DRUG MONITORING AND CLINICAL CHEMISTRY

Volume 5

Georg Hempel

Drug Monitoring and Clinical Chemistry

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Drug Monitoring and Clinical Chemistry

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Preface

Drug monitoring has been practised for about 30 years now. Initially, the concept was limited due to methodological problems in analysing low concentrations of drugs in biological fluids. In earlier times, only thin-layer chromatography and spectroscopic methods were available for analysing complex matrices. Now, as can be seen from the first chapters of this book, the methodology has clearly improved and a number of alternative methods are available for most analytical problems. Today, almost all drugs can be separated from complex matrices like human blood or other biological material and determined in very low concentrations. In the future, the availability of mass spectrometric detectors at a reasonable price will enable the analyst to solve more analytical problems in the clinic.

Selection of a suitable method for a given analytical problem is often not only made based on the question which method is scientifically most appropriate. The personal expertise of the analyst also plays an important role because some experience is still required for running chromatographic or electrophoretic methods when analysing drugs in biological fluids. Furthermore, the limited availability of capillary electrophoresis, which is one of the most suitable methods for drug analysis when not too low concentrations are present, means it is not used as often as chromatographic or immunochemical methods.

With most of the analytical problems solved, the question that arises is: are the measured concentrations of the drug in the biological fluid clinically relevant? Clinical relevance of the measured concentrations implies that there is a clear relationship between the concentration and the effect of the drug. If this is the case, the next step is to show that dose individualisation based on drug concentration measurements can standardise the individual's exposure to the drug. Even more important, one has to clarify if there is a clinical endpoint which can be positively influenced by individualising drug therapy.

For the therapeutic areas covered in this book, it has been shown that patients do benefit from therapeutic drug monitoring (TDM), although this is not the case in all therapeutic areas. However, with the limited financial resources in the clinic, even in the developed countries, one has to show that TDM is also cost-effective and the efforts in

the laboratory result in reductions in mortality, severe side-effects that would lead to longer hospitalisation and/or prolonged pharmacotherapy.

Another concept for dose individualisation, which was introduced in the late 1980s, is the area of pharmacogenetics. Pharmacogenetic methods have been shown to be an important tool for identifying patients at risk of experiencing severe toxicity or underdosing. However, there are still concerns if pharmacogenetics is effective from a pharmaco-economic point of view. In the future, methods for genotyping based on chip technology will become cheaper and easier to handle, so pharmacogenetics definitely has the potential to be a routine tool to optimise pharmacotherapy in the clinic. However, even when many more genes responsible for drug metabolism or distribution have been identified, this approach will not completely replace drug monitoring in the future.

Finally, I would like to thank all the contributors to this book for their efforts. Hopefully, the reader will find useful information for setting up or improving TDM in the clinic, for teaching purposes and to become up-to-date on recent developments in this field. I would like to thank Prof. Boos and all other colleagues from the department of Paediatric Oncology (University Children's Hospital Manchester) for the excellent collaboration and want to dedicate the book to my teacher Gottfried Blaschke, a great analyst, with many thanks for his support over the years.

Georg Hempel

Contents

G. Hempel (Ed.), *Drug Monitoring and Clinical Chemistry*
Handbook of Analytical Separations, Vol. 5

CHAPTER 1

Sample preparation for the analysis of drugs in biological fluids

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1.1 INTRODUCTION

Sample preparation is one of the essential processes for the analysis of various drugs in biological sample matrices [1–5]. Prior to the determination of the drugs in these complex mixtures, the elimination of undesirable interferences and the preconcentration of the analytes of interest is necessary to ensure precise identification and reproducible quantitation. A successful combination of the sample preparation techniques and the subsequent separation/determination methods is also an important topic that must be taken into account in order to select the most effective analytical procedure for a particular sample. An additional important requirement for the sample preparation method in drug analysis, especially in emergency clinical situations, is the processing time of the total analysis.

Liquid-liquid extraction (LLE) has been traditionally used as the sample preparation method for biological fluids [6–11]; however, a large amount of organic solvent is needed for the LLE technique. It also requires time-consuming manual procedures and has several other problems such as interferences from the sample matrix and emulsion formation [1]. Centrifugation of the sample matrix, typically with an addition of small amount of organic solvents and an appropriate buffer solution into the sample, has been also employed for protein removal before the preconcentration process. Due to the simplicity of the precipitation method, it is still used if the relevant compounds are not removed in the process. Solid-phase extraction (SPE) is an alternative sample preparation method for the analysis of various complex sample matrices and many applications have been published, including the combination with the subsequent separation systems [12–15], although the direct injection techniques for these complex mixtures have been also reported in several cases [16–21]. With the SPE technique the analyst has several advantages, e.g. cleaner samples, rapid and automated processing

with a good reproducibility as well as no emulsion formation and reduced solvent disposal [14,15].

Miniaturization in sample preparation has been also proposed. Solid-phase microextraction (SPME) was first developed for the analysis of volatile and semi-volatile analytes in a complex sample matrix [22–24], and recently the method was further developed for the coupling with liquid phase separations [4,24]. In the new method, in-tube SPME [25–27], a section of gas chromatographic (GC) capillary column is introduced as the extraction medium and the extraction is carried out by passing the sample matrix through the extraction capillary [25–33]. With regard to miniaturized sample preparation techniques, several other specially-designed extraction media have been reported such as fiber-packed capillary [34–42], porous hollow fiber [43–47] and polymeric resin disk [14,48].

In this chapter, various sample preparation methods for the analysis of drugs in biological fluids will be reviewed along with brief descriptions for the combination of the sample preparation techniques with the subsequent separation methods such as liquid chromatography (LC) and capillary electrophoresis (CE). The chromatographic separation method for drug analysis will be described in the following chapter.

1.2 CONVENTIONAL SAMPLE PREPARATION FOR BIOLOGICAL SAMPLES

1.2.1 Methods of protein removal

Deproteinization of the biological sample has been typically carried out by the addition of an organic solvent and/or an appropriate buffer solution [6,49–54], and could therefore be simultaneously conducted with the LLE process of the analytes of interest [53]. Because of the relatively easy operation, many kinds of centrifuge tubes with a membrane filter [55,56] have been developed and commercialized for proteins and other insoluble components in the sample matrix. The volume of the sample required for these centrifuge processes is less than about 1.0 mL, indicating its suitability when there is a limited amount of sample.

For the preconcentration of drugs by SPE, the protein removal process is sometimes needed before the application to the SPE cartridge to avoid clogging [1], although a good recovery of the analytes using a sonication followed by a dilution procedure in phosphate buffer (0.1 M; pH 6.0) has been reported for the analysis of whole blood samples [57]. Several other methods for protein removal before the SPE preconcentration step have been published [52].

With specially-designed stationary phases, direct chromatographic separation of the biological sample matrix has been accomplished, in which the deproteinization is simultaneously carried out during the separation of drugs in a single column [17–21]. The introduction of column switching techniques, where two or more columns are connected typically with the switching valve(s), allowed a wider applicability to be obtained [58–62], because different types of packing materials could be used for the pretreatment column(s) and the subsequent separation column.

1.2.2 Liquid-liquid extraction (LLE)

LLE is the traditional method for the extraction of drugs from biological matrices [10,15]. The extraction mechanism is based on the partition of the analytes between organic and aqueous phases, and the pH of the aqueous phase is normally adjusted so that the drug molecules are neutralized for effective extraction to the organic phase. Therefore, a suitable combination of non-polar organic solvent(s) and an aqueous sample matrix having favorable pH should be prepared, as well as a good volume ratio of these phases [63]. For the selection of a suitable organic solvent, a review article has been published for the determination of benzodiazepines in biological sample matrices [64]. One of the major advantages is the systematic selective extraction that enables the isolation of various drugs having different polarities [63]. As shown in Fig. 1.1, the feature makes the method suitable for the analysis of complex sample mixtures containing more than two drugs. With acidification, acidic (and neutral) drugs are extracted from the aqueous sample matrix to the organic (solvent) phase, while basic (and amphoteric) drugs are extracted with alkalinization of the aqueous phase [65–72].

