic study of danazol (Gad Kariem et al., 2000) are also worth mentioning.

It is worth mentioning that due to the poor UV absorption properties of bile acids, in the assay of their formulations, evaporative light scattering detector (Yan et al. (2005); Rajevic & Betto, 1998), or (after derivatization) coulometric electrochemical detector (Bousquet et al., 1997) were used.

LC is eminently suitable for *screening* purposes, e.g. RP-LC for anabolic steroids (Gonzalo-Lumbreras and Izquierdo-Hornillos, 2000), NP- and RP-LC for corticosteroids (Valvo et al., 1994), corticosteroids in topical pharmaceuticals using diode-array UV detector (Reepmayer, 2001). In Fig. 12.5 the baseline separation of 12 corticosteroid drugs within 15 min is shown using the latter system.

Especially useful is the coupling of *LC with mass spectrometry*: An LC/time-of-flight MS method was developed for screening anabolic steroids in illegal cocktails (Nielen et al., 2001), while for their determination in oily formulations RP-LC

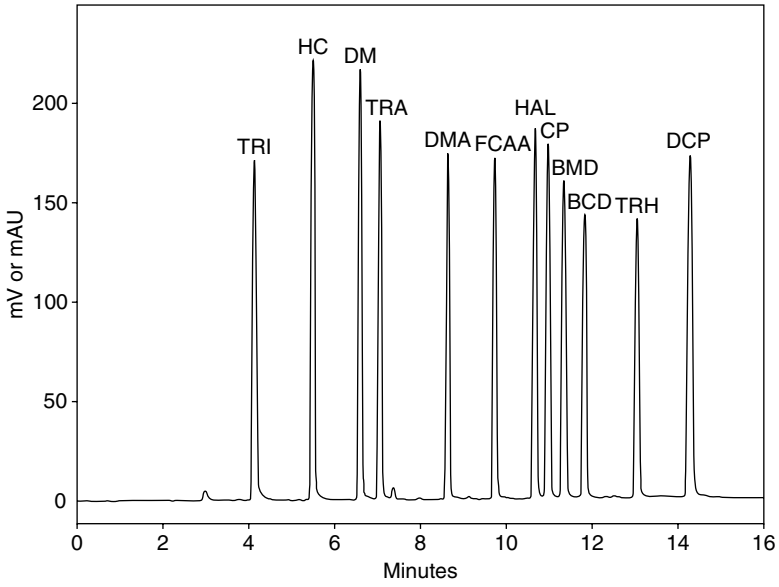

Fig. 12.5 LC separation of 12 corticosteroid drugs (from Reepmeyer, 2001, with permission)
Key: **TRI**: triamcinolone; **HC**: hydrocortisone; **DM**: dexamethasone; **TRA**: triamcinolone acetonide; **DMA**: dexamethasone 21-acetate; **FCAA**: fluocinonide; **HAL**: halcinonide; **CP**: clobetasol 17-propionate; **BMD**: betamethasone 17,21-diacetate; **BCD**: beclomethasone 17,21-dipropionate; **TRH**: triamcinolone hexacetonide; **DCP**: desoxycorticosterone pivalate.
Column: Symmetry C-18 75 × 4.6 mm; 3.5 µm. Eluent: acetonitrile–water, gradient: 0–2 min 18% acetonitrile, 2–14 min linear gradient from 18% to 82% acetonitrile; flow rate: 1 mL/min; wavelength: 240 nm

separation followed by off-line GC-MS analysis was used (Hooijerink et al., 1994). Betamethasone, dexamethasone and related steroids were detected by LC/ESI-MS in counterfeit drugs (Arthur et al., 2004). LC/MS is the most suitable method for rapid screening of high-throughput drug mixtures which are of great importance in drug discovery (Yu and Balogh, 2001). Capillary LC-MALDI-TOF-MS after post-column derivatisation with 2,4-dinitrophenylhydrazine was used for the screening of combinatorial libraries with seven corticosteroids as model compounds (Brombacher et al., 2003). Building LC-MS/MS libraries and its usefulness in rapid identification of impurities was demonstrated on the example of 19 steroid compounds (Joseph and Sanders, 2004) as well as budesonide and 23 related corticosteroids (Hou et al., 2005). LC-MS was also used for the rapid identification of the products of biotechnological transformations. The model for this study was the hydroxylation of progesterone to 9α-hydroxyprogesterone (Lindholm et al., 2003a,b). LC-UV was used as the in-process control method to follow this reaction at the industrial scale (Lindholm et al., 2003a, b).

Online *LC-NMR* – although only few data are available for its use in pharmaceutical steroid analysis – is certainly the method of the future in this field. For example, using this technique, estradiol and norethisterone acetate were detected in a galenic emulsion (Glaser and Albert, 2002). Further examples for the use of LC coupled with spectroscopic methods are presented in Sections 12.7.1 and 12.7.2.

In addition to the above uses of LC for the analytical investigation of bulk steroids and steroid formulations, it is a useful tool also in the hands of pharmaceutical technologists to investigate *drug release* from various formulations and devices for controlled drug delivery. Examples are the release of estradiol and its 3-acetate from intravaginal rings (Russel et al., 2000) and penetration of triamcinolone acetonide into skin from topical dosage forms measured in an enhancer cell (Rege et al., 1998).

As mentioned earlier, in the majority of cases, reversed-phase LC is used, mainly C_{18} columns with unbuffered binary or ternary mixtures of water with methanol, acetonitrile or tetrahydrufuran. Some innovations regarding *stationary phases* included use of ultra-performance liquid chromatography (UPLC) employing an ultra-microparticulate (1.5 μm) stationary phase, which with short columns provided shortened analysis time with excellent column efficiency. As Fig. 12.6 shows, the analysis time of a hydrocortisone cream with five components, could be reduced from 10 min with conventional LC to 2 min with UPLC (Novakova et al., 2006).

Monolithic columns were found to be useful tools for the ultrafast separation of steroids (Rim et al., 2003), such as in the assay of triamcinolone, prednisolone and dexamethasone tablets (Hashem and Jira, 2005) and separation of oestrogens and their degradation products (Mizuguchi et al.,2005). The sequential injection variant of monolithic silica-LC was used for separation and quantitation of the ingredients of a topical cream containing triamcinolone acetonide and preservatives (Satínský et al., 2003). Ultrafast analysis can also be achieved by decreasing the column length with simultaneous optimisation of the flow rate and gradient profile (ballistic gradients) as demonstrated in the separation of nine drugs, among them finasteride within 1.5 min (Romanyshyn and Tiller, 2001). Temperature-responsive stationary phases showing

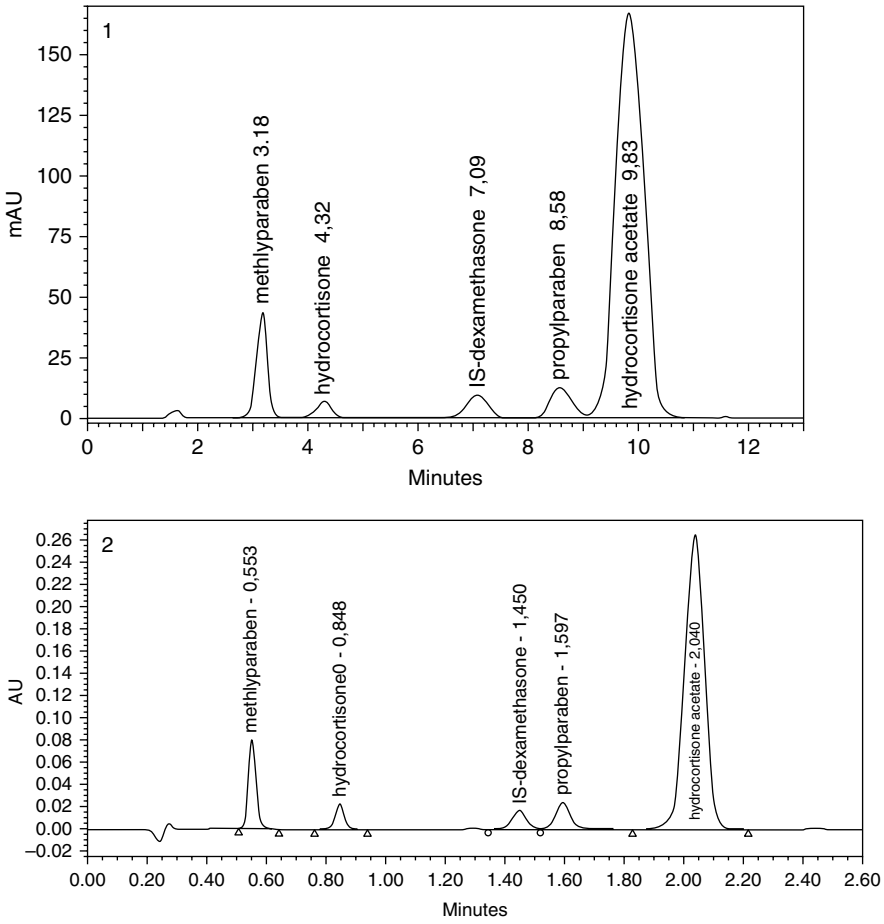

Fig. 12.6 Comparison of 1. LC and 2. UPLC analysis for the separation of the components of hydrocortison cream: hydrocortisone acetate active ingredient, hydrocortisone degradation product, methylparaben and propylparaben preservatives, dexamethasone internal standard
Eluent: methanol/acetonitrile/water 15:27:58 v/v/v. Detector wavelength 238 nm.
1. LC. Column: Discovery C-18 125 × 4 mm; 5 μm. Flow rate: 0.8 mL/min. Injected volume: 10 μL.
2. UPLC. Column: Acquity UPLC BEH C-18 50 × 2.1 mm; 1.7 μm. Flow rate: 0.7 ml/min. Injected volume: 2 μL

hydrophilic properties at lower and hydrophobic properties at higher temperatures, were prepared by covalently binding poly(*N*-isopropylacrylamide) to aminopropylsilica. This is a promising tool for the temperature-controlled separation of steroids (Kanazawa et al.,1996, 2002; Song et al., 2001). Excellent separation of the epimers of estradiol was achieved by modifying the surface of silica gel with the covalent binding of cholesterol (Buszewski et al., 2003). Molecularly imprinted artificial receptors as LC stationary phases were prepared among others for the screening of

corticosteroids (Baggiani et al., 2000) and oestrogens (Ye et al., 2001). Structurally closely related steroids, not separable by C-18 columns, were successfully separated using carbon-coated silica or porous graphitic carbon stationary phases (Reepmeyer et al., 2005). Owing to its electrical conductivity, the surface and thus the separation power of porous graphitic stationary phase can be changed if voltage is applied to the column during the chromatographic run. This technique, termed "electrochemically modulated liquid chromatography", was coupled online with electrospray MS. The method has been successfully applied to the improved separation of corticosteroids (Ting and Porter, 1997; Deng et al., 2000).

Several papers have been published dealing with the *mobile phase* selection and optimation in the LC analysis of steroid hormone drugs. The selectivity of the separation in RP-LC was improved by using ternary systems containing methyl or ethyl acetate as demonstrated by the separation of 9α-fluoroprednisolone acetate from its impurities (Bernal et al., 1992). Using 2,2,2-trifluoroethanol as a mobile phase component in ternary systems together with perfluorinated RP-LC stationary phases, unique selectivity was attained for several drugs, among them steroids, explicable by the strong adsorption of the cosolvent on the surface of the stationary phase (Valkó et al., 2001). Another fluorinated solvent, ethoxynonafluorobutane was introduced as an environmentally friendly and highly selective solvent in the NP-LC analysis of several compounds, among others steroids (Kagan, 2001). Cyclodextrins are known to be excellent chiral selectors in enantiomeric analysis (see Section 12.7.3). The effect of β-cyclodextrin on the retention of steroids and their separation in general (Zarzycki et al., 2002) and the effect of temperature on these characteristics (Zarzycki and Smith, 2001) were thoroughly studied.

The use of *micellar liquid chromatography*, a special branch of RP-LC where the eluent contains very low concentration of organic modifiers (mainly 1-pentanol and acetonitrile) and about 0.1 M sodium dodecyl sulphate (SDS) as the micelle-forming agent was applied to steroid analysis by Spanish groups. This technique enables the relatively rapid analysis of derivatives with wide range of polarity with minimum need for sample preparation. In addition to retention–structure relationship studies (Torres-Cartas et al., 2000a) and screening of steroids (Torres-Cartas et al., 2000, 2000b; Ruiz-Angel et al., 2002), the method was successfully used for the determination of anabolic steroids (Izquierdo-Hornillos and Gonzalo-Lumbreros, 2003) and several other steroid drugs. See Görög (2004) for a detailed list of applications in various formulations.

Although the principles and practice of *mobile phase optimisation* in LC were already set up in the early period of the history of LC, further studies are from time to time made, refining the optimisation with steroids and other compounds as the models. These include the description of a general quantitative relationship for column selectivity in RP-LC and the study of the effect of the change in conditions (Wilson et al., 2002), the use of experimental design for spherical coordinate representations of solvent composition in RP-LC (Morris et al., 2003), prediction of the RP-LC retention of steroids using solvatochromic parameters (Barron et al., 1995), the use of 2^3 factorial design and computer simulation (Gau et al., 1995), triangle optimisation for the separation of finasteride and related compounds (Cenderowska and Buszewski, 1999), the effect of the pH of the mobile phase for

the separation of ionisable steroids (Das Gupta and Mathew, 1995), the effect of column overloading on the separation of mometasone furoate and clotrimazole at widely different concentrations (Zhu and Coscolluella, 2000).

In the overwhelming majority of cases UV *detection* is applied in industrial–compendial steroid drug analysis (the role of LC-MS was discussed earlier in this section). To check and, if necessary, improve the selectivity, i.e. peak purity testing by detecting coeluting species and eliminating this effect is an important task. For example, in the case of danazol, this was done by subtracting the up-slope and down-slope diode-array spectra from the apex spectrum (Anderson et al., 1990). LC-MS is an excellent tool for estimating minor components under overlapping peaks, e.g. the identification and quantitation of as low as 0.01% prednisolone in hydrocortisone (Mulholland et al., 2000). Scanning UV spectra before and after UV irradiation in a post-column photochemical reactor and the evaluation of the differences was of diagnostic value in the identification of components in steroids drug mixtures including corticosteroids in creams (Di Pietra et al., 1996). Evaporative light scattering detector can be of importance in the case of UV-inactive steroids such as bile acids (Rajevic and Betto 1998; Yan et al.,2005). As already mentioned, native fluorescence of ethinylestradiol is used in the fluorimetric detection of the USP dissolution test of levonorgestrel – ethinylestradiol tablets (USP 29, 2006) while terbium(III)-sensitised fluorescence (exc: 245 nm/em: 545 nm) detection was described for various steroids in the course of their SDS micellar LC determination (Torres-Cartas et al., 2000b).

In addition to its analytical application, LC is a useful tool in other fields of steroid drug research, too. For example LC was used for the determination of apparent association constants of steroid-cyclodextrin inclusion complexes (Flood et al., 2001), lipophilicity profiling (Kerns et al., 2003), for studying drug-membrane interactions (Liu et al., 2002) and high-throughput log *P* measurements (Zhao et al., 2002). Calculations based on linear solvation energy relationship were found to be useful to find "open windows" for internal standards in RP-LC chromatograms of various compounds, among others steroids (Li, 2001) and these calculations were used to find suitable internal standard for the determination of estradiol and levonorgestrel in transdermal drug delivery formulations (Li and Shah, 2002). Biopartitioning micellar chromatography with polyoxyethylene (23) lauryl ether (Brij35) as the micelle forming agent was used for the prediction of human drug absorption (Molero-Monfort et al., 2001).