1.2.3 Solid-phase extraction (SPE)

Because the SPE method has several advantageous features over the LLE technique such as: (1) faster and more effective pretreatment; (2) significantly reduced solvent consumption; and (3) easy operation without complicated manual processing, a large number of applications have been reported, showing it to be one of the most effective and convenient sample preparation techniques for complex sample matrices [12–16]. Fig. 1.2 shows a typical SPE cartridge, in which an extraction medium (solid phase) is

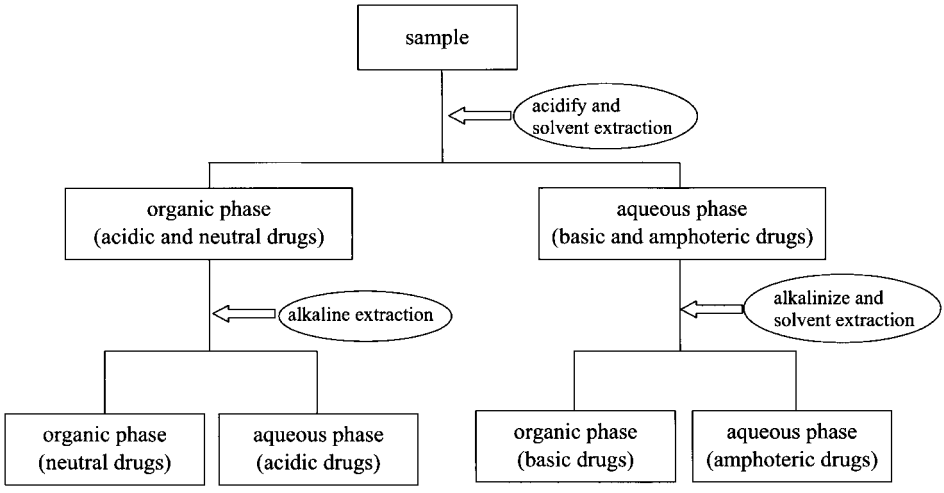

Fig. 1.1. Drug screening process by multiple LLE.

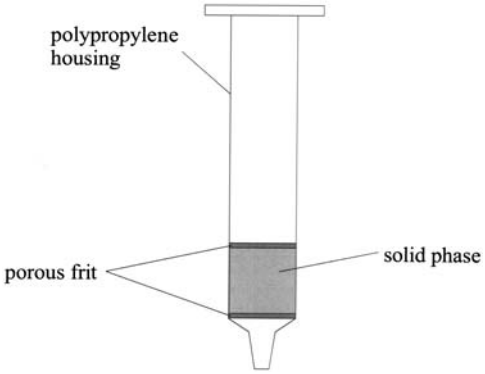

Fig. 1.2. Illustration of SPE cartridge most commonly used.

packed into a syringe-shaped housing made by polymeric materials such as polypropylene (PE). The volume of the sample that can be applied to these SPE cartridge ranges typically from 1 to 20 mL, where the loaded solid phase in these cartridges are from 0.1 to 5.0 g, respectively. To support the particles of the solid phase, a membrane frit with typical pore size of about 20 μm is placed at the top and bottom of the phase. Several kinds of membrane materials are available such as PE, polypropylene (PP) and polytetrafluoroethylene (PTFE). The particle size of the solid phase varies, but the average size is around 40–50 μm in diameter [14].

Since the solid phase sorbents have been developed based on the technology for stationary phase synthesis in LC, the theoretical principles for selecting the solid phase can be considered to be similar to stationary phase selection [12–14,73–75]. The scheme for the systematic selection of an appropriate extraction material and the conditions [73] are illustrated in Fig. 1.3. For most of the applications in drug analyses, however, hydrophobic extraction materials have been employed. This is primarily due to the relatively hydrophobic nature of the drug molecules in a biological sample matrix with a more hydrophilic nature.

Among the varieties of the solid phases that have been developed, octadecyl (C_{18}) bonded silica has been most commonly used for the extraction of single drug and/or single group of drugs having a similar structure, i.e. hydrophobicity [63]. For their successful extraction, the pH of the sample solution is quite important. With the widely used dilution by 0.1 M phosphate buffer (pH 6.0), at this pH the weakly basic (such as diazepam), the neutral and the weakly acidic drugs (such as barbiturates), present in the non-ionized form, are retained by the hydrophobic extraction material [1,14]. In order to avoid protein coagulation at lower pH values, an addition of 0.1 M phosphoric acid to blood samples was proposed [1]. The hydrophobic drugs extracted by the C_{18} bonded silica will be then separated in reversed-phase LC with an appropriate solvent polarity.

Mixed-mode bonded silica [1,57], where silanol groups are partially derivatized with alkyl chains having a medium length (or other ligands such as phenyl) and with cation exchange functionalities, can interact with at least two types of different mechanisms. Therefore, a selective extraction and elution of a certain class of drugs is possible.

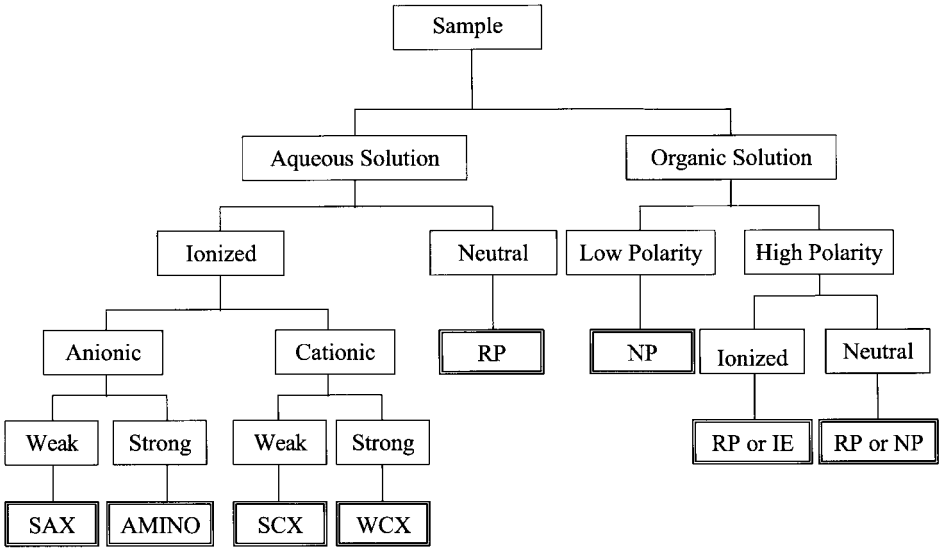

Fig. 1.3. Systematic selection of SPE conditions [73]. SAX, strong anion exchanger; SCX, strong cation exchanger; WCX, weak cation exchanger; RP, reversed-phase conditions; NP, normal-phase sampling conditions; IE, ion-exchange sampling conditions.
(Reproduced from Fig. 1 (appeared in page 20) in J. Chromatogr. A, 885 (2000) 17–39 by C.F. Poole et al.)

Franke and de Zeeuw et al. have demonstrated a drug screening process using mixed-mode bonded silica as the solid phase [1,57,76–79]. They introduced a commercially available mixed-mode SPE cartridge for systematic drug screening in biological fluids as illustrated in Fig. 1.4.

In their method, the SPE cartridge, having both hydrophobic and cation exchange functionalities, is preconditioned with phosphate buffer solution (pH 6.0) and methanol. During the extraction process, weakly acidic and neutral compounds are retained by the hydrophobic functional groups, while basic (protonated) compounds are also retained by the cation exchange groups. After the sequential washing processes with deionized water and 0.01 M acetic acid, and the drying procedures, the two-step elution processes are carried out. Acidic and neutral drugs are eluted in fraction A, and then basic drugs and other remaining neutral drugs are eluted in fraction B. Good recoveries of 80–100% have been reported along with acceptable RSDs of less than 10% [76]. The application of the method for drug screening in plasma [77,78], whole blood [77] and urine [78] has been shown. For further information on this technique, see the review article by the authors [1].

Polymer-based SPE media have been also developed and commercialized. In contrast to the silica-based material, polymeric materials can be used under virtually any pH conditions. Compared to the silica-based materials, the polymeric particles have higher specific surface area and no silanol groups therein. The extracted analytes will be completely and easily desorbed during the elution by a relatively small volume of the desorption solvent. Copolymer of styrene-divinylbenzene (PS-DVB or SDB) has been

widely employed with a typical particle size of 20–160 μm, although several materials can be obtained only with a diameter of 250–400 μm, which must be subjected to further grinding for an efficient extraction [14]. Using the PS-DVB resin, a wide range of drugs can be isolated from typical biological matrices, e.g. blood, serum, gastric content and tissues. The recommended SPE process with the polymer-based material can be found in [80], where 16 drugs in urine samples have been analyzed by GC with SPE sample preparation. Modification of the polymeric sorbents for SPE has been also proposed by Sun and Fritz et al. [81,82].

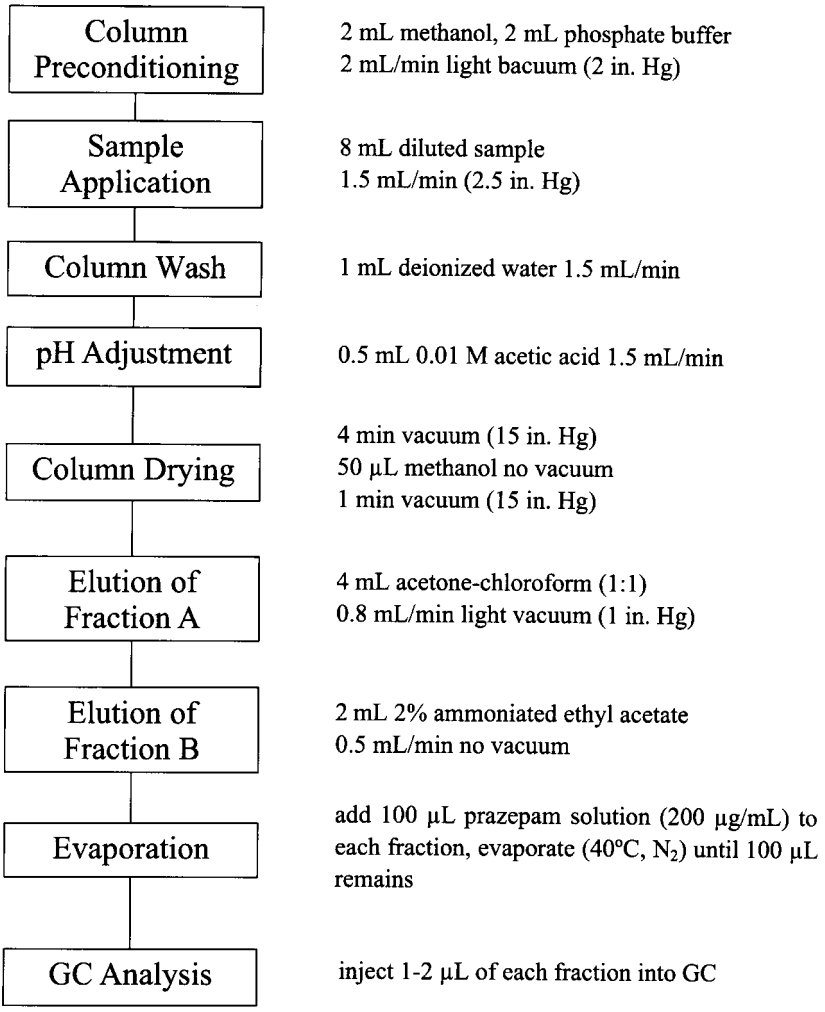

Fig. 1.4. SPE scheme for drug screening in typical biological sample matrices [76].
(Reproduced from Fig. 1 (appeared in page 73) in J. Chromatogr. B, 689 (1997) 71–79 by R.A. de Zeeuw.)

SPE sample preparation methods for particular classes of drugs such as benzodiazepines [10,52,57,63,77,83–86], tricyclic antidepressants (TCAs) [57,80,84], barbiturates [52,57,80, 84,85] and other drugs commonly encountered in drug abuse [10,52,54,57,80,84–88] have been published.

1.3 MICROEXTRACTION METHODS FOR BIOLOGICAL ANALYSES

1.3.1 Solid-phase microextraction (SPME)

SPME has been widely accepted for the sample preparation of complex sample matrices, such as biological and environmental mixtures. As the extraction device, a piece of fused-silica fiber coated with a polymeric material is employed. Fig. 1.5 compares the devices for three extraction techniques, where the extraction cartridges for the SPE and membrane extraction methods [14,89–92] are also illustrated. For the extraction the SPME fiber is immersed into the sample solution or inserted into the head space of the vials containing the sample solution. Several polymers typically used as GC stationary phases, such as polydimethylsiloxane (PDMS), have been introduced as the coating materials [24,27], because SPME was first developed by Pawliszyn et al. for the analysis of volatile/semi-volatile analytes that can be thermally desorbed in a GC injection device [22–24]. In contrast to the good compatibility with the GC system, however, a specially designed desorption device is needed for the analysis of non-volatile and/or thermally labile compounds in order to accomplish the effective coupling

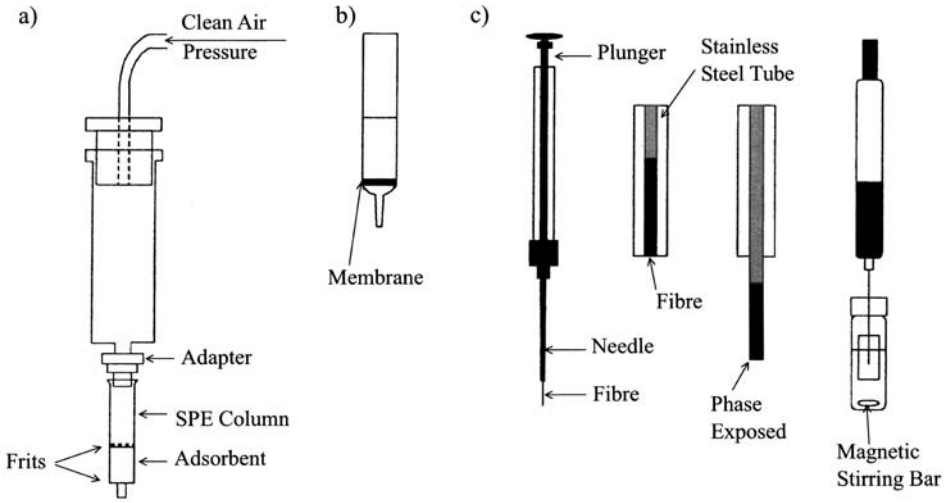

Fig. 1.5. Comparison of SPE and other commercialized microscale sample preparation methods [74]. (a) SPE; (b) membrane extraction; and (c) SPME.
(Reproduced from Fig. 1 (appeared in page 53) in J. Chromatogr. A, 885 (2000) 51–72 by C.W. Huck et al.)

of the SPME with liquid-phase separation systems, such as LC [93–103], supercritical fluid chromatography (SFC) [104,105] and electrodriven separation methods [106–108]. For the applications of SPME preparation in drug analysis, the recent trends have been reviewed by Snow *et al.* including the citations of typical reports for various class of drugs [4,109], and a book summarizing the wide range of real applications has been published [24,27].