12.5 Electrophoretic and Related Methods

12.5.1 Capillary Electrophoresis

Since the overwhelming majority of steroid drugs are neutral compounds, direct CE does not play an important role in their analysis. The direct application of this technique is restricted to ionisable derivatives. Bile acids are naturally among

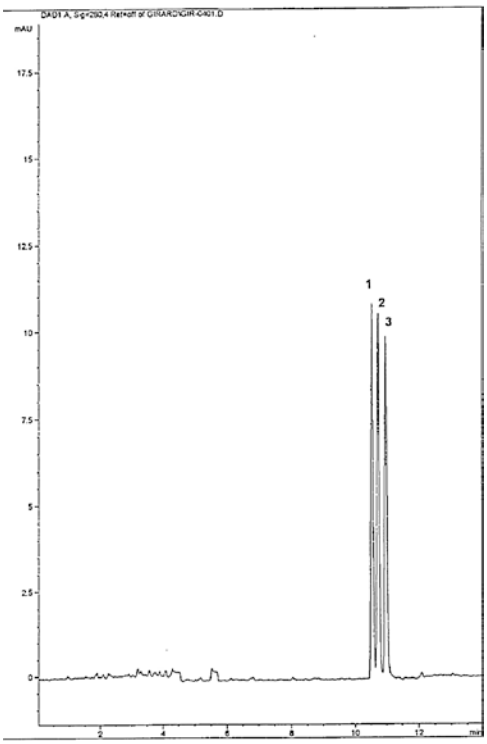

Fig. 12.7 Separation of nortestosterone (1), norethisterone (2) and norgestrel (3) by capillary electrophoresis after derivatisation with Girard-T reagent. pH 4.8; voltage 25 kV; temperature 15°C; injection time 3 s; UV detection 280 nm (from Görög et al., 1996, with permission)

these. Ursodeoxycholic acid was determined in pharmaceutical preparations at pH 8 using sodium *p*-hydroxybenzoate as the background electrolyte and the chromophoric agent in indirect UV detection (Chang et al., 2003). The simultaneous determination of hydrocortisone 21-hemisuccinate, oxytetracycline, and nystatin in pharmaceutical preparations was carried out at pH 11 (Lemus Gallego and Pérez Arroyo, 2002b). A possibility of enhancing the applicability of CE to non-ionisable steroids is covalent derivatisation with electrically charged reagents, e.g. ketosteroids with Girard P or T reagents. The Girard hydrazones of 4-ene-3-oxo- and 17-oxosteroids can easily be separated and quantificated. Figure 12.7 shows the separation of three structurally closely related oxosteroids, while Fig. 12.8 is the reaction equation (Görög et al., 1996).

Capillary electrophoresis can be a useful tool for preconcentration of different classes of steroids, making use of electro-osmotic flow and capillaries partially filled with various reagents, e.g. borate for complexation of steroids with proximal hydroxyls, high pH buffers for weakly acidic oestrogens, micelle-forming agents for hydrophobic steroids (Britz-McKibbin et al., 2003). The investigation of CE migration times of inclusion complexes formed between oestrogens and anionic

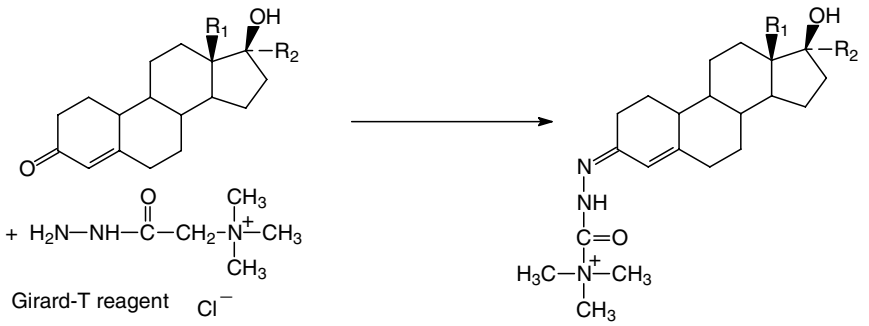

Fig. 12.8 Reaction equation of nortestosterone (R_1 = methyl, R_2 = hydrogen), norethisterone (R_1 = methyl, R_2 = ethinyl) and norgestrel (R_1 = ethyl, R_2 = ethinyl) with Girard-T reagent

sulfobutyl ether cyclodextrin derivatives was a useful tool in investigating the structures of these host-guest complexes (Deng and Huang, 2004).

12.5.2 Micellar Electrokinetic Chromatography

In this technique, negatively charged micelles are formed in the background electrolyte of the CE instrument. These are moving towards the anode while the solution in the capillary moves in the opposite direction due to the electro-osmotic flow. The micelles act as a quasi-stationary phase in RP-LC-like separation. MEKC, which is therefore suitable for the simultaneous separation of charged and uncharged molecules, is an ideal tool for the separation and quantification of lipophilic steroid drugs not containing ionisable functional groups that would enable their direct analysis by CE. These steroids (mainly corticosteroids) were among the first model compounds investigated after the invention of this new method (Nishi et al., 1990; Terabe et al., 1991; Vindevogel and Sandra 1991). A large number of papers have been published dealing with theoretical and practical aspects of the application of MEKC to steroid analysis. Due to the minimum requirements of sample pretreatment, low costs, high separation efficiency and speed MEKC can be a real alternative to LC in steroid analysis. This is demonstrated in Fig. 12.9 where LC and MEKC curves of betamethasone dipropionate, clotrimazole and their related substances as the model compounds are shown (Lin and Wu, 1999).

Some of the several applications of MEKC to pharmaceutical steroid analysis are listed here: the determination of ethinylestradiol and levonorgestrel (Berzas et al., 1999a) or gestodene (Berzas et al., 1999b) in oral contraceptives, hydrocortisone, and its acetate together with various antibiotics (Lemus Gallego and Pérez Arroyo, 2003b), prednisolone, Zn-bacitracin and phenylephrine in ointments and ocular drops (Lemus Gallego and Pérez Arroyo, 2003c), prednisolone acetate, sulphacetamide and phenylephrine in local pharmaceutical preparations (Lemus

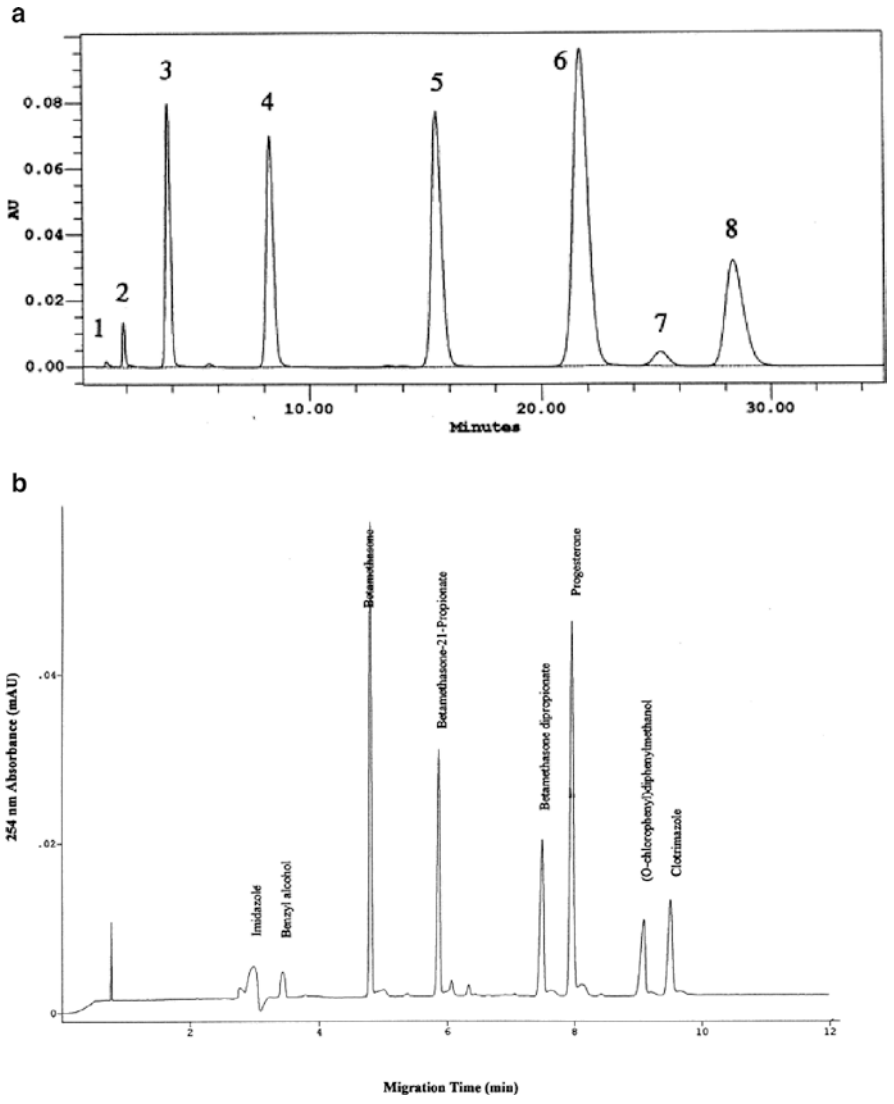

Fig. 12.9 Comparison between LC and MEKC of betamethasone dipropionate, clotrimazole and their related substances. (**a**) **LC**. Key: 1. imidazole; 2. benzyl alcohol; 3. betamethasone; 4. betamethasone 21-propionate; 5. progesterone; 6. betamethasone 17,21-dipropionate; 7. (*O*-chlorophenyl)diphenylmethanol; 8. clotrimazole
Column: Waters symmetry C_8 3.9 × 150 mm, 5 mm; eluent: methanol – 25 mM phosphate buffer of pH 7 (18:10 v/v); flow rate: 1.2 mL/min. (b) MEKC. Capillary: 41 cm × 75 mm i.d. fused silica; buffer: 50 mM SDS in 10 mM phosphate buffer pH 7.2, 5% butanol and 15% acetonitrile added; voltage: 15 kV; electrokinetic injection: 6 s; temperature: 30°C; detection: 254 nm (from Lin and Wu, 1999, with permission)

Gallego and Pérez Arroyo, 2003d), dexamethasone, trimethoprim and polymyxin B in various formulations (Lemus Gallego and Pérez Arroyo, 2001b), separation of and determination in tablets betamethasone, cortisone, prednisolone, 6α-methylprednisolone, prednisone and triamcinolone (Kuo and Wu, 2005), determination of betamethasone in dissolution tests (Lucangioli et al., 1997).

In the above-listed studies (and the majority of those not mentioned here) sodium dodecyl sulphate was used as micelle-forming agent in the background electrolyte. In some cases, bile salts (Nishi et al., 1990; Lucangioli et al., 1997; Kuo and Wu, 2005) and their mixtures with SDS (Amundsen et al., 2004) were also used. A comparison of the two systems using corticosteroids as the model compounds is also described (Noé et al., 1998). High concentrations of organic solvents (up to 50% acetonitrile or methanol) in the background electrolyte containing SDS enables ionic (benzalkonium chloride) and non-ionic (beclomethasone dipropionate, 2-phenylethanol) compounds to be determined within one electrokinetic run (Cifuentes et al., 1998). Using a related method, termed hydrophobic interaction electrokinetic chromatography seven active ingredients of an ointment (among them hydrocortisone) were separated and quantitated. The migration behaviour of the hydrophobic and ionic constituents was influenced by the concentration of tetradecyl ammonium bromide and ammonium chloride, respectively (Okamoto et al., 2001).

Various other neutral, anionic, cationic and zwitterionic additives can influence the characteristics of micelles, thus changing the migration velocity, migration order of analytes and online sample preconcentration leading to the increase of the sensitivity by 2–3 orders of magnitude. Examples of additives, successfully used in the analysis of steroids, are *N*-dodecyl-*N*,*N*-dimethylammonium-3-propane-1-sulfonic acid (Monton et al., 2003), negatively charged cyclodextrin derivatives (Urban et al., 2005) and various surfactants (Shen and Lin, 2006).

MEKC employing SDS with borate buffer, organic modifiers, and surfactants was successfully used in micromachined CE chips for the separation of oestrogens and their detection with an amperometric detector (Collier et al., 2005). Chiral aspects of steroid MEKC are discussed in Section 12.7.3.

12.5.3 Microemulsion Electrokinetic Chromatography

Microemulsion electrokinetic chromatography (MEEKC) technique is a variant of MEKC, where the quasi-stationary phase consists of oil droplets. To stabilise the emulsion, surfactants such as SDS or bile acid salts are added to the buffer solution and to achieve appropriate separation, organic solvents and various other additives are employed. Several papers deal with the comparison of the performances of MEKC and MEEKC using as model compounds among others steroid drugs (Vomastová et al., 1996; Hansen et al., 2001; Gabel-Jensen et al., 2001; Lucangioli et al.,2003). Although excellent separations were achieved in the above and other studies (Pedersen-Bjergaard et al., 2000; Pomponio et al., 2005) this method does not seem to have been used as extensively in steroid analysis as MEKC. A good correlation was

found between the retention characteristics of analytes and the respective log P_{ow} values: MEEKC is a good tool for the rapid estimation of log P_{ow} values of a wide range of compounds, among them steroid drugs (Poole et al., 2003).

12.5.4 Capillary Electrochromatography and CEC-MS

CEC can be considered to be the combination of LC and CE. The capillary is packed with fine particles of the solid support (usually 3 μm octadecylsilica), the driving force is electro-osmotic flow and the basis of separation is a typical RP-LC mechanism. This technique has been successfully applied to separation and quantitation of steroid drugs. The packing technique greatly influences the column efficiency. As high as 190,000 (Saevels et al., 1999) and 300,000 (Lynen et al., 2005) plates/meter were described using (among others) steroids as model compounds. Various wall-coating methods have also been described to improve separation of steroids, e.g. phospholipid coating combined with various diamines (Varjo et al., 2005). In addition to the generally used ODS phases used for steroid separations, the successful application of macroporous, spherical polystyrene-divinylbenzene stationary phase was also described (Liu and Pietrzyk, 2001). High-temperature CEC and temperature programming resulted in a considerable reduction of the separation run time of steroids, and the latter may be a good alternative to solvent programming (Djordjevic et al., 2000).

The performance of LC, MEKC and CEC was compared using six steroids (androst-4-ene-3,17-dione, testosterone, norethisterone, progesterone and the 17-OH and 20-OH derivative of the latter). Higher peak efficiencies were obtainable by MEKC and CEC (Taylor et al., 1999). Of the practical applications rapid analysis of norgestimate and its degradants (Wang et al., 1998), tipredane and related impurities (Euerby et al., 1997) and fluticasone propionate and related impurities (Smith and Evans, 1994), is mentioned. An example for chiral CEC is presented in the Section 12.7.3.

The very promising possibilities of the new technique CEC-MS have been reviewed with several examples from steroid drugs (Lane, 2000). The identification of impurities in fluticasone propionate (Lane et al., 1995) and the combination of CEC with nanospray MS with detection limits of 50 fg for corticosteroids (Warriner et al., 1998) merit special attention.

12.6 Electroanalytical Methods

Although electroanalytical (mainly polarographic and differential pulse polarographic, square-wave voltametry) methods based on the reduction of the 4-ene-3-oxo group were described in the early period of pharmaceutical steroid analysis (Görög and Szász, 1978; Görög 1983, 1989), these methods have never played important role in this field. Papers of this kind are still often published describing sufficiently sensitive and selective methods for the assay of some steroids in drug

formulations, (e.g. the determination of levonorgestrel in tablets – Ghoneim et al., 2004). However, no real advantages of these methods can be found compared with previously discussed methods (e.g. spectrophotometry) and due to its environmental hazard, there is a tendency to avoid electroanalytical methods based on the mercury electrode.

Direct potentiometric methods for the determination of the muscle relaxant quaternary ammonium compound pancuronium bromide, based on ion-selective membrane electrode containing tetraphenylborate or dipicrylaminate as counter ions in a PVC matrix (Aubeck et al., 1990) and carbon-paste electrode (Salem et al., 2005) are worth mentioning.

12.7 Selected Analytical Tasks

12.7.1 Impurity Profiling of Steroid Drugs

As described in Section 12.2.2, the pharmacopoeias usually prescribe semi-quantitative TLC or quantitative LC tests for purity control of bulk drugs and pharmaceutical preparations made therefrom. Since, in the majority of cases, the related impurities cannot be specified, even the LC data are of limited value as the impurity is expressed as the main component. It is necessary to improve the accuracy of determination of (potentially toxic) impurities (drug impurity profiling), to improve the safety of drug therapy. Impurity profiling (i.e. detection, identification/structure elucidation and quantitative determination of impurities) usually requires the complex application of one or more separation methods and at least two to three spectroscopic techniques (usually UV, MS, NMR) either off-line or preferably online (Görög, 2000, 2003a).

Of the innumerable published studies, a few, resulting in interesting structures, are listed here beginning with corticosteroids. Six synthesis-related impurities were identified in a synthetic prednisolone sample (Görög et al., 1998). Amongst minor impurities, 9α-bromo analog was found as the main impurity in triamcinolone acetonide (Cavina et al., 1992). The structure elucidation of impurities in fluticasone propionate (among others an interesting –S–S-bridged dimer) is a good example for the application of new techniques such as CEC-MS (Lane et al., 1995) and online LC-NMR (Mistry et al., 1997).

No less than 13 (potential) impurities of spironolactone were isolated by column chromatography from the mother liquor, after crystallisation. In addition to spectroscopic methods, single-crystal x-ray analysis was also applied in their structure elucidation (Chen et al., 2004, 2006). By-products of the ethinylation step in the synthesis of contraceptive gestogens (norethisterone, norgestrel) were identified (Görög et al., 1993; Horváth et al., 1997). In addition to the well-known 3α-epimeric impurity, *Z* and *E* isomers of the 17-(3'-acetoxy-2'-butenoate) and 17-(3-oxo-butenoate) identified in ethynodiol diacetate are by-products of the acetylation of the 17-OH group (Görög et al., 1991; Babják et al., 2002). Impurities found in allylestrenol originate from its total synthesis (Görög et al., 1997). In the course of

impurity profiling of estradiol, LC-UV played predominant role (Görög et al., 1995a). Several impurities in nortestosterone originating from the Birch reduction step in its synthesis were identified by the use of combined application of chromatographic and spectroscopic techniques with GC-MS as the most informative method (Görög et al., 1995b, 1998). This method was successfully used for the impurity profiling of cholesterol as well (Laukó et al., 1993). An isomeric impurity identified in danazol was formed in the course of the ring closure reaction of its isoxazole moiety (Balogh et al., 1995).

12.7.2 Analytical Aspects of Drug Stability Studies

Degradation products formed during the storage of bulk drugs and drug formulations are closely related to impurities. Drug stability studies need stability-indicating analytical methods. These are almost exclusively chromatographic (in the majority of cases LC) methods. In order to prove the stability-indicating nature of a chromatographic method, the drug or pharmaceutical preparation should undergo stress-degradation conditions (exposure to heat, humidity, light, low, and high pH, oxidising agents). The degradation products formed under these conditions should be separated from the main component thus ensuring that the assay really measures the undecomposed drug. For example, two recently published LC methods for the assay of estradiol (Nygaard et al., 2004) and medroxyprogesterone acetate (Burana-osot et al., 2006) formulations are stability indicating and so are the majority of TLC, LC, and various electrophoretic methods described in the preceding sections. It is worth mentioning that in some cases the stability-indicating methods elucidated the exact reaction kinetics analysis of the degradation reaction, such as in the case of mometasone furoate (Teng et al., 2003), to be carried out. From the methodological point of view, the structure elucidation of the degradation products is very similar to methods used for structures of drug impurities. In stability studies, the objective is not only to prove the stability-indicating nature of the analytical method but also to describe the mechanism of degradation.

Corticosteroids, with their sensitive side chain at C-17, have been the subject of several stability studies. For example, in the course of the stress stability test of mazipredone (21-deoxy-21-*N*-methylpiperazinyl derivative of prednisolone) in acidic and alkaline media, a complex, multistep degradation mechanism was found with several degradants, mainly products of intramolecular oxido-reduction reactions (Gazdag et al., 1998; Görög, 2003b). For the scheme of this degradation mechanism see Fig. 12.10. Several synthesis-related impurities and degradants were identified in dexamethasone (Spangler and Mularz, 2001; Conrow et al., 2002) – of these, the 17β-carboxy-17α-formyloxy derivative, formed under storage conditions via a mixed anhydride, is especially interesting (Conrow et al., 2002). The light-induced degradation of methylprednisolone suleptamate leads to interesting, unpredictable structures (Ogata et al., 1998).

6α- and 6β-hydroxy derivatives were identified as auto-oxidation products in norethisterone, norgestrel and nestorone (Görög et al.,1995, 1998). LC-NMR and

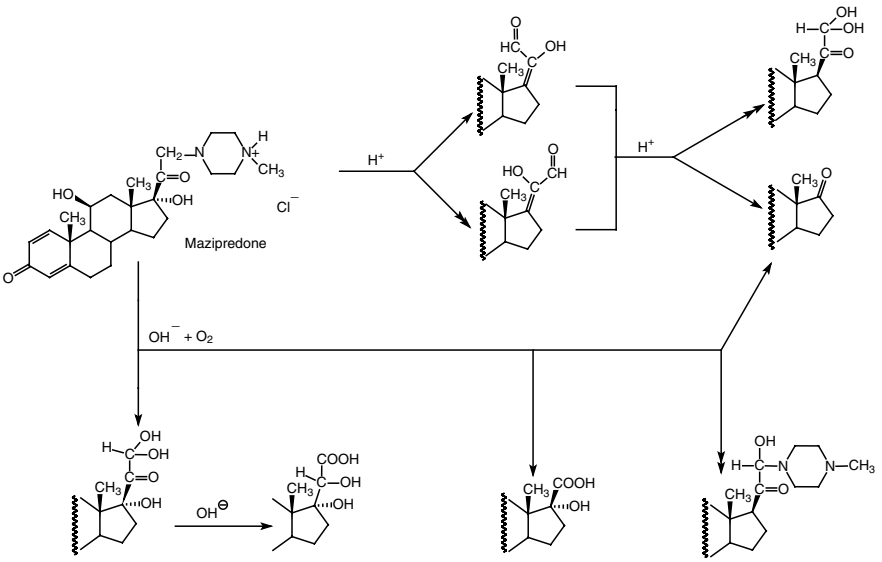

Fig. 12.10 Reaction scheme of the degradation of mazipredone in acidic and alkaline media determined by LC/UV/MS (Gazdag et al., 1998)

LC-MS were employed to determine five interesting structures (a 9-hydroperoxide and four different dimers) in the course of oxidative and light-stress studies of ethinylestradiol (Segmuller et al., 2000) and several oxidative degradation products in tipredane (Euerby et al., 1996, 1997). An oxidative degradation product was identified in pipecuronium bromide (Görög et al., 1997; Görög, 2003b) and quantitated by NP-ion-pairing LC (Gazdag et al., 1991). For the structures see Fig. 12.11.

12.7.3 Enantiomeric Analysis

Although steroid hormone drugs usually contain six to seven chiral centres, their configuration is generally fixed in natural steroids and the semi-synthetic drugs prepared from them. For this reason, in the overwhelming majority of cases, enantiomeric analysis is not necessary. The only important exception is the totally synthetically prepared norgestrel, which is mainly administered as enantiomerically pure levonorgestrel, but the racemate is also recorded in USP 29. Several papers have been published on the separation of the enantiomers of norgestrel. Only a few of these deal with the determination of the enantiomeric purity of levonorgestrel. In the first validated method RP-LC with γ-cyclodextrin in the mobile phase was used and a limit of quantitation of 0.1% dextronorgestrel was achieved (Gazdag et al., 1988). With β-cyclodextrin fairly good separation is achievable at 0°C only (Lamparczyk et al., 1994). Using α_1-acid glycoprotein (Chiral AGP) column for the LC separation, good separation was obtained (Blom et al., 1993; Szász et al., 1998), but the limit of quantitation (0.6%) was inferior to that achieved using γ-cyclodextrin.

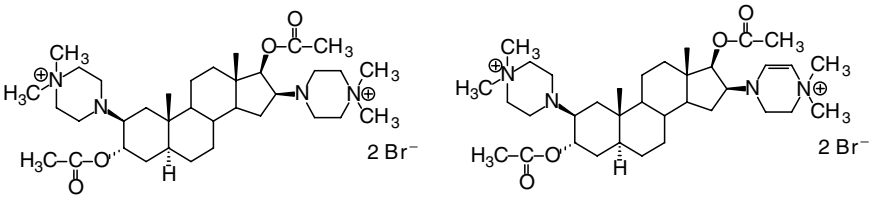

Fig. 12.11 Structures of pipecuronium bromide and its UV-active impurity (Görög et al., 1997; Görög, 2003b)

Norgestrel is an excellent model compound for testing the separation efficiency of various enentioselective chromatographic and related techniques. For this reason, many papers contain data for norgestrel, usually among many other model compounds. Acceptable to excellent separation was achieved using Chiralcel-OJ, cellulose tris(3,5-dichlorophenylcarbamate), amylose tris(3,5-dimethylphenylcarbamate) (Chankvetadze et al., 2002a), and covalently bonded β-cyclodextrin (Berthod et al., 1990), even bonded to a monolithic silica column (Lubda et al., 2003). SFC using immobilised polysiloxane-anchored permethyl-β-cyclodextrin (Chirasil-Dex) (Jung and Schurig, 1993) and teicoplanin or its aglycone (Chirobiotic T or TAG) (Liu et al., 2002) was also used to separate the enantiomers of norgestrel. The above-mentioned cellulose tris(3,5-dichlorophenylcarbamate) and amylose tris(3,5-dimethylphenylcarbamate) were successfully used for CEC separation of the enantiomers (Chankvetadze et al. 2001, 2002b). A comparison of the latter in the LC and CEC modes revealed significant advantages of CEC. This chiral selector was successfully used also for the LC separation of as many as 26 steroid enantiomeric pairs including estradiol, estrone, ethinylestradiol, nandrolone, etc. (Kummer et al., 1996; Kummer and Werner, 1998).

^{1}H-NMR was found to be an excellent tool for modeling interactions between norgestrel enantiomers and cyclodextrins, thus creating the possibility of finding a correlation between the NMR and RP-LC data (Tárkányi, 2000, 2002). In addition to this, NMR spectroscopy using γ-cyclodextrin (Tárkányi, 2000, 2002) or the lanthanide shift reagent praseodymium tris[3-heptafluoropropylhydroxymethylen e)-(+)-camphorate] (Blom et al., 1993) was found to be suitable for estimating the enantiomeric purity of levonorgestrel without separation. The limit of detection is somewhat higher than that achievable by LC.

12.8 Summary

The aim of industrial steroid analysis is to increase the *safety*, *reproducibility and economy* of the production of bulk steroid drugs and formulations made thereof. This is achieved by using strict analytical control at all stages of production and formulation. The results of these efforts, together with requirements of the analytical

experts of the Regulatory Authorities greatly contribute to satisfying the increasing demands for quality of steroid drugs and resulting improved *safety of steroid drug therapy*.

When *comparing* the areas of *biological–clinical* and *industrial–compendial analyses* similarities and differences are equally present. Of course the target analytes are steroid drugs in both areas, but the industrial analysis deals with the intermediates in their production, the impurities and degradation products of the drugs, while the biological–clinical analysis includes the determination of metabolites and intermediates in the biosynthesis of steroid hormones. From the methodological point of view high-performance liquid chromatography (HPLC or LC) is the predominant method in both fields (in biological–clinical analysis usually coupled with mass spectrometry) but almost all methods of modern analytical chemistry play a more or less important role. Matrix effects cause less pronounced difficulties in industrial–compendial than in biological–clinical analysis. Because of the practically unlimited sample size in the former field, sensitivity is not as crucial issue here as it is in biological–clinical analysis. However, the demands for accuracy and precision are higher.

The state of the art in compendial (and to a great extent also in industrial) steroid analysis can be characterised by inspecting the monographs in the principal pharmacopoeias. As for the assay methods of bulk steroid drugs, LC is also here the most important method, but non-selective methods such as UV spectrophotometry and in some cases titrimetry still play an important role, especially in the *European Pharmacopoeia* and to lesser extent also in the *United States Pharmacopoeia*. The Related Impurity Test that best characterises the quality of a bulk drug, is almost exclusively carried out by semi-quantitative thin-layer chromatographic (TLC) or quantitative LC tests in both pharmacopoeias. For the assay of drug formulation selective, stability-indicating methods are necessary. For this reason LC (in the majority of cases reversed-phase LC) plays an even more important role here than in the case of bulk steroid drugs.

In non-pharmacopoeial steroid drug analysis *UV spectrophotometry* is still a widely used method for spectrophotometrically active unsaturated three-oxosteroids and oestrogens with a phenolic A ring. The limited selectivity of this method can be improved by a combination of various chemometric, multiwavelength and derivative spectrophotometric methods. At the same time, the importance of colorimetric methods based on chemical reactions has almost completely vanished. The importance of UV spectrophotometry as a tool for structure elucidation of impurities and degradation products has somewhat increased due to easy availability online of UV spectra using, for example, LC diode-array detectors and TLC densitometers.

Fluorimetry based on native fluorescence of oestrogens is moderately important especially if used for sensitive LC detection. Fluorescence induced by strongly acidic reagents has completely lost its importance as a quantitative tool but these reagents are still widely used as TLC spray reagents.

The main application fields of *infrared, near infrared* and *Raman spectroscopies* are the identification of bulk steroids, characterisation of their polymorphic modifications and the in-process control of the production of steroid formulations.

The main application of *mass spectrometry* in industrial–compendial steroid analysis is structure elucidation either off-line or increasingly attached online to LC.

NMR spectroscopy is of fundamental importance in the structure elucidation of steroids but there are examples for its use for screening purposes also. A relatively new application field is the use of solid state NMR spectroscopy for the characterisation of polymorphic modification and solid dosage forms of steroid drugs.

Chemiluminometry is of little importance in industrial steroid analysis. *Circular dichroism* (*CD*) *spectropolarimetry* is a fairly important tool in structure elucidation of steroids but a few analytical applications (among them using CD spectropolarimeter as LC detector) are also available.

Of the *chromatographic* methods *thin-layer chromatography* (*TLC*) is widely used for various purposes. The main application field is the purity check of bulk steroid drugs and steroid formulations. In the pharmacopoeias, semi-quantitative limit tests are prescribed but the importance of quantitative methods for the determination of impurities and degradation products by densitometry is increasing. The latter technique is often used also for the assay of steroid drug formulations. Of the special techniques within TLC, *high-performance thin-layer chromatography* (*HPTLC*) and *overpressured thin-layer chromatography* (*OPLC*) are increasingly used for all of the above purposes. Further techniques such as temperature-controlled programmed TLC, programmed multiple development TLC with a new modification of the horizontal sandwich chamber, ultra-thin-layer chromatography (UTLC) using a monolithic structure based on a silica-gel matrix and tandem TLC-LC are interesting but as yet less widely used.

Despite its great tradition and the fine results obtainable (e.g. for screening purposes) the importance of *gas chromatography* (*GC*) has decreased in industrial–compendial steroid analysis mainly as the consequence of increasing interest in high-performance liquid chromatography. *Supercritical fluid chromatography* (*SFC*) has never played important role in this field. *High-performance liquid chromatography*(*HPLC* or *LC*) is the most important method in contemporary industrial–compendial steroid analysis. This technique is generally used in various pharmacopoeias for the assay of bulk steroid drugs and steroid formulations as well as for purity check of both. An especially important feature of this method is that – if properly optimised – it is stability-indicating enabling stability studies of steroid drug formulations to be carried out.

The coupling of LC with mass spectrometry (*LC-MS*) is an important tool for screening purposes of among others high-throughput steroid drug mixtures and the identification/structure elucidation of impurities and degradation products. Another important field of application is the analytical control of drug release from various formulations and devices for controlled drug delivery. In the overwhelming majority of cases reverse-phase LC with ODS columns is used. Innovations as regards stationary phases, not yet widely used are the introduction of ultrafine particles (down to 1.5 μm; ultraperformance liquid chromatography [UPLC]) and the use of monolithic columns and new stationary phases such as silica with covalently bound cholesterol, supports with electrochemically modulated surface, etc. Steroids are often among the model compounds of computer-assisted mobile phase optimisation studies.

Good results were obtained using micellar liquid chromatography which is a special branch of RP-HPLC. Liquid chromatography plays an important role in non-analytical aspects of steroid drug research from determination of log *P* values and modeling drug-membrane interactions to the prediction of human drug absorption.

Due to the lack of electric charge in the majority of steroid drugs the use of capillary electrophoresis (CE) is restricted to bile acids and quaternary ammonium derivatives. Although electric charge can be introduced by derivatisation, this technique does not play an important role in steroid analysis. Much more interest has been focused on the use of related methods. Of these, micellar electrokinetic chromatography (MEKC) using sodium dodecyl sulphate or bile acids (or their mixture) as the micelle-forming agents has been successfully used for the separation and quantification of various groups of steroid drugs. The same applies – although to a lesser extent – to the related technique microemulsion electrokinetic chromatography(MEEKC). Although as yet only a limited number of applications to steroid drugs have been described, *capillary electrochromatography* (*CEC*) and its coupled version with mass spectrometry (CEC-MS) are very promising techniques.

Of the *application fields* within industrial and compendial steroid analysis three merit special discussion.

The *impurity profiling* of steroid drugs, i.e. detection, identification/structure elucidation, and quantification of impurities in bulk drugs and formulations made thereof requires the complex application of at least two chromatographic or related separation method, mainly TLC and LC, in the latter case preferably coupled online with mass spectrometry but the first examples of the promising technique LC-NMR have also been published.

From the methodological point of view the same applies to *drug stability studies,* where the degradation products formed in the course of storage of the drugs or forced degradation studies should be detected, identified and quantified.