1.3.2 In-tube solid-phase microextraction (In-tube SPME)

For an effective on-line coupling of the sample preparation and LC separation system, and the automation of the hyphenated system, in-tube SPME has been proposed by Pawliszyn et al., where a section of open-tubular GC capillary column was employed as the extraction device [25–31]. An overview of the typical in-tube SPME/LC/MS system is illustrated in Fig. 1.6 [32]. During the extraction, the sample solution is repeatedly drawn and ejected by the metering pump *via* the extraction capillary until the equilibrium between the sample matrix and the stationary phase coating is established. Then, the extracted analytes are desorbed by the flow of the desorption solvent or the mobile phase solvent. With the desorption by the mobile phase, the desorption and injection of the preconcentrated analytes can be processed simultaneously. For the typical applications, an extraction capillary of 0.25 mm i.d. $\times$ 50 cm with the optimal draw/eject flowrate of 50–100 μL/min was used. Several classes of drugs in biological

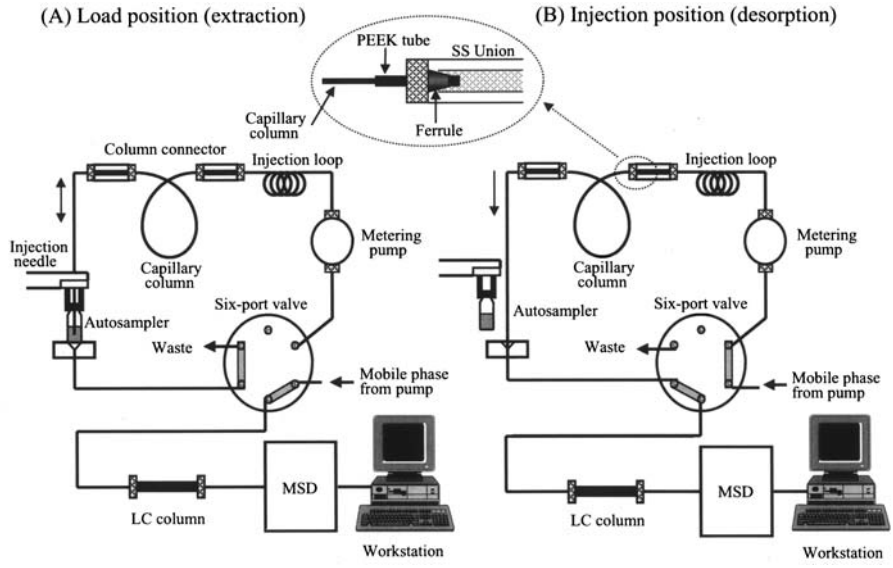

Fig. 1.6. In-tube SPME-LC-MS system [32]. (A) Load position (extraction); and (B) injection position (desorption). (Reproduced from Fig. 4 (appeared in page 41) in J. Chromatogr. A, 880 (2000) 35–62 by H. Kataoka et al.)

sample, such as benzodiazepines in urine and serum [110], β-blockers in urine and serum [29,31,111], and amphetamine and methamphetamine in urine [30], have been reported along with a review of drug analysis with in-tube SPME [33].

1.3.3 Other novel miniaturized extraction techniques

For the effective on-line coupling of microscale sample preparation and microcolumn separations, fibrous synthetic polymers have been proposed by Jinno et al. [34–42]. In the newly developed microextraction method, fiber-in-tube solid-phase extraction (FIT-SPE), the extraction is processed in a short capillary, which is packed longitudinally with several hundred polymer filaments as the extraction medium. Because of the parallel arrangement of the filaments to the outer tubing (extraction tube), a large number of coaxial narrow channels can be formed in the extraction capillary as shown in Fig. 1.7, where in-tube capillary [25] and wire-in-tube capillary [112] are also illustrated [113].

Compared with the conventional in-tube capillary, i.e. a section of open-tubular GC column, the wire-in-tube capillary has a considerably reduced internal volume by inserting a stainless steel wire therein; this allowed an improved extraction power for trace analysis [36]. The fiber-in-tube method, however, allows further reduction of the internal void volume to be accomplished, while the capillary shows a reduced pressure drop during the extraction and desorption compared with the conventional particle-

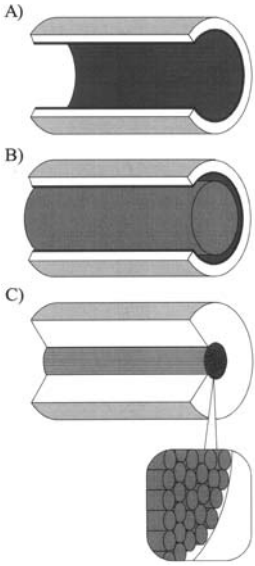

Fig. 1.7. Illustrations of three types of microextraction capillaries. (A) In-tube [25,26], (B) wire-in-tube [112] and (C) fiber-in-tube [34,35]. (Reproduced from Fig. 4 (appeared in page 59) in J. Chromatogr. A. 1000 (2003) 53–67 by Y. Saito and K. Jinno.

packed SPE cartridge. Furthermore, any undesirable plugging from insoluble and/or particulate materials in real sample matrices can be significantly reduced in the FIT format. Recent studies also demonstrated that the effective interaction of the sample solution with a number of the fine fibrous extraction media in the extraction capillary could enable further miniaturization to a microscale sample preconcentration device [35–38]. Polymer-coated fibrous material [40] has been also introduced as the extraction medium for the on-line sample preparation of TCA drugs [41].

Rasmussen et al. have developed the so-called liquid-phase microextraction (LPME) technique, in which a porous polypropylene hollow fiber was employed as an extraction device [43–47]. LPME was performed in a conventional vial (4 mL) containing a small piece of polypropylene hollow fiber (600 μm i.d. × 8 cm) with a wall thickness of 200 μm and a pore size of 0.2 μm. The analytes of interest are extracted from the sample into an organic solvent, which is immiscible with water and immobilized in the pores of the hollow fiber, and then transferred into an aqueous solution (acceptor) filled inside the hollow fiber. For typical applications with subsequent electrophoretic separation, the sample volume and the acceptor volume were 25 μL and 1 to 4 mL, respectively. Then, a higher phase ratio than with conventional SPE can be accomplished. A good compatibility with other chromatographic separations, such as GC and LC systems, was also reported for the analysis of several classes of drugs in biological matrix as well as the coupling with CE separation system [45].

Another microscale sample preparation method was proposed by Tomlinson et al. [114–116]. This research group developed the so-called "preconcentration-CE" (PC-CE) method as an on-line coupled sample preconcentration process to CE separations. The concept is similar to that of conventional SPE, and the preconcentration was carried out in a small section of packed tubing, typically a few millimeters length of C_{18} particle-packed section in a PTFE tubing (400 μm i.d.), which was directly connected to the CE separation capillary. They have further developed another miniaturized sample preparation technique, specially designed for on-line coupling to CE separation [117,118]. The method can be regarded as a kind of membrane extraction [14,48], and a piece of a commercially available polymeric membrane PS-DVB was employed as the adsorptive membrane [119–125].