Since the configuration of the six to seven chiral centres in natural steroids and the semi-synthetic drugs prepared from them is fixed, *enantiomeric analysis* is not among the most important issues in drug analysis. Nevertheless, separation of the enantiomers of the total synthetically prepared drug *norgestrel* has been thoroughly studied. Norgestrel is an excellent model compound to check and compare the suitability for enantiomeric analysis of LC with chiral mobile phase additives, LC, SFC, CEC with various chiral stationary phases, and ^{1}H-NMR spectroscopy.

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Chapter 13
Quality Management of Steroid Hormone Assays

Jonathan G. Middle

13.1 Introduction

This chapter describes the principles and practices of quality management of the steroid hormone in clinical laboratory service, and the assessment of the quality of that service by the author's EQA programme (UK NEQAS for Steroid Hormones).

The whole area of 'quality' has undergone a major expansion in the last decade with the advent of international standards and formal accreditation for laboratory medicine. However, the emphasis seems to have been on process quality rather than analytical quality. Where steroid hormones are concerned, the latter still leaves a lot to be desired, but there is hope that the introduction of bench-top mass spectrometry techniques may overcome the inadequacies of immunoassay, and change our perspective on the true values of some steroid analytes.

There has been a major change in routine steroid analysis in the last decade, with the widespread use of automated platforms that have transformed highly specialised assays into those that are 'routine' for any laboratory to offer, whether clinically desirable or not. At the time of writing (late 2007) there has been rapid and unprecedented restructuring of the diagnostics market, with several well-known companies being bought and absorbed into a single global electronics conglomerate. Concurrently, there has been widespread discussion of alternative clinical laboratory service delivery models involving more testing at the point of care and networking of laboratories.

13.2 Quality Management

ISO defines "QUALITY" as "the totality of characteristics of an entity that bears on its ability to satisfy stated and implied needs" and "QUALITY ASSURANCE" as "all those planned and systematic activities necessary to provide adequate confidence that a product or service satisfies given requirements for quality".

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The quality management of clinical laboratories is governed by the recently revised ISO 15189:2007 – "Medical laboratories – Particular requirements for quality and competence". This Standard is based on the parent Standard ISO 9001:2000 – "Quality management systems – Requirements", which places emphasis on satisfying customer requirements and continuous quality improvement. It also relates to ISO/IEC 17025:2005 – "General requirements for the competence of testing and calibration laboratories". Clinical laboratories may now apply for accreditation against these and related Standards through regular assessment inspections which ensure efficient operation and management of a quality management system. Indeed, in some countries, accreditation is legally and/or financially mandatory.

Emerging from these standards is the strong sense that clinical laboratories must set defined *quality goals* for their service and *quality specifications* for their assays that meet the requirements of requesting clinicians and the patients they serve. This philosophy comes under the heading of clinical governance (a term mainly used in the UK, a nice definition of which is: "Framework through which NHS organisations are accountable for continuously improving the quality of their services and safeguarding high standards of care thus creating an environment in which excellence in clinical care will improve."). This is not a new concept; it was elaborated in Stephen Jeffcoate's seminal book, which emphasised the critically important concept that proficiency in quality assurance procedures is not seen as an end in itself, but just one step towards the real goal of improved patient care (Jeffcoate, 1981). The ability to produce accurate and precise results is one thing; offering the right test at the right time in the right clinical setting, with interpretative comments designed to maximise diagnostic effectiveness, is quite another.

Whilst the ultimate responsibility for quality management lies with the Head of Department, all staff must be encouraged to play their part and facilitate quality improvement. Poor quality of results means at the very least some additional pain and distress for the patient upon repeat sampling, and at the worst, incorrect or delayed diagnosis and/or treatment, with potentially fatal consequences. The human and economic costs of poor-quality results can be large, and many orders of magnitude greater than any apparent 'savings' made by employing inadequate quality assurance procedures. Getting it right the first time is the most cost-effective answer to quality management (Westgard et al., 1984).

Having said that, in the modern world there are associated financial and political pressures which require laboratories to meet efficiency and value-for-money targets set by Government as a part of healthcare delivery policies that improve outcomes but do not increase costs. Squaring this circle against a background of increasing workloads and decreasing staff is a major challenge for laboratory directors.

13.3 The Particular Need for High-Quality Steroid Assays

While the above comments apply universally across all analytes and pathology disciplines, it might be argued that there is a special need for quality in steroid endocrinology, due to the biological characteristics of these hormones. In contrast to analytes which are maintained at set points under tight control, steroid hormone

concentrations can vary in a pulsatile or circadian fashion, or over a longer time course as in the menstrual cycle, and this variability can encompass a wide range of analyte concentration. Single measurements are often of little clinical value in diagnosis; steroid assays are performed as part of dynamic function tests, or in carefully timed series, often in conjunction with other investigations. This places great emphasis on minimising both within-assay and between-assay imprecision, and, in our increasingly mobile society, between-laboratory variation.

The clinical utility of a steroid hormone may exist over a very wide range of concentrations, as exemplified by oestradiol, where low concentrations (<150 pmol/L) may be investigated in the context of ovarian failure, but very high concentrations (>10,000 pmol/L) may be monitored during the stimulation phase of assisted conception (Ratcliffe et al., 1988). This range is too great for conventional 'competitive' immunoassay, and two separately optimised assays might be more appropriate from a scientific, but not necessarily a practical, standpoint.

Steroid assays are performed extensively in the field of research, both at the academic level in universities, and in commercial laboratories for the purpose of understanding the mechanisms of drug action and for clinical trials. The emphasis here must be on trueness (accuracy), as measurement of only the analyte of interest and not other related substances and metabolites might be crucial to hypothesis testing, and also on long-term reproducibility, where the results of experiments or trials need to be compared over many years, and be reproducible between centres.

It should not be forgotten that the clinical setting may be one of high patient anxiety and deep emotions concerning gender assignment, sexual proficiency and fertility, or the presence of life-threatening endocrine or malignant disease. The time-to-diagnosis can be long for endocrine pathology, so that patients may present after an extended history of multiple investigations and treatments. The analyst might pause to consider these facts from time to time, and should need no further stimulus for the pursuit of a high-quality service.

13.4 Analytical Validity and Calibration of Steroid Assays

We can assert that a good-quality clinical assay should:

- Have optimal trueness and imprecision across the working range appropriate for its clinical application
- Be robust in the hands of different operators
- Provide appropriate diagnostic sensitivity and specificity
- Have good value for money in terms of additional patient benefit resulting from the availability of accurate and reliable results
- Be safe and environmentally 'friendly'

The key concepts now in widespread use and embodied within international standards and directives are 'trueness', 'traceability' and 'uncertainty', which are defined as follows:

- **Trueness**: Closeness of agreement between the average value obtained from a large series of results of measurements and a true value (from ISO 3534-1:1993, definition 3.13).
- **Traceability**: Property of the result of a measurement or the value of a standard whereby it can be related to stated references, usually national or international standards, through an unbroken chain of comparisons all having stated uncertainties (VIM:1993, definition 6.10).
- **Uncertainty**: Parameter associated with the result of a measurement that characterises the dispersion of the values that could reasonably be attributed to the measurand (VIM:1993, definition 3.9).

With regard to trueness and uncertainty, the reader is referred to a particularly useful guest-essay by Fuentes-Aderiu on the Westgard.com website (www.westgard.com/guest15.htm) for further elaboration of these concepts.

With regard to traceability, the important concept is the Traceability Chain, shown in Fig. 13.1. Here, the key elements are the definition of the analyte (measurand) in SI units and a hierarchical cascade of primary, secondary and working reference materials and methods which are used to underpin calibration of the final field method. When this chain is intact and fully documented in the technical specification of the method, then results can be said to be fully traceable. The graphic on the right-hand side of the diagram indicates the magnitude of the combined standard of uncertainty – $\mu_c(y)$ – which clearly increases as we go down the traceability chain.

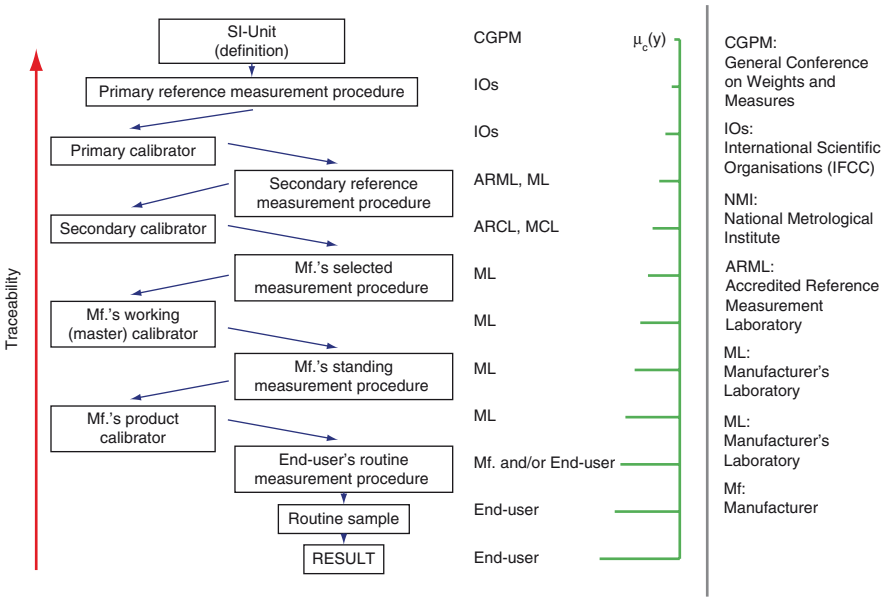


Fig. 13.1 Traceability chain indicating organisations having responsibility and combined standard uncertainty $\mu_c(y)$

Also indicated on the diagram are the organisations considered responsible for each level of the traceability chain. Note that the complete traceability chain is valid only for those measurable quantities that can have a value expressed in SI units. When primary or secondary calibrators are not available, the traceability chain for many measurands in laboratory medicine ends at a lower level, e.g. at the manufacturer's standing measurement procedure.

For the establishment of trueness and traceability, essential requirements are the existence of reference materials containing known amounts of the analyte in question and reference methods, which fulfil appropriate criteria for unbiased measurement within a reference measurement system. Two important and influential reviews on this topic are Thienpont et al. (2002) and Panteghini (2007). No one (in the author's humble opinion) has done more for the promotion, recognition and establishment of these important concepts than Professor Linda Thienpont, who has published widely on the role of networks of reference laboratories for steroid hormones in particular. Another important contributor in the field of reference methods (isotope dilution gas chromatography mass spectrometry) for steroid hormones is Professor Lothar Siekman, whose work in this area stretches over almost four decades. In a contribution to the IFCC e-journal (http://www.ifcc.org/ejifcc/vol13no3/130301002n.htm) he makes the crucial point that "an inevitable precondition for the establishing of traceable results to calibrators and control materials is the specificity of the measurement procedures applied. Results of measurement cannot be traceable when the procedure applied partially detects components which are not consistent with the definition of the measurand".

The European Union has embodied traceability requirements and the use of reference measurement systems by manufacturers to assign values to calibrators and controls, in its *In Vitro* Diagnostic Devices Directive (EU, 1998). To support this requirement, an international organisation has been set up – the Joint Committee for Traceability in Laboratory Medicine (JCTLM) – to create databases of established reference materials and methods. Their website at http://www.bipm.org/en/committees/jc/jctlm/ contains the results of the current working groups and the databases under construction.

There is an important caveat here, which might be pure pedantry on the author's behalf, but maybe the reader should take note of it. The traceability element of the EU IVD Directive seems to suggest that only values assigned to calibrants and controls need to be traceable to reference methods of higher order, and not the final result. In the author's opinion, this leaves the door open for manufacturers to apply 'adjustment factors' to results, where suboptimal specificity leads to values which are different to those of other methods (or EQA data), and the manufacturer wants results to be similar. Such factors may be revealed by recovery exercises (see UK NEQAS data below).

The standardisation of immunoassays in general was the subject of the third and fourth Bergmeyer Conferences. From the third Conference, the reader is referred to the overview by Ritchie (1991), the discussion of the philosophy of measurement systems by Büttner (1991), the description of the problems caused by heterogeneous analytes by Ekins (1991a) and, the most relevant in the context of this chapter,

the standardisation of hapten immunoassays by Gosling (1991). From the fourth Conference, the section on the standardisation of Cortisol assays (Gosling et al., 1993) describes recommendations on how the analytical validity of these assays might be improved by proper assessment of cross-reactivities, recovery and linearity studies, use of the reference method (isotope dilution gas chromatography mass spectrometry) and clinical validation using a panel of fresh frozen human serum from normal and pathological cases. As a direct result of the latter consideration, a panel of sera for cortisol was prepared in the author's laboratory, had values assigned by members of the steroid reference laboratory network and the samples lodged at the Institute for Reference Materials and Measurements (IRMM) under product code ERM-DA451/IFCC in 1998. It is believed that a number of manufacturers have used these samples in the calibration of their assays.

The author (Middle, 1998) has published a personal view on the standardisation of steroid hormone assays, in which the performance of oestradiol was critically reviewed and used as an exemplar for the many issues involved. Strong recommendations were made as to how the steroid reference measurement system should be used to improve trueness and method comparability:

- A network of steroid reference laboratories
- Availability of reference materials based on unprocessed human serum from normal and pathological states, with steroid hormone values assigned by the network
- A forum for facilitating information exchange between manufacturers
- A clear path for establishing traceability of EQA target values with the reference method network

These recommendations were repeated, almost with the same emphasis, in a review of testosterone measurement in women (Kane et al., 2007), indicating that very little had changed in the intervening decade. Indeed, the measurement of testosterone in females became a cause célèbre after the publication of a strongly critical method comparison (Taieb et al., 2003) and an accompanying editorial in Clinical Chemistry (Herold and Fitzgerald, 2003), where it was stated that results from current immunoassays were no better than chance. It is probably not by chance, however, that there has been a flurry of papers in recent years on the development of tandem mass spectrometry methods for testosterone (Cawood et al., 2005; Kushnir et al., 2006), and the publication of a position statement on testosterone measurement by the US Endocrine Society (http://www. endo-society.org/news/press/2007/EndocrinePositionStatementCallsStandardizedTestosteroneAssays.cfm) in February 2007, calling for 'standardised assays'. Three of their recommendations were

- Know the type and quality of the assay that is being used and the properly established and validated reference intervals for that assay. Reference intervals should be established by each laboratory in collaboration with endocrinologists, using well-defined and characterised populations.
- In the absence of other information, direct assays (those performed on whole serum) perform poorly at low testosterone concentrations (i.e. in women, children and hypogonadal men) and should be avoided. Assays after extraction and

chromatography, followed by either mass spectrometry (MS) or immunoassay, are likely to furnish more reliable results and are currently preferred.
- Assays for testosterone may behave differently in controls and affected individuals, perhaps reflecting differences in the endocrine milieu of patients.

At the time of writing, another commentary has just been published (Stanczyk et al., 2007) which reinvents the proposals discussed above, i.e. to develop standard pools of human serum for cross-comparison of methods on an international basis.

While it would seem that the creation of properly calibrated, traceable assays for steroid hormones is a straightforward matter, because all the elements of the traceability chain have been in existence for some time, why is it that methods are so poor? In contrast to many peptide hormones, where analyte heterogeneity is a major problem for traceable measurement, the chemical entity is precisely defined, highly pure 'standard' is readily obtainable 'off-the-shelf' and powerful reference methods exist in well-managed and specified networks of reference laboratories. However, antibody specificity and other 'matrix effects' can radically affect assay characteristics, and lead to considerable variability in the results obtained. The presence of specific (and non-specific) binding proteins for steroids in serum (Hammond, 1990; Selby, 1990) adds complexity to direct assay systems, in that reagents must be added to release the hormone from binding proteins, and then stop free hormone from binding to the assay tube. The steroid will also be in the presence of precursors and metabolites, the concentrations of which may vary with sex, age, time of day and physiological/pathological state, so the assay system has to be capable of 'seeing' endogenous analyte against a complex and variable background. Added to this, there is the issue of constraints imposed by multi-analyte immunoassay platforms where common incubation times, temperatures and reagents prevent individual analyte optimisation. Since every aspect of an immunoassay affects antibody specificity, this feature is crucial. Given these problems, it is perhaps not so surprising that we see such variation between methods.

In terms of analytical quality specifications for steroid hormones, the following is the author's view of a minimal set for any laboratory to require and any manufacturer of instruments and reagents to fulfil:

- Satisfactory trueness, specificity and baseline security confirmed by regression and the difference plots of the manufacturer's data for single donations of unprocessed clinical samples with values assigned by a member of the steroid reference laboratory network. The slope m of the regression line ($y = mx + c$) indicates correct calibration; the scatter about the regression line (S_{yx}) indicates analytical specificity and the intercept c is a guide for baseline security. The shape of the difference plot indicates concentration-dependent bias which may not be immediately obvious from the regression line.
- Clearly stated traceability back to primary standards, with all elements of the traceability chain fully and openly described and documented, with no arbitrary 'factors' applied to final results.
- Adequate recovery of added pure hormone (100% ± 10%) according to a clearly defined protocol involving concentrations of analyte relevant to clinical practice and authentic matrices.