1.4 FUTURE PROSPECTS IN BIOLOGICAL SAMPLE PREPARATION METHODS

Various sample preparation methods have been reviewed, especially for drug analysis in biological sample matrices, including LLE, SPE and some other miniaturized extraction techniques, such as SPME. The general shift from the traditional LLE technique to the miniaturized methods, via the innovations in SPE, will be accelerated significantly based on the recent requirements for analytical procedures such as high-speed analysis with high efficiency but without a large solvent consumption, and highly selective analysis for a particular class of compounds. Miniaturization of the sample preparation process and the instrumentation is one of the most promising solutions for these

requirements, and the more effective and rapid drug analysis systems will be developed and commercialized in the next few decades.

The automation of the miniaturized system will also be required for the reproducible and precise identification and quantification of the particular drug molecule from the huge number of candidates in the library.

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CHAPTER 2

Chromatographic methods for the analysis of drugs in biological fluids

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2.1 INTRODUCTION

Analysis of drugs in biological fluids is very important and can give a wealth of information relating to pharmacokinetics, pharmacodynamics, pharmacology, toxicology and for therapeutic drug monitoring (TDM) purposes. To date, a wide variety of analytical methods has been developed and adapted for determining drugs in biological fluids, i.e. blood, urine, milk, etc.

This chapter focuses on the utilization of high-performance liquid chromatography (HPLC) and gas-chromatography (GC) and their hyphenated methods with mass spectrometry (MS), i.e. LC-MS and GC-MS, for the analysis of drugs in biological fluids. The more recent results on achiral drug analyses are selected and described with emphasis on the useful and practical methods. Recently, the hyphenated chromatographic methods for biomaterials were reviewed [1].

This chapter compromises two parts. Part I discusses the analysis of drugs in biological fluids, summarized in Tables 2.1-1 to 2.1-5 while Part II describes chromatographic method of analysis of chiral drugs in biological fluids with some examples summarized in Tables 2.2–1 to 2.2–9.

Among the separation techniques employed for drug analysis in recent years, HPLC has proven to be the most popular because of its excellent capability for the determination of aqueous samples. Drugs as well as endogenous components in biological fluids are commonly non-volatile polar compounds, and thus HPLC is more suitable than GC for their separation. For analyte detection with HPLC, a wide array of detection techniques with comprehensive and excellent features can be connected to it on-line, i.e. ultra violet (UV), fluorescence (FL), chemiluminescence (CL), electrochemical (EC), mass spectrometric (MS) detectors, etc.

References pp. 69–75

The combination of MS with a proper means of separation is ideal for the analysis of complex biological samples. To be detectable by MS, analytes should be introduced in the gaseous phase. GC is suitable for volatile compounds; however, non-volatile compounds can be applied to GC after derivatization to increase their volatility. MS was first interfaced to GC because analytes eluting from the column or capillary are in the gaseous phase and further volatilization is not required. Therefore, hyphenating GC with MS has been widely used for the analysis of various kinds of substances, including drugs. On the other hand, HPLC hyphenated with MS has become more popular in the last decade because of its intrinsic selectivity and sensitivity when dealing with aqueous samples. In addition, it has become apparent that a tandem LC with MS/MS detection system is the best technique for trace amounts of substances in biological fluids.

Other separation techniques such as capillary electrochromatography (CEC) are powerful and attractive, but the number of recent reports describing their utilization in drug analyses in biological fluids is rather few. The main reason for the limited applications of CEC in the biomedical field is that the method lacks sufficient sensitivity. Regarding separation, in general CEC has no advantages over HPLC or capillary electrophoresis.

PART I. CHROMATOGRAPHIC METHODS OF ANALYSIS OF DRUGS IN BIOLOGICAL FLUIDS

2.2 HPLC

In addition to being routinely employed in practical TDM analysis of drugs, HPLC is also utilized in clinical, pharmacological, toxicological and forensic studies. For the successful detection of the target analytes, their physicochemical properties such as UV-absorbance, fluorescence (FL), redox potential, etc. are evaluated and exploited. If compounds do not have any of these properties, a derivatization reaction is recommended in order to introduce such a characteristic in the molecule. Provided that the target compound has a reactive moiety, a UV- or FL-labeling reaction is commonly utilized, and this is achieved via the selective reaction between the functional groups of the analyte and that of the labeling reagent. Derivatization serves to improve the sensitivity, selectivity and the chromatographic behavior. A chemical conversion via a photochemical reaction to produce a strongly UV-absorbing or fluorescing derivative from non-UV absorbing or non-FL molecules is also advantageous and indispensable for the selective analysis of drugs.

2.2.1 UV detection

An HPLC-UV method is simple and practical for detecting a substance which has a chromophore with absorption bands in the UV or visible (Vis) region; fortunately, many drugs fulfill this criterion, somewhere between 200–800 nm. The selection of the monitoring wavelength is based on the absorptivity of the analyte in question; the maximum wavelength is often chosen. At shorter wavelengths in particular, the selected

TABLE 2.1-1
HPLC-UV DETECTION

Drug	Category	Method	Sample	Determination Range	Simultaneously Determined Compound	Reference
isoniazid	tuberculosis	HPLC-UV, 275 nm	plasma	0.5–8 μg/ml regression slope = 1.03	acetylisoniazid	11
quinapril	ACE inhibitor anti-hypertension	HPLC-UV, 215 nm	plasma	10–500 ng/ml	quinaprilat	24
metaformin	antihyperglycemia, type-2 diabetes	HPLC-UV, 236 nm	plasma milk (breast)	20–4000 μg/ml r2 > 0.99	buformin	8
omeprazole	H + /K + − ATPase inhibitor	HPLC-UV, 302 nm semimicro-column	plasma	10–2000 ng/ml r > 0.999	omeprazole sulfone	38
ranitidine	histamine H2-receptor agonist	HPLC-UV, 320 nm	plasma	20–1000 ng/ml r > 0.999		12
fluconazole	antifungal drug	HPLC-UV, 210 nm	plasma	0.195–100 ng/ml r > 0.9999		32
zidovudine	HIV	HPLC-UV, 265 nm ion-pair	plasma	57.6–2880 ng/ml r > 0.999	lamivudine nevirapine	29
amphotericin B	antifungal, antibiotic	HPLC-UV, 405 nm	plasma	0.5–5.0 μg/ml 0.005–0.5μg/ml r > 0.999	liposomal amphotericin B amphotericin B colloidal dispersion	26
praziquantal	trematodes, cestodes schistosomiasis	HPLC-UV, 217 nm	plasma	100–2000 ng/ml r = 0.999		13
clobazam	anticonvulsant epilepsy	HPLC-UV, 235 nm	plasma	0–500 ng/ml r = 0.9995		27
linezolid	antibacterial agent	HPLC-UV, 254 nm	serum	0–30 mg/l r = 0.999		
carbamazepine	epileptic seizures trigeminal neuralgia psychiatric disorder	HPLC-UV, 237 nm	plasma	0.5–15.0 μg/ml r = 0.9990	10,11-dihydro-10,11-dihydroxy-carbamazepine,10,11-dihydro-10,11-epioxycarbamazepin, etc.	28
ketorolac	NSAID, analgesic efficiacy	HPLC-UV, 313 nm	plasma	0.05–10.0 μg/ml r2 = 0.998		25
loratadine	antihistamine, urticaria, allergic rhinitis	HPLC-UV, 200 nm	plasma	0.5–50 ng/ml r > 0.999		18

TABLE 2.1-1
CONTINUED

Drug	Category	Method	Sample	Determination Range	Simultaneously Determined Compound	Reference
risperidone	antipsychotics, symptoms of schizophrenia	HPLC-UV, 278 nm	plasma	5–100 ng/ml r = 0.998	9-hydroxy-risperidone	14
erthromycin	antibiotics, bacterial infections	HPLC-UV, 236 nm HPLC-ED	plasma, tissues (animals) milk (cow)	0.25–20 μg/ml (plasma) 0.125–10 μg/ml (fat, liver, muscle, kidney) 0.025–2.0 μg.ml (milk) r > 0.98		15
carboplatin	antineoplastic	HPLC-UV, 230 nm HPLC-UV, 290 nm (post-column)	plasma	0.05–40 mg/ml r2 > 0.999		5
omeprazole	gastric ulcer	HPLC-UV, 302 nm	plasma	5–500 ng/ml r = 0.993		20
cefepine	empirical antibiotic,	HPLC-UV, 263 nm	serum	0.5–200 μg/ml	cefpirome	31
rifampicin	tuberculosis	HPLC-UV, 254 nm	urine, plasma	2–20 μg/ml r > 0.999	desacetylrifampicin, rifapentine	6
indinavir	HIV, protese inhibitor	HPLC-UV, 210 nm	plasma	25–2500 ng/ml r2 > 0.99		23
ampicillin	chemotherapy	HPLC-UV, 220 nm	serum	0.19–9.41 μg/ml r = 0.999		50
metformin	diabetes NIDM	HPLC-UV, 232 nm	plasma	0.1–40 mg/ml r = 1.000		30
paclitaxel	cancer, malignances	HPLC-UV, 230 nm	plasma	10–500 ng/ml r = 0.9998		16
vancomycin	antibiotic, gram-positive bacteria	HPLC-UV, 282 nm FPIA (flourescence polarization immuno-assay	plasma, tissues sternum	0.5–75 mg/ml r = 0.9966		22

TABLE 2.1-1
CONTINUED

Drug	Category	Method	Sample	Determination Range	Simultaneously Determined Compound	Reference
carbamazepine	antiepileptic, tonic-colonic seizure	HPLC-UV, 210 nm	plasma	0–12 mg/ml r = 0.9998	carbamazepine-10,11-epoxide	10
cisplatin	anticancer, solid tumors	HPLC-UV, 210 nm	plasma	60–600 nM r > 0.994	monohydrated cisplatin	7
gentamicins C1	antimicrobial, infections	HPLC-UV, 365 pre-column	plasma, urine	0–50 mg/l r2 = 0.999	gentamicin C1a gentamicin C2	3
viriconazole	antifungal	HPLC-UV automated, direct injection column-switching	plasma	10–3000 ng/ml r = 0.9985	UK-115794(II) (IS)	33
meropenem	antibiotic, respiratory infection	HPLC-UV, 296 nm direct injection column swithcing	serum, bronchial secretions	0.5–40 μg/ml r2 = 0.9993	ceftazidime	34
thiacetazone	tuberculosis, ribonucreotide reductase inactivator	HPLC-UV, 322 nm direct injection ISRP column	plasma, (human, rat)	0.17–2.8 μg/ml r2 = 0.9998		35
propafenone	supraventricular and ventricular arrhytmias	HPLC-UV, 246 direct inhection column switching	serum	0.025–4 μg/ml r = 0.998	5-hydroxypropafenone, LU46532 (IS)	37
propentofyline	glutamate release inhibitor	HPLC-UV, 270 nm direct injection solumn switching	serum (human, rat)	1–100 nmol/ml r = 1.000 (humna) r = 0.999 (rat)	A720287, A802751, A802831	39
celecoxib	COX-2 inhibitor, NSAID, inflammation, pain	HPLC-UV, 260 nm normal-phase column column switching	plasma	25–2000 ng/ml	analogue of celecoxib (IS)	21

TABLE 2.1-1
CONTINUED

Drug	Category	Method	Sample	Determination Range	Simultaneously Determined Compound	Reference
aceclofenac	NSAID, analgesic	HPLC-UV, 278 nm direct injection column switching	plasma	50–10000 ng/ml r2 > 0.999	diclofenac	41
amoxicillin	antibiotic	HPLC-UV, 234 nm direct injection column switching	serum	0.35–21.0 μg/ml r > 0.99	amoxicilloic acid	45

References pp. 69–75

TABLE 2.1-2
HPLC-FL

Drug	Category	Method	Sample	Determination Range	Simultaneously Determined Compound	Reference
ranitidine	gastric acid secretion inhibitor	HPLC-FL post-column o-OPA, 2-ME Ex = 350, Em = 450 nm	plasma urine	0.1–4 μg/ml r = 0.9999	ranitidine S-oxide ranitidine N-oxide	74
fluoxetine	antidepressant, serotonin reuptake inhibitor	HPLC-FL Ex = 230, Em = 290 nm	plasma	8–200 ng/ml r = 0.9995	norfluoxetine	51
ciprofloxacin	antibacterial agent, treatment for gram-negative pathogens	HPLC-FL direct and column-switching Ex = 278, Em = 418 nm	plasma Mueller-Hinton broth	0.07–1.25 μg/ml r = 1.0000 (plasma) r = 0.9999 (M.-H.B.)		52
methotrexate	anticancer, leukemia, osteosarcoma, etc	HPLC-FL automated precolumn, column-switching Ex = 367, Em = 463 nm	plasma	50–500 ng/ml r = 0.999	pteridine carboxalaldehyde, pteridine carboxylic acid	68
morphine	opiate	HPLC = FL direct injection, column-switching on-column derivatization	plasma	10–100 ng/ml r = 0.9996		73
ganciclovir	herpesvirous replication inhibito	HPLC-FL column-switching Ex = 278, Em = 380 nm	plasma serum	0.4–4.0 μg/ml r2 > 0.997		54
SSRIs	antidepressant, selective serotonin reuptake inhibitor	GC-NPD HPLC-FL pre-column Ex = 340, Em = 520	serum	GC 20–400μg/l r2 > 0.998 HPLC 5–1000 μg/l r2 > 0.999	fluoxetine Norfluoxetine fluvoxamine citalopam desmethylcitalopam sertraline	67

TABLE 2.1-2
CONTINUED

Drug	Category	Method	Sample	Determination Range	Simultaneously Determined Compound	Reference
					desmethylsertraline venlafaxine milnacipram paroxetine	
mirtazapine	antidepressant	HPLC-FL Ex = 290, Em = 350 nm	plasma	0.5–100 ng/ml r > 0.9900	demethylmirtazapine	55
gabapentin	antiepileptic, partial onset seizure	HPLC-FL Ex = 320, Em = 450 nm	serum	60–15 μg/ml r = 0.996–0.999		56
atenolol	anti-hypertension action on β1-receptor	HPLC-FL Ex = 222, Em = 300	plasma	10–1000 ng/ml r = 0.99500–0.99968	chlorthalidone(UV) salbutamol	44
MDMA 3,4-methylenedioxy-methamphetamine	stimulant designer drugs	HPLC-FL Ex = 288, Em = 324 nm	blood serum vitreous humor urine	2–1000 mg/l blood, serum, vitreous humor 0.1–5.0 mg/l urine	3,4-methylenedioxyethylamphetamine 3,4-methylenedioxyamphetamine 57	
5-aminosalicylic acid	anti-inflammatory, inframatory bowel disease	HPLC-FL Ex = 311, Em(cut-off) = 449 nm pre-column	plasma urine	0.1–8 μg/ml r2 > 0.996	*N*-acetyl-5-aminosalicylic acid	66
verapamil	calcium channel antagonist, hypertension and angina pectoris	HPLC-FL Ex = 204, Em = 314 nm	plasma	5–250 ng/ml r = 0.999	norverapamil	58
gemfibrozil	hypolipidaemic, coronary heart disese	HPLC-FL Ex = 242, Em = 300 nm	plasma	0.05–0.5 mg/ml r = 0.9999		59
levofloxacin	antibacterial agent, treatment of infections	HPLC-Fl, Ex = 280, Em = 450 nm HPLC-UV, 293 nm	plasma	50–10000 ng/ml (UV) 20–5000 ng/ml (FL) r2 > 0.999	ciprofloxacin moxifloxacin trovafloxacin cinoxacin	60