- Satisfactory imprecision (%CV of repeat measurements) across the working range of the assay, with values at critical clinical decision points that are low enough to detect significant clinical change against a background of biological variation.
- Minimal reagent lot-to-lot variability that does not compromise maintenance of reference ranges and clinical decision making.
- Freedom from interference by related substances, metabolites, drugs and other substances which might confuse the clinical picture. All significant interferents should be tested at several realistic concentrations and their effects tabulated in a consistent systematic manner which allows easy comparison between methods (Middle, 2007).
- Satisfactory robustness in the hands of different operators and minimal instrument to instrument variability as evidenced by tight grouping in EQA services (particularly important when laboratories use multiple instruments or modular systems).

13.5 Methodological Issues Relevant to the Clinical Laboratory Service and Its Quality Assessment

The history of steroid analysis will be discussed in other sections of this book. Here the focus will be on the current (late 2007) methodology in routine clinical use in the UK. Figure 13.2 shows the pattern of methods used by clinical laboratories registered in the UK NEQAS for Steroid Hormones in late 2007 (shaded columns) compared with mid 1995 when the first edition of this book was published (open columns), for cortisol, oestradiol and testosterone (female matrix). Firstly it can be seen that there has been a marked increase in participation – particularly for testosterone – as more laboratories find they can offer steroid analysis on their multi-analyte immunoassay platforms. Secondly, there has been a marked shift away from single analyte radio-immunoassays towards the near-universal use of multi-analyte immunoassay platforms and modular systems. Very few laboratories employ extraction protocols for their steroid immunoassays now, except for urinary free cortisol. Thirdly, the diagnostics market has consolidated considerably with currently only a few major companies involved (in 2007 Siemens took over Bayer Diagnostics, Diagnostic Products Corporation and Dade Behring).

All steroid immunoassays in routine clinical use continue to employ a ‘limited reagent’ or ‘competitive’ design, where analyte in the sample competes with labelled analyte for a limited number of antibody binding sites (Ekins, 1991b). Assay designs using combinations of anti-idiotypic antibodies (Barnard and Kohen, 1990) raised the possibility of ‘excess-reagent’, ‘non-competitive’ design hapten assays, with advantages of (perhaps) greater specificity and extended dynamic range, but these have not come to market. Neither has micro-array technology (e.g. Ekins et al., 1990) had any impact. What is likely, is that many more laboratories will move away from immunoassay and employ liquid chromatography tandem mass spectrometry (LC-MS/MS), especially for those applications (urinary free cortisol, testosterone in the female matrix, 17hydroxyprogesterone) where immunoassay specificity is a problem.

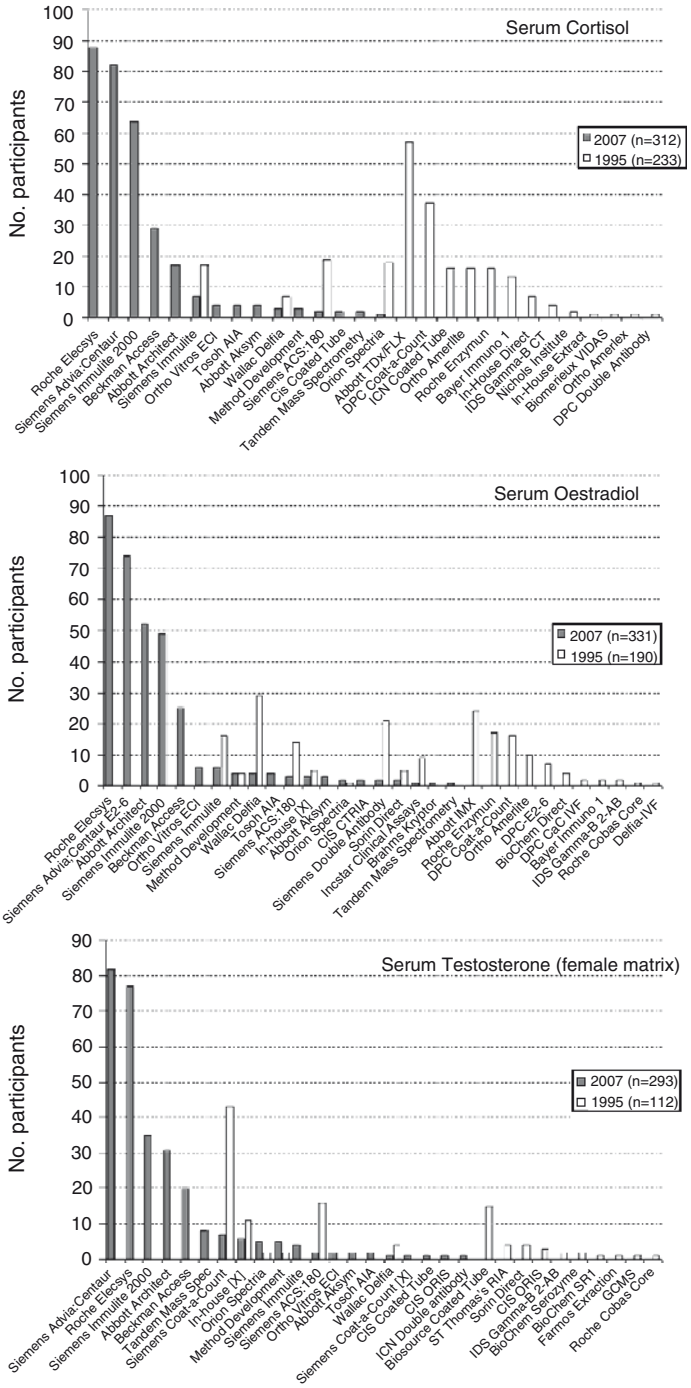


Fig. 13.2 Patterns of method use in the UK NEQAS for Steroid Hormones

Very few clinical laboratories undertake formal evaluations of individual steroid hormone assays any more. Rather they enter into a tendering process with a major diagnostic instrument platform supplier for a wide range of analytes, possibly across several different 'blood sciences' instrument modules. The advent of 'total solutions' suppliers, where a single company will provide a wide range of equipment, consumables and services, means that individual choice for many assays is impossible. A major problem is that those who fund procurement (service commissioners) may make the tacit assumption that the analytical quality of all (steroid) assays is the same, and that results are correct, comparable and interchangeable. This is definitely something to be striven for, but as we shall see below in the EQA data, it is far from reality. The emerging electronic healthcare record also requires long-term stability and comparability of test results; otherwise data on method, calibration and reference range will have to be included with each result.

It is critically important to appreciate that commercial methods can and do change. Raw materials may vary from batch to batch, and the effects on assay results can be subtle and unexpected. Machine-to-machine variability with fully automated systems is also a reality. Periodically, antisera (especially if these are from a small animal source), have to be replaced, and these may have different characteristics. Manufacturer's tolerance limits in their quality control programmes can be wider than would be expected by the user, and products with markedly altered characteristics released with little warning and sometimes with a very short overlap period for the new method to be evaluated. Laboratories have to be vigilant about this and pre-empt problems with appropriate terms in their contracts with suppliers.

13.6 Internal Quality Control (IQC) of Steroid Hormone Assays

It is probably not necessary to go into great detail on this topic, as it is the 'bread and butter' of clinical laboratory quality control and practised with due diligence in the vast majority of well-managed laboratories. However, there are some principles which deserve highlighting and some elements that are particularly relevant to steroid assays.

Internal quality control (IQC) is real-time process control, which is designed and managed to ensure that 'bad' results do not leave the laboratory and adversely affect patient care. Along the way, information is gathered on within-run imprecision and between-run reproducibility (both expressed as a coefficient of variation (CV)). Each assay is probed (sampled) at regular intervals with a stable control material which mimics a patient sample. This material has a known value for the analyte concentration (assigned or derived), and each result that is obtained for the control is compared with this value and an assessment made as to whether the assay is 'in' or 'out' of control. If 'in' the assay process continues; if 'out' the assay process is halted and the cause of the poor performance identified and fixed before it is allowed to continue once again. Clearly there is a cost involved (the control material

itself, the additional reagents consumed and the time required for the staff to manage the process), but this must be balanced against the cost of an error.

In designing an IQC programme, the questions that arise are: which materials, how many different ones, what analyte concentrations, how often should they be run, how should the data be presented, what rules should be followed to assess if the assay is in control, what should be done if out of control? The formal way to answer these questions is to set a quality specification for each assay, which takes into account its analytical properties (analytical sensitivity and specificity, working range defined by an imprecision profile (Ekins, 1983)), physiological properties (concentration, within and between individual biological variation) and clinical properties (diagnostic sensitivity and specificity, clinical decision points, clinically significant change in analyte concentration). Mathematical rules may then be applied to decide how many IQC materials with different concentrations should be run in any given period of time, and how different rules should be applied to yield a given probability of error detection and false rejection. (The reader is referred to the westgard.com website on all of this.) Westgard and Darcy (2004) have recently criticised the lack of rigour in applying these concepts to analytes where there are national guidelines for interpretation and clinical action. They conclude that "evidence based medicine must employ scientific methodology for translating test interpretation guidelines into practical, bench-level, operating specifications for the imprecision and inaccuracy allowable for a method and the QC necessary to detect method instability."

An important concept is that of the 'analytical goal' discussed by Fraser (1983) and further elaborated by Hyltoft Petersen and Horder (1992), Fraser and Hyltoft Petersen (1993a), and de Verdier et al. (1993). Analytical goals for trueness and imprecision should be combined, and, since the analytical goal for inaccuracy is zero, the goal for total analytical error should therefore be that for imprecision based on biological variation, so that for individual testing and serial measurement,

$$CV_{Analytical} > \tfrac{1}{2}\, CV_{Intra\text{-}individual}$$

For monitoring of patients, the ideal performance should aim at stability with low imprecision relative to within-subject variation. For diagnosis, maximal trueness should be achieved, so that common reference intervals may be used when populations are homogeneous for a given analyte. Ricos and Arbos (1990) have indicated analytical goals for cortisol (7.6%) and oestradiol (11.8%). The former is probably achievable, but the latter remains elusive. Steroid hormones were not included in the latest listing by Ricós et al. (2007) of collated data on within-subject biological variation, probably because they vary so much during the day or in different physiological states. It is likely that the analytical goal concept will gradually be replaced by that of combined uncertainty, as this term is now being incorporated in international standards.

The materials used in IQC require careful consideration, as the requirements for steroid hormones are more stringent than for some general clinical chemistry analytes,

where non-human materials may suffice. IQC samples must be of human origin, and as close in composition to real patients' samples as possible, so as to ensure matching of the matrix. 'Spiking' to raise the steroid concentration to an appropriate level may be necessary, but is undesirable, as possible interfering substances (precursors/metabolites) that would normally be present in native serum will not be in appropriate concentrations.

The concentration of analyte should be chosen to target the main clinical decision points of the assay, and, because the dose–response relationship in immunoassay is non-linear, multiple levels (usually three) should be used to control the whole of the assay working range.

The material must be stable over its intended life-time, which should be sufficient to cover a substantial number of assays. This implies that volumes will be quite large, and that stabilisation (sodium azide, lyophilisation) and/or special storage may be required. For steroid hormones, frozen liquid serum maintained at −40° in a dedicated or infrequently used freezer is to be preferred. Storage in the same freezer as patient samples is not to be recommended, as the thermal shock of repeated temperature fluctuations may lead to deterioration of the material. Careful consideration must be given to the overlap period between different batches of materials, so that imprecision data for the new batch is established before the old one is exhausted.

IQC material for steroid hormones is often included with the immunoassay system reagent packs. This may be convenient for the user, but it must be emphasised that this material might not be ideal in terms of analyte concentration or matrix, and have characteristics that are carefully 'tuned' to the analytical system. Externally sourced, third party IQC materials are much to be preferred. At the time of writing, two such sources of commercial material with a wide range of steroid hormone values are in common use – Bio-Rad Laboratories and Randox Clinical Diagnostics Solutions. The author endorses neither but refers the reader to their websites at www.bio-rad.com and www.randox.com, respectively.

Most of the literature on IQC refers to 'batches' of analyses and 'batch rejection rules', but routine analysis of clinical samples may now increasingly be performed in 'random' mode, where successive samples may be sent to one of several different analytical modules, all capable of performing a range of different analyses. How should IQC be undertaken in this scenario? Clearly, each module should be independently assessed for each analysis that is performed on it, using appropriate materials and sampling frequencies, and the resultant data integrated and analysed inter alia for systematic differences between modules. It is the author's experience from discussions with UK NEQAS participants, that this often leads to certain modules or one of a pair of instruments being 'preferred' for certain analytes.

Whatever system is used, control rules should not be regarded as absolute, and each laboratory should draw up its own set of objective criteria for rejecting assay batches, which can then be used with sufficient flexibility to take into account clinical needs and avoid unnecessary repeat sampling. The data should be examined by an experienced member of staff who is not immediately involved in the assay, so as to maintain objectivity (Seth and Hanning, 1988). Assays which breach control rules may then be examined in detail, and, with the use of other information, a decision

made and recorded. All IQC data should be summarised and reviewed as part of an audit of laboratory effectiveness, ideally in conjunction with EQAS data, which can reveal longer-term changes in assay performances. Indeed, internal audit of all processes is now an important feature of quality management and continuous quality improvement.

13.7 The External Quality Assessment of Steroid Hormone Assays

External Quality Assessment (EQA) – in the USA the term Proficiency Testing (PT) is preferred – is a completely separate and distinct process from IQC. It involves a retrospective analysis of laboratory performance, using samples distributed on a regular basis by an EQA centre. The samples distributed (close to but not necessarily identical to those from patients) must be assayed 'blind' exactly as if they were patients' samples. Results of surveys are processed to give target values (see fuller description below), against which each participant's results are assessed. Reports for each distribution and at the end of each cycle (generally six-monthly or annual) are provided. Laboratories are usually scored against acceptable limits of performance laid down by professional bodies or legislation, but practice varies depending on the country and scheme. In the UK, such limits are advisory, but their persistent breach will signal intervention by professional bodies. However, in Germany for example, laboratories are required by law to meet certain performance standards for the common analytes based on deviation from a reference method value.

EQA is primarily concerned with 'trueness' (bias from 'true' value) rather than imprecision, although a limited assessment of the latter can be derived from the variability of the bias, and repeat distributions of the same material. By encouraging the use of unbiased methods and their harmonisation with reference methods, comparability of patients' results may be improved at the national level, and collaboration amongst the EQAS centres of different countries offers the prospect of global harmonisation.

EQAS have an important investigative role, in that analytical parameters such as recovery and linearity, baseline security, cross-reactivity and interference may be probed. As well as providing independent, objective data on performance to participants, they serve as 'clearing houses' for information about the strengths and weaknesses of methods. Organisers often liaise with manufacturers of instrumentation and reagents in respect of current problems and new developments. They also work closely with international and national reference centres and laboratories.

In the UK, laboratories participate in EQAS on a *quasi* mandatory basis, in that *de facto* mandatory laboratory accreditation standards require participation in EQA. In other countries (e.g. USA, Germany), participation may form part of mandatory proficiency testing that is regulated by legislation, so that laboratories cannot receive re-imbursement for their work unless satisfactory EQA performance is demonstrable. Usually only the Organiser of each scheme knows the identity of participating laboratories, which are given unique code numbers. Organisers of schemes may be

heads of department, senior clinical scientists or technical staff, depending on the history and location of the EQAS centres. For the major schemes, where the Organiser has departmental responsibilities, scheme Managers may also be employed. Steering Committees composed of experts in the field serve to advise organisers on the day-to-day running of their schemes, and help resolve technical or administrative problems; they are not concerned with the individual performance of the participants. Advisory Panels (one for each pathology discipline composed of nominees of the professional bodies) have the responsibility for setting criteria for an acceptable performance in consultation with the Steering Committee and Organisers. Panels have an independent monitoring role on behalf of the professions, and may contact individual laboratories if the Organiser cannot resolve performance problems. Such regulation by national professional bodies is common throughout Europe, but by different procedures.

EQA services are themselves becoming subject to international standards and accreditation in exactly the same way as clinical laboratories. Clinical Pathology Accreditation (UK), Ltd. – CPA (UK) – (see www.cpa-uk.co.uk) accredits all EQA schemes providing services in the UK against its EQA standards, which include elements of the ILAC G13 document (revised in 2007) and ISO Guide 43 – soon to become a full ISO standard – ISO 17043. Such standards cover all aspects of EQA management, personnel, infrastructure, design, process, evaluation and continuous quality improvement. Crucially important are the elements covering communication with participants and meeting their requirements.

The role of participation in EQA schemes in laboratory accreditation varies again with different countries. In the UK, participation in all relevant recognised schemes is required as one of the standards of the CPA (UK), Ltd., but a legal framework describing how EQA schemes themselves should be licensed or regulated, and formalising the relationships between the various professional groups, is not in place. It is the author's opinion that in order to obtain the most educational benefit from EQA, it should be separately directed and managed from accreditation, so that laboratories are encouraged to use analytically valid methods, whether or not they happen to perform 'well' in official EQA or PT programmes. Such separation obviates the temptation to cheat, and prevents commercial interests influencing the design of EQA and PT programmes.

13.7.1 Principles and Practice of Steroid Hormone EQA

The author's UK NEQAS service will be used to illustrate various elements.

Participation All public and private sector laboratories offering a clinical service for steroid hormones in the UK are strongly recommended and actively encouraged to participate in recognised EQAS. Research institutions and commercial companies in the field of diagnostic reagents are welcomed and overseas laboratories and companies may also participate. Participants are required to assay EQAS samples in exactly the same way as they do patient specimens, and return results within a reasonable time from sample despatch; indeed timeliness and completeness of

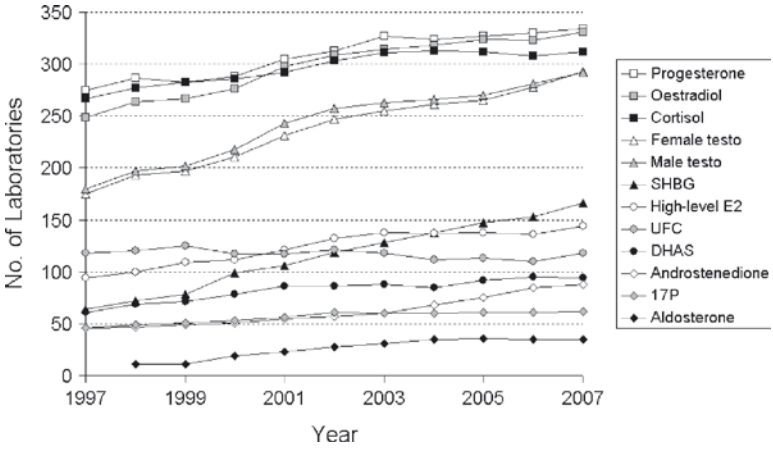


Fig. 13.3 Trends in analyte participation in the UK NEQAS for Steroid Hormones

participation are aspects of performance that are monitored. Current participation is indicated in Fig. 13.3.

Method of Survey The frequency of distributions and the number of samples in each is a compromise between what is needed to ensure adequate assessment of a significant proportion of assay runs, and what can be provided from limited amounts of serum available with appropriate hormone levels. There are also statistical considerations in respect of obtaining enough data points in the cumulative analysis period for a reasonable degree of confidence in performance figures to be achieved. Mature assays with few problems require only a minimal monitoring effort, whereas immature assays with large differences between methods and a high degree of variability, need more intensive surveys, and detailed probing of assay validity.

UK NEQAS for Steroid Hormones operates on a monthly distribution frequency that is five samples for cortisol, progesterone, oestradiol, androstenedione, dehydroepandrosterone sulphate (DHEAS) and 17α-hydroxyprogesterone, plus four samples for urinary free cortisol, high-level oestradiol and SHBG and three each for male and female testosterone and aldosterone, are sent each month. Pools are repeated between four and eight times over a period of up to 2 years, thus providing additional information on assay stability. Table 13.1 shows the data for repeat pools in the cortisol scheme.

Sample Type It is important that the material used for EQA should be as close as possible to that normally assayed in clinical practice, so as to ensure commutability. This ensures that the matrix is appropriate, and that a full range of accompanying substances and metabolites that may be present in clinical samples are there. For some general clinical chemistry analytes, non-human matrices may be acceptable, but for hormones, there is no substitute for human material. Serum is preferred to plasma because of its superior storage qualities, and is used for all analytes

Table 13.1 Stability of the ALTM (nmol/L) for cortisol pools distributed ≥3 times between January 2006 and September 2007

Pool	M/F	*N*	Mean	SD	%CV
C387	F	4	97.85	1.19	1.22
C402	M	3	98.03	0.74	0.76
C362	M	4	154.21	1.00	0.65
C403	M	3	185.81	2.27	1.22
C396	F	4	259.50	1.75	0.68
C364	M	3	268.01	1.19	0.44
C386	M	3	295.02	3.04	1.03
C389	F	5	320.49	5.53	1.73
C361	M	4	334.98	2.01	0.60
C395	M	3	373.61	0.54	0.14
C385	M	4	387.83	2.74	0.71
C404	M	3	468.38	2.92	0.62
C401	F	3	569.15	2.39	0.42
C384	F	3	783.07	4.73	0.60

M/F indicates male or female matrix.

in the UK NEQAS for steroid hormones. It is usually obtained from normal healthy donors through the UK National Blood Transfusion Service, but clinical material may be used wherever available. Separate pools are made for each of the analytes, according to the range of concentration required for assessment. Pools containing added hormone are prepared from time to time to extend the range of concentrations and for specific recovery experiments. All sera are tested for infectious agents at the individual donation level prior to use and are maintained at −40° until the day of despatch. The above 'purist' approach to EQA material preparation is very costly and not always possible for schemes with large numbers of participants, or those that buy in their materials from manufacturers of commercial control materials.

Sample Stability It is an obvious concern that samples used in the assessment of laboratory performance should be stable and free of any unwanted interference with assay systems. Serum used in the UK NEQAS for steroid hormones is filtered down to 0.5 micron. Figure 13.4 shows the stability of repeat distributions of pools for progesterone over a 12 month period.

Hormone Concentrations Assessed These are generally based on clinical requirements. In the UK NEQAS for serum cortisol, performance assessment is normally restricted to 100–900 nmol/L, with the occasional low sample to assess dexamethasone suppressed values, and high sample to mimic Synacthen stimulation. In the urinary free cortisol scheme, levels range between 100 and 800 nmol/L. For progesterone, assessment is made in the range 10–40 nmol/L, which includes the most useful range for the monitoring the adequacy of the luteal phase. For oestradiol, the main scheme range is 100–1,000 pmol/L, thus covering post menopausal levels, and concentrations found within the normal menstrual cycle. These samples are

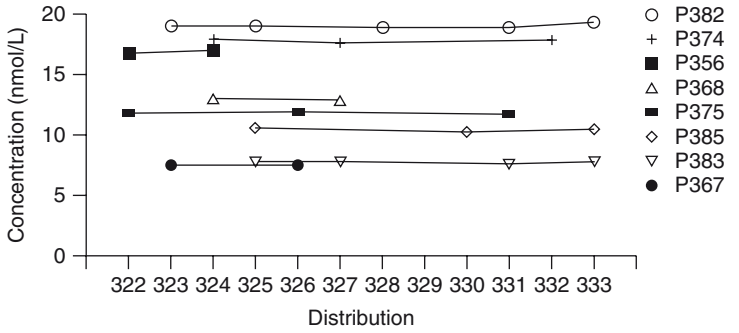


Fig. 13.4 Stability of the ALTM for progesterone pools

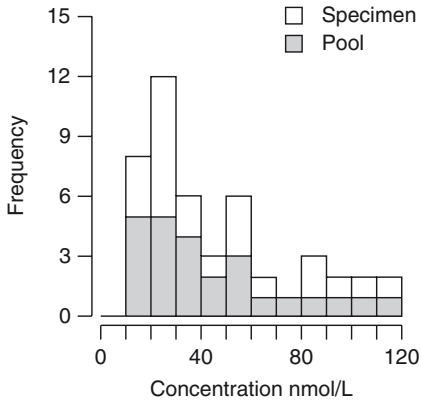


Fig. 13.5 Analyte concentration of pools and specimens distributed in the SHBG scheme

inadequate for the assessment of assays used in stimulated cycles for assisted conception, so a separate scheme for high-level oestradiol, where concentrations in the range 1,000–10,000 pmol/L are employed, addresses this problem. For testosterone, female and male samples are distributed separately. Female concentrations range typically from 1.0 to 5.0 nmol/L and male from 8 to 40 nmol/L. For the 17α-hydroxyprogesterone scheme, concentrations in the range from the normally very low normal adult up to 100 nmol/L are distributed. This covers the clinically important 20–30 nmol/L area where CAH patients on treatment are monitored, as well as the higher levels associated with diagnosis of this condition. The ranges for androstenedione and DHEAS are both around 2.0–30 nmol/L and that for SHBG is about 10–130 nmol/L; all of these analytes include both male and female matrix samples. For aldosterone the range distributed is about 200–1,400 pmol/L. Figure 13.5 shows a typical distribution of specimens and pools in the SHBG scheme.

Derivation of Target Values The target value in any EQAS is of fundamental importance, as against this is performance assessed. In an ideal world, targets

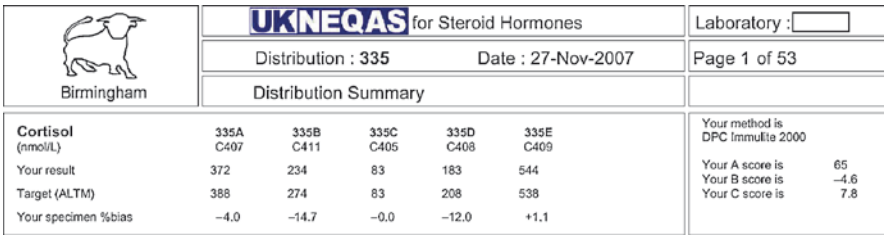
would be derived using reference methods. While the technical expertise for rigorous quantitative analysis of serum steroids by ID-GCMS is available at several centres, the very considerable financial resources required to fund targeting of every pool used by UK NEQAS are not. The approach now taken in the author's schemes is to conduct limited ID-GCMS targeting exercises for a few pools, which are then used to assess the state of the art of routine assays at a single point in time. Results of some of these will be described below.

In the absence of target values traceable to a reference value, UK NEQAS employs a consensus value, the All Laboratory Trimmed Mean (ALTM) for all analytes except 17 hydroxyprogesterone, which uses a Grouped Laboratory Trimmed Mean (GLTM) of extraction methods. Trimmed means are derived after deletion of the highest and lowest 5% of the data set for each sample – a treatment designed to remove outliers. The standard deviation of the trimmed data set is then calculated with weighting factors appropriate to the number of data points deleted, according to the method of Healy (1979).

The method mean is often used as the target value in many EQA schemes. While it is very useful for the laboratory to know how well it compares with its peer group, an uncritical use of such a target value can lead to a false sense of security where methods give a wide range of results. This approach also defeats one of the main objects of EQA, which is to encourage harmonisation of methods so that patients' results are comparable between centres.

Performance Evaluation This is undertaken on a rolling six-monthly basis each month, using the UK NEQAS Birmingham 'ABC of EQA' scoring system. As the latest distribution's data are added, the oldest set is lost. Each result in the six-month time window is assessed against the target value and a simple percentage bias is calculated. After suitable trimming, the mean of these biases is calculated and termed the B score. The standard deviation of the B score is termed the C score, which represents the variability of the bias from distribution to distribution, sample to sample and according to analyte concentration. It is not the same as imprecision. The A score is a derived parameter which enables analytes with widely differing performance characteristics to be compared in a common currency. The sample percentage bias is multiplied by a factor which is derived from the relationship between the coefficient of variation of the ALTM and analyte concentration. Where variability is large, the factor is small; where variability is small, the factor is large. Thus there is more of a penalty to have a high bias where comparability between laboratories is good. All A scores were normalised to a median value of 100 at a bench mark point of January 2000, so that at that time, a score of 100 meant average for the state of the art, and all analytes could be compared. As time has progressed, a reduction in the median A score reflects an improvement in the state of the art, and an increase reflects a deterioration. The UK National Quality Assurance Advisory Panel for Chemical Pathology sets criteria for acceptable performances for B and C scores for each analyte in the Scheme.

Each month, participants receive a report which is structured in a way that enables different staff members to focus on the information they need. A series of pages:


Birmingham

UKNEQAS for Steroid Hormones

Laboratory :

Distribution : 335 Date : 27-Nov-2007

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Distribution Summary

Cortisol (nmol/L)	335A C407	335B C411	335C C405	335D C408	335E C409
Your result	372	234	83	183	544
Target (ALTM)	388	274	83	208	538
Your specimen %bias	−4.0	−14.7	−0.0	−12.0	+1.1

Your method is DPC Immulite 2000

Your A score is 65
Your B score is −4.6
Your C score is 7.8

Fig. 13.6 UK NEQAS distribution summary page

(a) Confirm the results received and give the scheme statistics for each sample – target value and resultant percentage bias. A section of the page is shown in Fig. 13.6.

(b) Show box and whisker plots of the distribution of A, B, and C scores for the method used by the participant, with the participant's score overlaid, for each analyte in which they participate. These have the working name 'Graphic Equaliser Plots' – examples are shown in Fig. 13.7.

(c) Show plots of B versus C scores which indicate performance limits, 5th–95th centile domains for all laboratories and the method used by the participant, individual performance coordinates for each user, the score coordinate for the participant. These have the working name 'Penalty Box Plots' and examples are given in Fig. 13.8.

(d) An analyte-specific page for each sample in that month's distribution, showing method-related data in tabular form, the spread of all results as a histogram, and the derived statistics. An example is given in Fig. 13.9.

(e) A table which shows for each sample for the six distributions in the scoring time window the result obtained, target value and percentage bias. Pools are ranked in order of concentration to highlight concentration dependent effects. At the foot of the table, average monthly biases and performance scores are given. This table may be used to assess the comparability of results obtained when pools are repeated. An example is given in Fig. 13.10 where a participant underwent a method change at distribution 333.

(f) Box and whisker plots of B and C scores for methods for the analyte and trend graphs for the participant. Shading on these plots indicates acceptable performance limits. Examples are given in Fig. 13.11, once again showing a method change. This example (oestradiol) illustrates very well the large differences in bias (B score) between methods for this analyte.

(g) Tables for method related statistics and scores, so that these may be compared. Only the most popular methods are shown. An example is given in Fig. 13.12.

(h) A 'snapshot' page showing a series of plots of distribution and scoring time window-related statistics. The examples given below show a difference plot and bias plot according to the concentration. Some examples of two of the 12 plots on the page are given in Fig. 13.13.