TABLE 2.1-2
CONTINUED

Drug	Category	Method	Sample	Determination Range	Simultaneously Determined Compound	Reference
fenoterol	b2-serective adrenergic agonist, tocolytic agent	HPLC-FL pre-column Ex = 287, Em = 345 nm	plasma	338–1688 pg/900 µl r = 0.990		65
tramadol	analgesic, acute and chronic pain relief	HPLC-FL Ex = 202, Em = 296 nm	plasma	10–400 pmol/ml r = 0.9992	*O*-desmethyltramadol	61
amoxicillin	antimicrobial, *Helicobactor pylori* infection	HPLC-FL Ex = 365, Em = 445 nm internal conversion to fluorescent compound	rat plasma, gastric juice and tissue	0–15 mg/ml (juice) 0–200 mg/ml(plasma) 0-100 µg/ml (tissue) r2 > 0.9975	ampicillin	69
efavirenz	NNRTI, non-nucleoside reverse transcriptaze inhibitor, HIV-1	HPLC-FL Ex = 310, Em = 390 nm post-column photochemical derivatization	plasma r > 0.999	50–1000 ng/ml		72
ciprofloxacin	antibiotics, antibacterial	HPLC-FL Ex = 300, Em = 458 nm	serum, urine	0.005–1.0 µg/ml (serum) 0.05–2.0 mg/ml (urine) r > 0.9995	anthranilic acid	53
captopril	ACE inhibitor,	HPLC-FL Ex = 400, Em = 480 nm pre-column	plasma	12.5–150 ng/ml r = 0.9992		64
5-chloro-3-(4-methanesulfonylphenyl)-6'-methyl-[2,3']bipyridinyl	COX II inhibitor, NSAID	HPLC-FL Ex = 260, Em = 375 nm post-column photochemical derivatization	plasma urine	5–500 ng/ml r2 > 0.99		70
flecainide	antiarrhythemic agent, ventricular and supraventricular arrhythmia	HPLC-Fl Ex = 300, Em = 370 nm	serum	10–2000 ng/ml r = 0.9998	*N*-(2-piperidylmethyl)-2,3-bis(2,2,2-trifluoroethoxy)-benzamide	62

TABLE 2.1-2
CONTINUED

Drug	Category	Method	Sample	Determination Range	Simultaneously Determined Compound	Reference
itraconazole	fungal infections	HPLC-FL Ex = 265, Em = 363 nm	plasma	10–1000 ng/ml	hydroxyitraconazole	63
methotrexate	cytostatic, rheumatoid arthritis	HPLC-FL Ex = 370, Em = 417 nm photochemical, direct injection	plasma r = 0.998	4.5–2.27 mg/ml	7-hydroxymethotrexate	71

TABLE 2.1-3
LC/MS

Drug	Category	Method	Sample	Determination Range	Simultaneously Determined Compound	Reference
tolterodine	muscarinic receptor antagonist, incontinence	LC/MS/MS column switching	plasma	100 pM–100 nM r = 0.994–0.9997	5-hydroxymethyltolterodine	86
sumatriptan	serotonin receptor agonist, migraine	LC/MS/MS API	plasma urine	0.2–20 ng/ml r = 0.9998	D3-sumatriptan metabolites (GR49336,GR112504)	93
simbastatin	cholesterol-lowering agent,	LC/MS/MS direct injection	plasma	0.5–200 ng/ml	simvastatin acid lovastatin (IS)	92
hypercholesterolemia	APCI			lovastatin acid (IS)		
bosentan	non-peptidic endothelin receptor antagonist, anti-hypertension, chronic heart disease	LC/MS/MS narrow bore, column switching ESI	plasma, serum, bile liver (man, dog, rat)	1–10000 ng/ml	hydroxy-, phenyl-, hydroxy-phenyl metabolites	88
simvastatin	cholesterol-lowering agent	LC/MS/MS API	plasma r2 > 0.9998	0.05–50 ng/ml	b-hydroxy acid metabolite	81
flecainide	supression of ventricular arrhythmias	LC/MS APEI	serum	0.05–2.0 mg/ml r2 > 0.999	2,3-bis(2,2,2-trifluoroethoxy)-N-(2-pyridinemethyl)benzamide (IS)	76
vancomycin	antibiotic, bactericidal activity	LC/MS/MS direct injection TIS	serum urine (rat, dog, primate)	0.001–10 μg/ml	teicoplanin (IS)	95
busulfan	haemotological malignancy, bone marrow transplantation	LC/MS TIS	plasma	5–2500 ng/ml	busulfan-d8 (IS)	79
famotidine	histamine H2 receptor inhibitor, peptic ulcer disease	LC/MS/MS TIS	plasma urine	0.5–500 ng/ml (plasma) 0.05–50 μg/ml (urine)	[4-({[2-(5-amino-4H-1,2,4,6-thiatriazin-3-yl)ethyl]thio}methyl)-2-thiazolyl]guanidine S,S-(thiatriazine)dioxide (IS)	96

TABLE 2.1-3
CONTINUED

Drug	Category	Method	Sample	Determination Range	Simultaneously Determined Compound	Reference
oxcarbazepine	pre- and postsynaptic voltage-dependent sodium channels brocker, seizure, trigemius neuralgia	LC/MS APCI	plasma	0.1–50.0 μg/ml r2 = 0.9991	10-hydroxycarbazepine	78
olanzapine	antipsychotic, schizophrenia	LC/MS/MS ESI	plasma	5–300 μg/ml r > 0.991	clozapine N-desmethylclozapine	85
methyldopa	antihypertensive agent, α-adrenergic agonist	LC/MS/MS ESI	plasma	20–5000 ng/ml r = 0.992–0.997	dopa-phenyl-D3 (IS)	84
ketoconazole	antifungal	LC/MS/MS TIS	plasma	20.0–10000 ng/ml r > 0.9985	R51012 (IS)	94
	olanzapine antipsychotic, schizophrenia	LC/MS/MS ACPI	plasma	5–500 ng/ml r > 0.998	LY170158 (IS)	89
amiodarone	antiarrhythmic, supraventricular and ventricular arrhythmia	LC/MS/MS ESI	plasma	50–5000 μg/ml r2 > 0.996	desethylamiodarone tamoxifen (IS)	83
	haloperidol antipsychotic,	LC/MS SSI direct injection	plasma urine	10–800 ng/ml r = 0.995 (plasma) 400–800 ng/ml r = 0.997 (urine)	4-(4-chlorophenyl)-4-hydroxy-piperidine, reduced haloperidol, 4-[4-(4-chlorophenyl)-4-hydroxy-1-piperidinyl]-(4-chloro-phenyl)-1-butanone (IS)	77
	butorphanol anlgesic, μ-opioid receptor agonist/antagonist	LC/MS/MS TIS	plasma	13.7–1374 pg/ml r2 > 0.99	BC2605 (IS)	82
fexofenadine	H1 antihistaminic, allergic rhinitis, urticaria	LC/MS ESI	plasma urine	0.5–200 ng/ml r > 0.998 (plasma) 1.0–200 ng/50 Ml r > 0.998 (urine)	MDL026042 (IS)	75