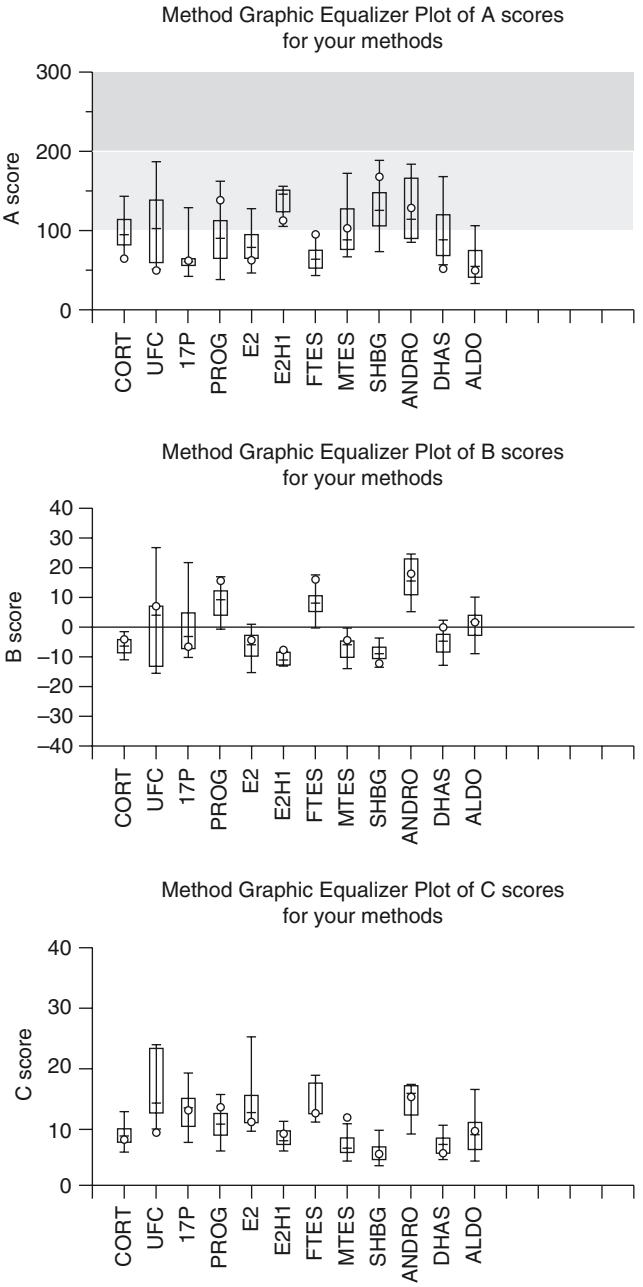

Fig. 13.7 Graphic Equaliser Plots of A, B, and C scores

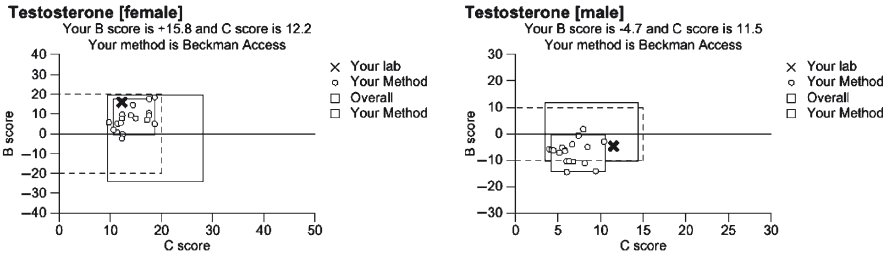

Fig. 13.8 Penalty Box Plots for male and female matrix testosterone

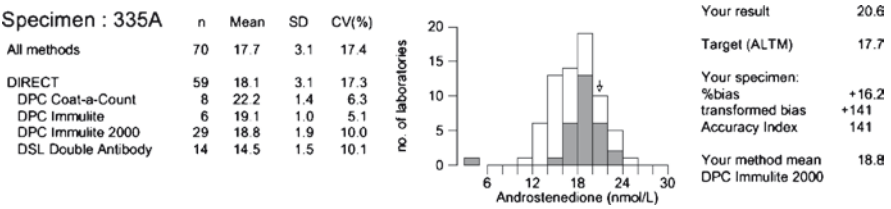

Fig. 13.9 Extract from the androstenedione analyte distribution report

Pool (exclusion) [Type]	Distribution 330 26-Jun-2007 result	target	%bias	Distribution 331 24-Jul-2007 result	target	%bias	Distribution 332 21-Aug-2007 result	target	%bias	Distribution 333 25-Sep-2007 result	target	%bias	Distribution 334 30-Oct-2007 result	target	%bias	Distribution 335 27-Nov-2007 result	target	%bias
E417 [F,V]				<50.0	**49**													
E412 [F,R,V]	85	**87**	−2.6															
E407 [F,V]													127	**125**	+1.9			
E392 [F,V]										100	**135**	−25.9						
E336 [F,V]							148	**154**	−3.9									
E390 [F,V]				155	**167**	−7.2												
E403 [F,V]										146	**176**	−17.0						
E408 [F,V]													204	**190**	+7.4			
E402 [F,V]							201	**209**	−3.7									
E413 [F,R,V]	200	**221**	−9.6															
E349 [F,V]							223	**239**	−6.6									
E381 [F,V]				214	**242**	−11.7												
E418 [F,V]										245	**281**	−12.8						
E409 [F,V]													344	**337**	+2.1			
E414 [F,R,V]	360	**368**	−2.2															
E380 [F,V]							341	**410**	−16.8									
E410 [F,V]													545	**538**	+1.2			
E338 [F,V]							458	**539**	−15.0									
E420 [F,V]																501	**540**	−7.3
E421 [F,V]																510	**540**	−5.5
E422 [F,V]																501	**541**	−7.4
E419 [F,V]										491	**543**	−9.6						
E423 [F,V]																498	**546**	−8.8
E424 [F,V]																498	**548**	−9.1
E394 [F,V]				489	**572**	−14.6												
E405 [F,V]										542	**590**	−8.2						
E415 [F,R,V]	630	**708**	−11.0															
E411 [F,V]													961	**1005**	−4.4			
E393 [F,V]				1070	**1316**	−18.7												
E416 [F,R,V]	1250	**1424**	−12.2															
Method	PH2			PH2			PH2			AB13			AB13			AB13		
mean			−7.5			−13.1			−9.2			−14.7			+1.6			−7.6
A score	78			80			76						52			58		
B score	−10.4			−11.5			−10.8						−5.8			−6.3		
C score	10.4			8.8			8.1						10.6			7.0		

F female only pool
R Recovery
V no preservative

Fig. 13.10 Six distribution summary table for oestradiol (main scheme)

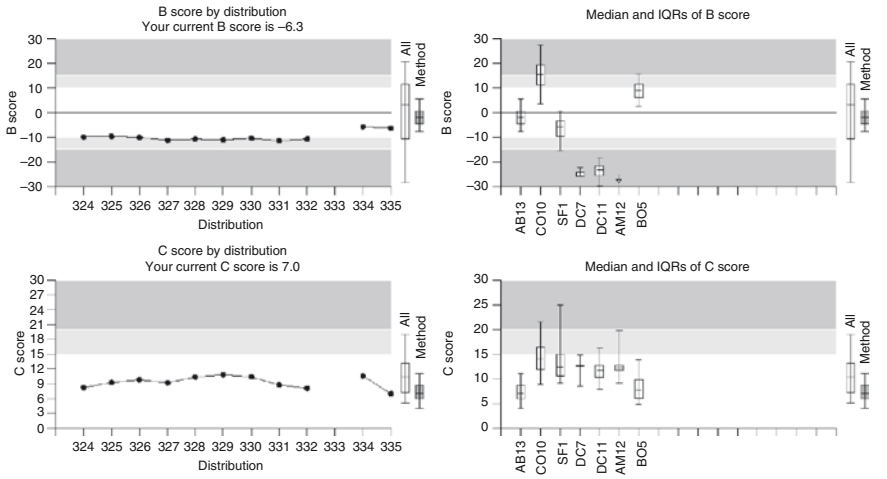

Fig. 13.11 B and C score trend graphs and method-related box and whisker plots for oestradiol

		335A			335B			335C			335D			335E		
	n	Mean	SD	CV(%)	Mean	SD	CV(%)	Mean	SD	CV(%)	Mean	SD	CV(%)	Mean	SD	CV(%)
All methods	77	23.6	2.6	10.8	19.5	2.7	13.7	10.8	1.3	12.5	25.5	2.7	10.5	10.0	1.4	14.1
DPC Immulite	9	24.6	2.1	8.4	19.4	1.8	9.4	10.4	0.5	4.5	25.4	0.7	2.8	9.4	0.5	5.1
DPC Immulite 2000	47	22.6	1.7	7.6	18.3	1.4	7.5	10.2	0.8	7.9	24.5	1.5	6.1	9.5	0.9	9.6
Roche Elecsys	13	30.7	8.1	26.5	24.2	2.3	9.7	13.1	1.6	12.0	36.9	9.1	24.6	11.1	2.5	22.7

			A score			B score			C score		
		n	Median	Interquartile Range		Median	Interquartile Range		Median	Interquartile Range	
All methods		85	101	70	167	−2.7	−8.1	+2.5	7.6	5.6	8.8
DPC Immulite	DC7	9	72	56	83	−2.9	−5.9	−0.4	6.1	4.8	7.7
DPC Immulite 2000	DC11	52	89	69	121	−5.0	−8.9	−2.6	7.0	5.6	8.0
Roche Elecsys	BO5	13	297	238	359	+23.5	+19.2	+29.0	7.9	7.6	11.3

Fig. 13.12 Method-related distribution data and score summary for DHEAS

13.7.2 Current Performance Issues

Performance characteristics change with time, so it is not appropriate in a text book to go into great detail. Current data are always freely available from the author. However, some recent data will serve to highlight some of the analytical quality issues mentioned above.

13.7.2.1 Low Level Cortisol and Oestradiol Performance

Good performance at low concentrations is required clinically for the interpretation of dexamethasone suppression tests. Table 13.2 shows an analysis of a low level pool (sample 328A) distributed in early 2007. Methods with CVs less than 10% and narrow inter-quartile ranges at these levels are clearly superior. The Roche methods remain exemplary in this respect. Abbott Architect is clearly not as good as other methods here.

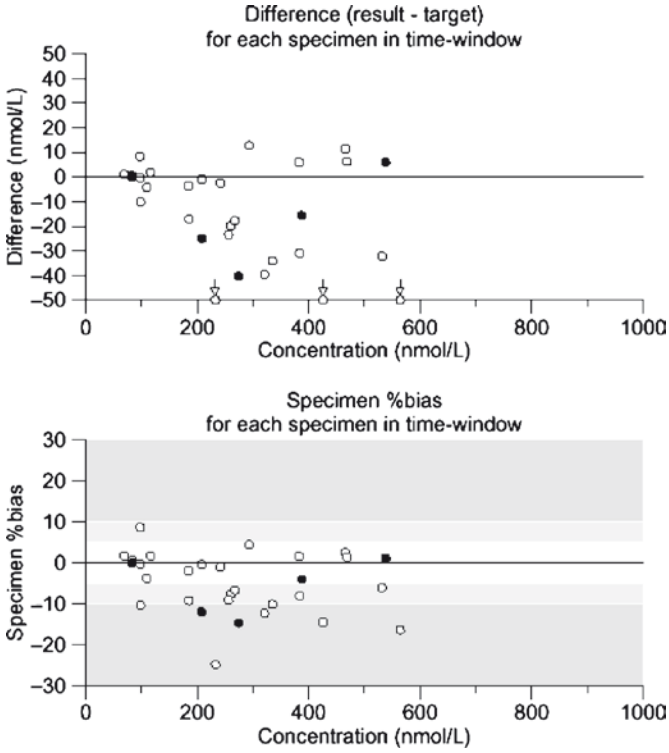

Fig. 13.13 Some examples of plots from the 'snapshot' page

Table 13.2 Data (nmol/L cortisol) from a low-level sample study

	All	BO5	E170	CO10	SF1	DC11	DC7	AB13
n	282	22	60	79	26	60	5	16
Min	43	62	60	61	55	43	61	49
5th	56	62	60	65	61	56	63	49
25th	65	65	64	70	65	64	72	52
50th	70	68	68	77	71	71	75	57
75th	75	71	69	80	78	74	77	62
95th	85	74	73	87	81	82	87	76
Max	136	74	75	99	81	100	89	90
Mean	70	68	67	76	71	69	75	59
SD	9.9	3.7	3.8	7.5	7.2	9.1	10.2	10.7
%CV	14.1	5.5	5.7	9.9	10.2	13.1	13.6	18.1

CO10 = Bayer Centaur; E170 = Roche Modular; DC11 = DPC Immulite 2000; SF1= Beckman Access; BO5 = Roche Elecsys; AB13 = Abbott Architect; DC7 = DPC Immulite 1.

Table 13.3 Data (pmol/L oestradiol) for a low level sample study

	All	DC11	AM13	AB13	E170	BO5	SF1	CO10
Numeric	130	5	5	13	47	17	8	25
"<"	145	37	0	27	15	1	14	35
"<" Low	<7.3	<51	n/a	<37	<44	<37	<7.3	<26
"<" High	<200	<75	n/a	<100	<200	<37	<200	<200
Min	10.0	60.0	23.0	39.0	23.3	20.0	17.0	10.0
5th	18.1	62.6	24.2	40.7	32.3	20.8	18.8	10.6
25th	36.3	73.0	29.1	45.8	41.4	38.0	32.5	28.0
50th	46.5	73.0	29.7	53.5	46.0	42.2	73.0	49.0
75th	63.8	73.4	31.1	65.3	59.5	54.0	87.5	61.0
95th	84.4	74.7	33.0	74.5	80.9	78.6	110.0	85.4
Max	110.0	75.0	33.5	75.0	97.0	89.0	110.0	100.0
Mean	50.2	70.9	29.3	55.8	51.8	47.2	65.1	46.9
SD	21.3	6.1	3.9	13.9	17.0	20.1	36.8	24.5
%CV	42.3	8.7	13.3	23.1	32.8	42.6	56.6	52.4

DC11 = Siemens Immulite 2000/2500; AM13 = Ortho Victros Eci; AB13 = Abbott Architect; E170 = Roche E170 Modular; BO5 = Roche Elecsys; SF1 = Beckman Access/Dxi; CO10 = Siemens Advia Centaur

Table 13.3 shows a similar exercise for oestradiol. Note here the very large number of laboratories which reported a 'less than' result – i.e. lower than their limit of reporting – and the large range of values which labs quote as their limit. The variability of results is very marked for several methods – a tenfold difference between lowest and highest for one.

13.7.2.2 Recovery Studies

Recovery studies are performed at regular intervals for all analytes. A base pool is made with serum donations containing low endogenous concentrations of analyte. To portions of this are added doubling amounts of pure steroid in ethanolic solution. The example shown in Table 13.4 is for oestradiol and illustrates the problems of under-recovery observed for this and other analytes. For the four columns on the left of the table, SPEC = specimen identifier; ALTM = all-laboratory trimmed mean; "added" = amount of oestradiol added to the spiked pools; "recovery" = % recovery of the amount spike. Under each method code is the number of users returning results, method mean results for each sample and the % recovery of the amount spiked. The average recovery is the mean of the individual recovery figures obtained for the separate samples. It can be seen that only two methods show adequate recovery, and for many, the recovery is non-linear. The only interpretation that can be made from these results is that manufacturers are trying to compensate for poor specificity by adjusting calibration.

Table 13.4 Recovery exercises for oestradiol main scheme at distribution 330

				AB13		AM13		BO5		CO10		DC11		DC7		PH2		SF1	
				n=42		*n*=4		*n*=79		*n*=58		*n*=47		*n*=5		*n*=5		*n*=23	
Sample No	ALTM (pmol/L)	Added (pmol/L)	Recovery (%)	(pmol/L)	(%)	(pmol/L)	(%)	(pmol/L)	(%)	(pmol/L)	(%)	(pmol/L)	(%)	(pmol/L)	(%)	(pmol/L)	(%)	(pmol/L)	(%)
330A	87	0		87		48		94		91		81		73		88		67	
330B	221	181	73.6	227	77.4	119	39.0	238	79.8	268	98.0	154	40.5	139	36.6	208	66.1	196	71.5
330C	369	362	77.8	373	79.0	194	40.4	405	86.0	423	91.7	275	53.5	283	58.0	331	67.1	345	76.8
330D	707	724	85.6	669	80.4	384	46.4	796	97.1	819	100.5	572	67.9	575	69.3	582	68.2	622	76.7
330E	1,425	1,448	92.4	1,386	82.8	797	51.7	1,664	108.4	1,652	107.8	1,159	74.4	1,178	76.3	1,170	74.7	1,191	77.6
		Average	82.3		79.9		44.4		92.8		99.5		59.1		60.1		69.0		75.6

AB13 = Abbott Architect; AM13 = Ortho Vitros Eci; BO5 = Roche Elecsys/E170 modular; CO10 = Siemens Centaur; DC11 = Siemens Immulite 2000/2500, DC7 = Siemens Immulite 1; *n*= Number of participants using the method; PH2 = Wallac Delfia; SF1 = Beckman Access/Dxi; TO1 = Tosoh AIA.

Table 13.5 Oestradiol pools distributed for the ID-GCMS exercise in August 2007 (oestradiol values in pmol/L)

Sample	332B	332C	332D
POOL	E336	E349	E338
ALTM	154	239	539
GCMS	139.1	247.4	591.0

13.7.2.3 ID-GCMS Exercises

Here, routine pools are sent to Professor Thienpont's steroid reference laboratory in Gent, Belgium for value assignment. These are then distributed to participants as part of the routine UK NEQAS service. The resultant data enables the Organiser to assess the validity of the ALTM as a target value and gives objective information to participants and manufacturers about the relationship of routine methods with the reference method.

The example below is for oestradiol, distributed in August 2007 at distribution 332. Table 13.5 indicates the sample and pool identifiers together with the observed ALTMs and ID-GCMS assigned values. Figure 13.14 shows regression and difference plots for all laboratories and various methods. The 25th, 50th, and 75th centiles are plotted. These data reveal quite clearly where the problems of poor specificity and incorrect calibration lie.

13.7.2.4 Interference Studies

Table 13.6 shows an extract of the data for an interference exercise to assess the effect of physiological amounts of DHEAS on testosterone assays (full exercise in Middle, 2007) for a series of female matrix pools. The method, number of users (n), median testosterone results for each sample and the regression parameters: slope, intercept (intcpt) and correlation coefficient (r^2) when these medians were plotted against the amount of DHEAS added to the pool (mmol/L) are indicated. The table also gives DHEAS values for the pools obtained by the service laboratory, showing that the spiking was broadly nominal. For the method data sets, also given are the inter-quartile ranges (25th–75th centiles) of the testosterone results and the paired t-test p-values obtained when the spiked data were compared with the base pool.

The data for all laboratories show the combined effect on testosterone measurement for UK NEQAS participants. The effect was an increase of about 0.03 nmol/L testosterone for every 1 mmol/L of DHEAS. The effect on Abbott Architect was 0.1063 nmol/L testosterone per mmol/L DHEAS. Other methods were affected to a similar extent, notably Roche Elecsys, Roche E170 Modular and Beckman Access/DxI. Minimal effects were observed for Bayer Advia Centaur and DPC Immulite 2000/2500. This exercise revealed effects that were not clear from the manufacturers' information.

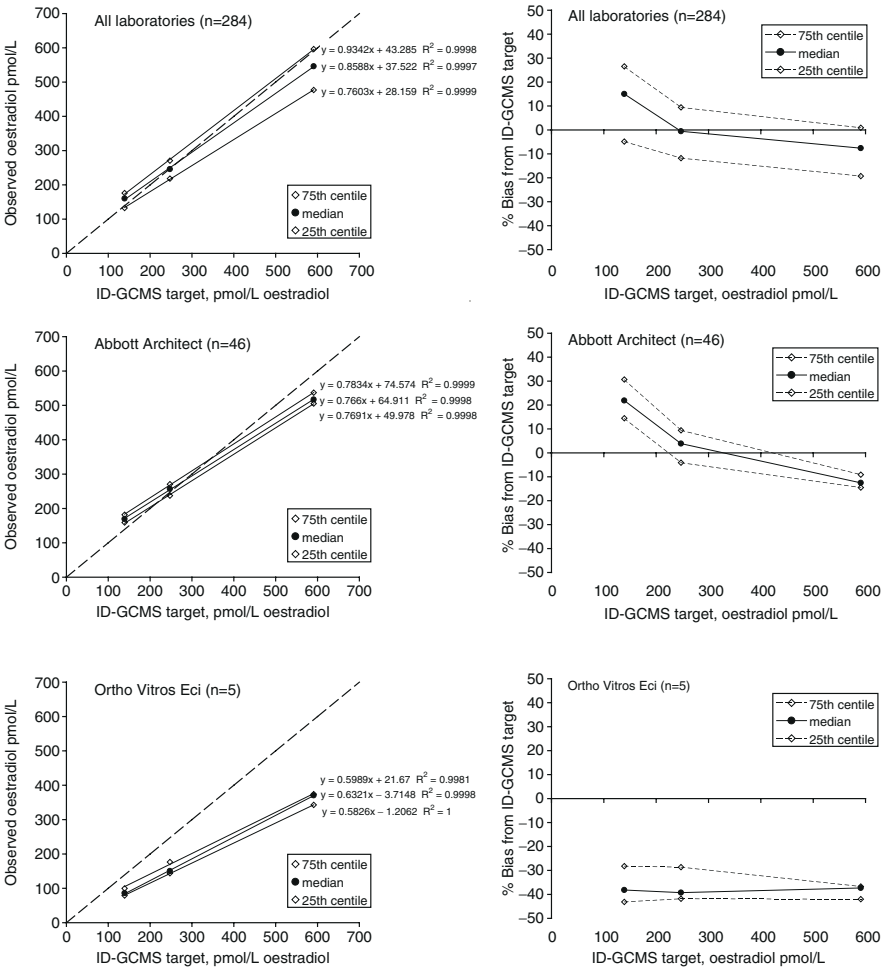

Fig. 13.14 Regression and difference plots for the oestradiol ID-GCMS exercise distributed in August 2007

13.8 Other EQAS Services for Steroid Hormones

13.8.1 UK-Based

UK NEQAS is of course not the only EQA service for steroid hormones. In the UK, the Wales External Quality Assessment Scheme (WEQAS) operated from Cardiff has an immunoassay subsection which includes steroid hormones. The analytes in this service are serum cortisol (150–1,000 nmol/L), serum progesterone (1–40 nmol/L), serum oestradiol (90–1,270 pmol/L) and serum testosterone (1–25

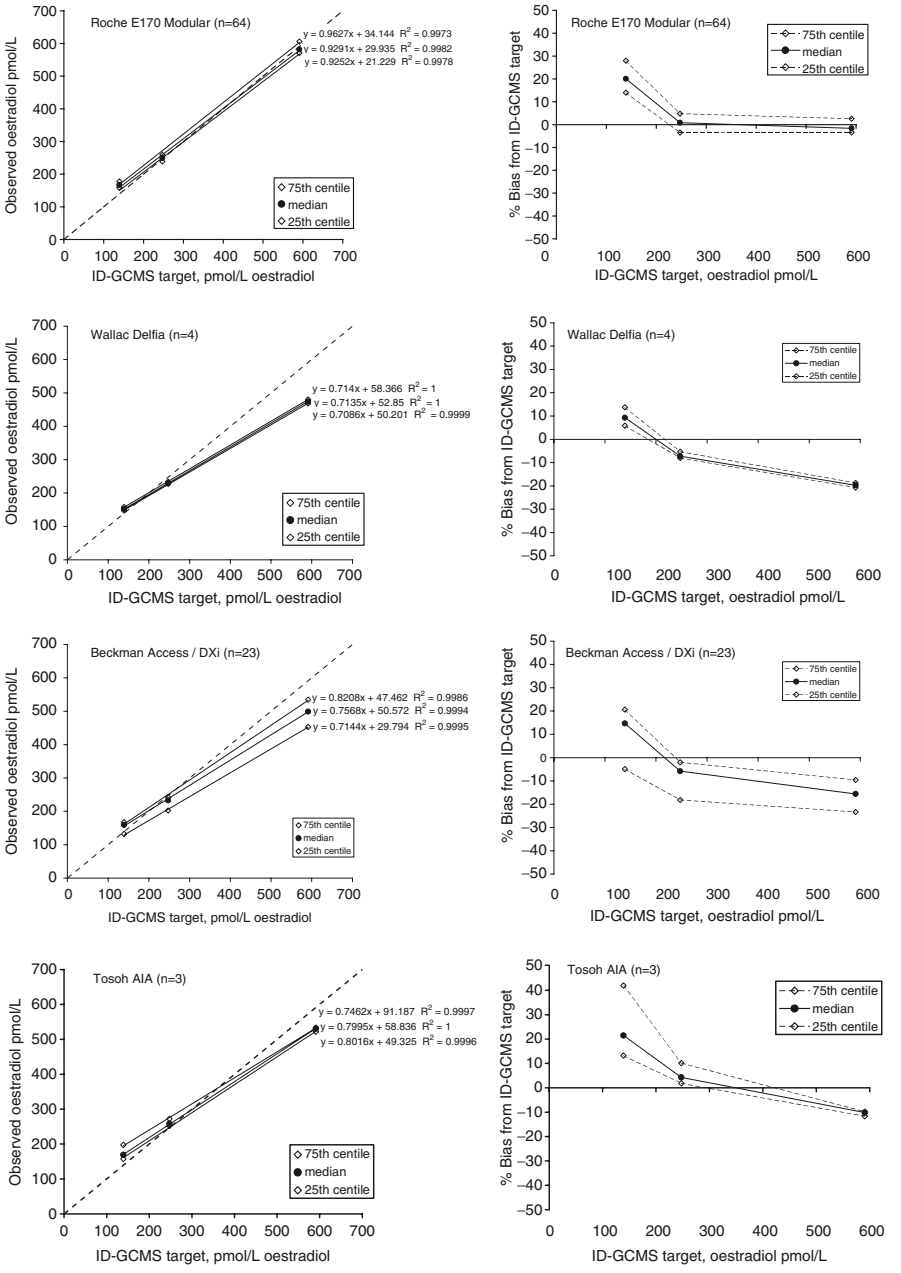

Fig. 13.14 (continued)

Table 13.6 Effect of DHEAS on testosterone assays in the female matrix

Sample identifiers		319A	319B	319C			
DHEAS added (µmol/L)		0.00	10.07	20.14			
Assayed DHEAS (µmol/L)		4.5	13.8	24.8			
	n	Median testosterone (nmol/L) (inter-quartile range) [2-way paired *t*-test *p*-value]			Slope	Intcpt	r^2
All laboratories	230	1.60 (1.40–1.80)	2.00 (1.67–2.50)	2.20 (1.70–3.60)	0.0298	1.63	0.9643
Roche E170 Modular	46	1.50 (1.40–1.70)	2.60 (2.46–2.80) [$p < 0.0001$]	3.80 (3.60–4.10) [$p < 0.0001$]	0.1142	1.48	0.9994
Abbott Architect	18	1.85 (1.77–1.90)	2.96 (2.78–3.13) [$p < 0.0001$]	3.99 (3.80–4.11) [$p < 0.0001$]	0.1063	1.86	0.9994
Roche Elecsys	22	1.40 (1.30–1.50)	2.45 (2.24–2.59) [$p < 0.0001$]	3.50 (3.30–3.69) [$p < 0.0001$]	0.1043	1.40	1.0000
Beckman Access/DxI	18	1.65 (1.49–1.70	2.35 (2.15–2.50) [$p < 0.0001$]	2.99 (2.77–3.10) [$p < 0.0001$]	0.0665	1.66	0.9991
DPC Immulite 2000/2500	18	1.65 (1.45–1.89)	1.66 (1.50–1.99) [NS]	1.80 (1.50–1.98) [NS]	0.0077	1.62	0.8218
Bayer Advia Centaur	80	1.71 (1.60–1.91)	1.79 (1.53–1.95) [NS]	1.80 (1.60–2.03) [$p = 0.013$]	0.0047	1.72	0.8620

nmol/L). The latter is not divided by female and male matrix as in UK NEQAS. WEQAS provides GCMS target values for cortisol, progesterone and testosterone. It is believed that materials are prepared in a similar fashion to those employed by UK NEQAS and are distributed on a monthly basis. Further information on WEQAS may be found at http://www.qualitylab.org/index.html.

EQA for Vitamin D is provided by the Vitamin D EQA Scheme (DEQAS) operated from Charing Cross Hospital in the UK (see http://www.deqas.org/). Samples of liquid serum are provided quarterly; the analytes included are 25 hydroxy Vitamin D and 1,25 dihydroxy Vitamin D. Selected pools have GCMS target values assigned.

13.8.2 Europe-Based

EPTIS (see http://www.eptis.bam.de/) maintains a database of many European PT/EQA services in all sectors. It should be noted that its database is not comprehensive.

The Dutch Foundation for Quality Assessment in Clinical Laboratories (SKML) based in Nijmegen in the Netherlands (see http://www.skml.nl/) is listed in EPTIS as providing steroid hormones in their Special Chemistries service.

Labquality based in Finland (see http://www.labquality.fi/in_english/) provide EQA for steroid hormones in their 2301 Hormones B programme. Androstenedione, aldosterone, cortisol, DHEAS, oestradiol, progesterone, 17-hydroxyprogesterone, SHBG, testosterone and free testosterone are listed.

The Swiss Centre for Quality Control (CSCQ) based in Geneva (see http://www.cscq.ch/f/manuel/programmes/hormonologie.htm) lists serum cortisol, progesterone, oestradiol and testosterone in its Hormone service.

SEKK based in the Czech Republic (see http://www.sekk.cz/index_en.htm) list aldosterone, cortisol, DHEAS, 17-hydroxyprogesterone, oestriol (unconjugated), oestradiol, progesterone and testosterone in its E1-Hormones 1 programme.

In Germany, the DGKL (Deutsch Vereinte Gessellschaft für Klinische Chemie und Laboratoriumsmedizin) has a programme (Hormones Group 1) within its RFB section (Referenzinstitut für Bioanalytik – see http://www.dgkl-rfb.de/index_E.shtml) for steroid hormones. Analytes included are aldosterone, cortisol, DHEAS, oestradiol, oestriol (unconjugated), progesterone, 17-hydroxyprogesterone and testosterone. This service provides GCMS target values for many of its steroid analytes.

13.8.3 North America-Based

The College of American Pathologists (CAP) PT service (see http://www.cap.org/apps/cap.portal?_nfpb=true&_pageLabel=home) lists cortisol in its Ligand Assay (General) program, and 11-deoxycortisol, 17-hydroxyprogesterone, androstenedione, DHEAS, oestradiol, unconjugated oestriol, progesterone, testosterone, testosterone (free), testosterone (boiavailable) and SHBG in its Ligand Assay (Special) service.

13.8.4 South America-Based

ProgBA (Programa Internacional Buenos Aires de Control de Calidad Externo en Análisis Clínicos) based in Argentina (see http://www.cemic.edu.ar/investigacion/inv_unidadesyprog_cirheprogba.asp) has participants throughout South America. Its steroid hormone service includes oestradiol, progesterone, androstenedione, cortisol, testosterone, free testosterone, DHEAS, 17-hydroxyprogesterone and SHBG.

13.8.5 Australia and South Pacific-Based

The Royal College of Pathologists of Australasia (RCPA) Quality Assurance Programmes (see http://www.rcpaqap.com.au/) offers EQA services across all sectors of laboratory medicine. Its Endocrine Programme includes 17-hydroxyprogesterone, aldosterone, androstenedione, cortisol, DHEAS, oestradiol, total oestriol, unconjugated oestriol, progesterone. SHBG and testosterone.

13.8.6 International EQA Schemes

The Randox International Quality Assessment Scheme (RIQAS) (see http://www.randox.com/English/products.cfm?ccs=631&cs=681) provides human-based lyophilised serum and includes cortisol, DHEAS, oestradiol, 17-hydroxyprogesterone, progesterone and testosterone in its monthly immunoassay programme.

13.9 Concluding Remarks

The major analytical quality issues for clinical steroid hormone measurement remain:

- Lack of agreement with the reference method despite the existence of all elements of the traceability chain
- Poor comparability between methods, reflecting poor coordination amongst the diagnostic industry
- Under-recovery of added hormone by many methods
- Poor low-concentration performance where this is clinically significant by some methods
- Poor specificity as revealed by interference studies

EQA schemes must constantly bring these issues to the notice of the professions involved with Quality Assurance, manufacturers and participants, so that producers of instruments and reagents may be encouraged to critically examine assay parameters, rather than optimise against market leaders or those methods that appear to do well in EQAS. The network of steroid reference laboratories which laboratories and manufacturers can rely on to provide accurate and reliable target values for standards, calibrants and control materials must be used.

On a more positive note, what is encouraging is the development and use of routine tandem mass spectrometry methods, which moves us away from the problems of direct immunoassays on large automated instruments. This technology may reveal the true concentrations of clinically important steroid hormones and their metabolites and generate new knowledge that will be of benefit to patients. Figure 13.15 shows the December 2007 performance of the tandem mass spectrometry group for Urinary Free Cortisol (UFC) and testosterone (female matrix). The dotted

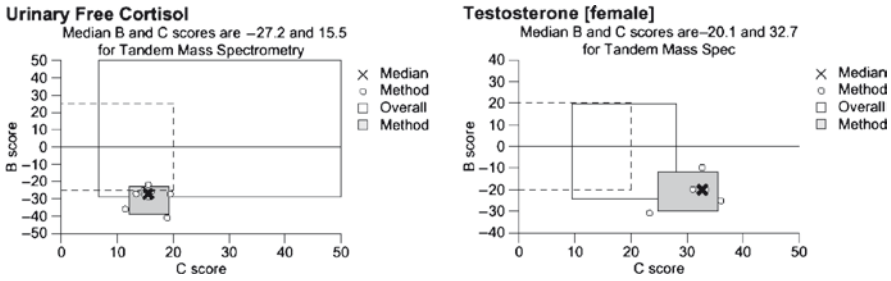

Fig. 13.15 Tandem mass spectrometry group performance for urinary free cortisol and testosterone (female matrix)

lines indicate acceptable performance criteria, the open rectangle is the 5th–95th centile of scores for all laboratories, and the shaded square is the 5th–95th centile of scores for the method group. Each circle is a laboratory in the method group and the cross is the median for the group. For UFC the B score (bias) has a high negative value compared to the ALTM, which is dominated by immunoassay methods, but this reflects the much greater specificity of the method. Recovery of this method is close to quantitative. For testosterone (female matrix) negative, bias is also seen as well as higher variability of the bias (C score). However, the latter is due to the method giving much lower results for endogenous hormone specimens – in line with its greater specificity – but higher results for spiked specimens, reflecting its near quantitative recovery.

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It should be noted that Siemens Healthcare Medical Solutions have now absorbed Bayer Diagnostics and the Diagnostic Products Corporation. Therefore all references to method names such as Bayer Centaur and DPC Immulite should be understood now to mean Siemens Centaur and Siemens Immulite.

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