TABLE 2.1-4
GC AND GC/MS

Drug	Category	Method	Sample	Determination Range	Simultaneously Determined Compound	Reference
clemastine	antihistamine, allergic rhinitis, urtucaria	GC-NPD dimethylpolysiloxane	plasma	0.1–12.8 ng/ml r > 0.99	orphenadrine (IS)	99
fluoxetine	serotonin re-uptake inhibitor, antidepressant	GC-NPD OV-1	plasma	5–3000 ng/ml r = 0.996	norfluoxetine	100
lidocaine	local anesthetic	GC-FID HP-5	plasma	25–2000 ng/ml r = 0.998		101
cocaine	local anesthetic	GC/MS EI	whole blood	10–2000 ng/mlr r2 > 0.98	benzoylecgonine, ecgonine methylester, cocaethylene	114
lorazepam	anxiolytic, sedative	GC/MS/MS EI	plasma urine	0.1–500 ng/ml r = 0.991 (plasma) r = 0.995 (urine)	oxazepam-d5 (IS)	117
sertraline	antidepressant, SSRI	GC/MS EI, SIM	plasma	0.2–10 ng/ml r = 0.999	ethylsertraline (IS)	115
chloral hydrate	sedative	GC/MS EI, SIM	plasma	0.1–250 μM r = 0.9990–0.9996	monochloracetate, dichloroacetate, trichloroacetate, trichloroethanol	127
ketotifen	H1-receptor blocking agent, antiasthmstic	GC/MS EI, SIM	plasma	0.5–10.0 ng/ml r2 = 0.999	pizotifen (IS)	103
nalmefen	opioid antagonist	GC/MS NCI, SIM	plasma	0.5–200 ng/ml	6β-naltrexol-d7 (IS)	110
oxymetholone	synthetic steroid, bone marrow stimulant	GC/MS EI, SIM pre-column	plasma	1–40 ng/ml r2 > 0.995	methyltestosterone (IS)	109

TABLE 2.1-4
CONTINUED

Drug	Category	Method	Sample	Determination Range	Simultaneously Determined Compound	Reference
naltrexone	opioid μ-receptor antagonist	GC/MS EI, SIM pre-column	plasma milk (human, sheep)	2–20 μg/l (human plasma) 2–60 μg/ml (human milk) 2–150 μg/l (sheep plasma) 10–600 μg/l (sheep milk) r > 0.99	6,β-naltrexol naloxone	104
buprenorphine	analgesic, post-operative pain	GC/MS EI, SIM pre-column	plasma	0.1–20.0 ng/ml r2 = 0.993	norbuprenorphine	111
memantatine	Parkinson's disease, NMDA receptor blocker	GC/MS NCI pre-column	plasma	0.117–30 ng/ml r2 = 0.9999	adamantine (IS)	112
midazolam	sedative-hypnotic, anxiolytic	GC/MS SIM	plasma	1.5–300 ng/ml r = 0.999	pinazepam (IS)	102
methylphenidate	attention deficit disorder (ADD)	GC/MS NCI stable isotope dilution	plasma	0.14–18.25 ng/ml r2 = 1.0000		128
tizanidine	muscle relaxant	GC/MS SIM pre-column	plasma	0.5–10.0 ng/ml r2 = 0.999	N-acetyltizanidine	108
paroxetine	SSRI inhibitor, antidepressant	GC/MS NCI stable isotope dilution	plasma	0.094–12.0 ng/ml r2 = 0.9998	[2H6]-labeled paroxetine (IS)	106
busulfan	antineoplastic, marrow ablation	GC/MS SIM	plasma	0–4 mg/l r = 0.9979	pusulfan (IS)	107

References pp. 69–75

TABLE 2.1-5
OTHER METHODS

Drug	Category	Method	Sample	Determination Range	Simultaneously Determined Compound	Reference
fluconazole	antifungal, candidiasis, meningitis, aspergillosis	MECC-UV, 190 nm direct injection	plasma	0.125–25 μg/ml r > 0.999	UK-54373 1,7-dimethylxanthine (IS)	119
levodopa	Parkinson's disese	HPLC-EC, amperometry column switching	plasma, urine	0.02–4.0 mg/ml r2 > 0.993	carbidopa 3-OMD, 3-OMC	123
isoniazide	tuberculosis	HPLC-EC, coulometry direct injection	alveolar cells, broncoalveolar lavage, plasma	0.1–10 mg/ml r = 0.998 (plasma)	diphenylcarbazide (IS)	124
buspirone	psychotropic, anti-anxiety	HPLC-EC, coulometry column switching	plasma	0.1–25 ng/ml r = 0.998	prazosin (IS)	125
manidipine	calcium antagonist	HPLC-EC, amperometry column switching	serum	0.5-10 ng/ml	nilvadipine (IS)	126
naproxen	NSAID, antipyretic, analgesic	MECC LIF (HeCd laser, 325 nm), direct injection	plasma	0.5-25 mg/l	salycylate (IS) naproxen-protein conjugates, naproxen-lysine	120

wavelength may affect the chromatogram's appearance and intensity due to the presence of many other components in the biological fluid that absorb in this region. On the other hand, if the analyte has no or poor absorption in the UV-Vis region, a labeling reaction to introduce a suitable chromophore is usually adopted. To date, many labeling reagents have been developed and tailor-made to serve in the determination of drugs and biological substances. Fig. 2.1 shows representative labeling reagents for UV-Vis [2]. Several pre-column derivatization methods with UV-labels have been examined; Gentamicins C1, C1a, and C2 were derivatized with 1-fluoro-2,4-dinitrobenzene in a weak cation-exchange solid-phase extraction (SPE) cartridge, in which the cartridge was conditioned with methanol followed by Tris buffer (pH 10). After application of a plasma or urine sample followed by washing with the same Tris buffer, the retained analytes were derivatized with a mixture of derivatizing reagents [3]. 4-(4,5-Diphenyl-1*H*-imidazol-2-yl)benzoyl chloride (DIB-Cl), designed to react with primary and secondary amine and phenol hydroxyl groups to introduce a high extinction coefficient chromophore, was used to improve the detection sensitivity of fenfluramine and phentermine [4]. A post-column derivatization with sodium bisulfite was proposed for carboplatin [5].

Commonly, pretreatment of biological fluid samples is required before injection into the HPLC system. The simplest pretreatment is deproteinization with organic solvents. Examples of this practice include the application of methanol for rifampicin [6], ethanol for cisplatin [7], and acetonitrile for metformin, linezolid and carbamazepine [8–10]. Strong acids have also been used for deproteinization of plasma or serum samples; 10% trichloroacetic acid for isoniazide [11] and 60% perchloric acid for ranitidine [12]. A mixture of 0.2 *M* zinc sulphate-acetonitrile was used for praziquantel [13]. After deproteinization, the mixture is centrifuged and the supernatant is subjected to the analysis. A deproteinization method is advantageous as it is quicker and more economical compared to a liquid-liquid extraction and SPE methods.

Liquid-liquid extraction is another alternative for sample clean-up. Water-immiscible organic solvents are used for extraction of analytes from biological fluids. A mixture of diisopropyl ether-isoamylalcohol (99:1, v/v) was used for the extraction of risperidone and 9-hydroxyrisperidone in human plasma [14]. Chloroform for extraction of erythromycine from a biological matrix [15], mixtures of acetonitrile-n-butyl chloride (1:4, v/v) for extraction of paclitaxel in plasma [16] and chloroform-isopropyl alcohol (9:1, v/v) for extraction of benzodiazepines in serum [17], 2-methylbutane-hexane for extraction of loratadine [18] and ethyl acetate for extraction of mazindol [19] and sympathomimetic amines [4] in plasma have been reported.

SPE has become more popular for drug analyses in biological fluids due to the selective retention of the analyte(s), reduced sample and solvent requirements, high throughput and the possibility of automation. A C18 (octadecylsilyl silica gel, ODS) cartridge such as Bond-Elute (100 mg) is commonly used in SPE of human plasma and serum, which is pre-conditioned with methanol and water, successively, followed by applying the samples. After washing the interfering compounds out with aqueous methanol, the target analyte is eluted with aqueous acetonitrile. Examples on SPE include omeprazole using on-line SPE [20], celecoxib with a disk cartridge [21], vancomycin [22], indinavir [23], quinapril [24], ketorolac [25], and amphotericine B

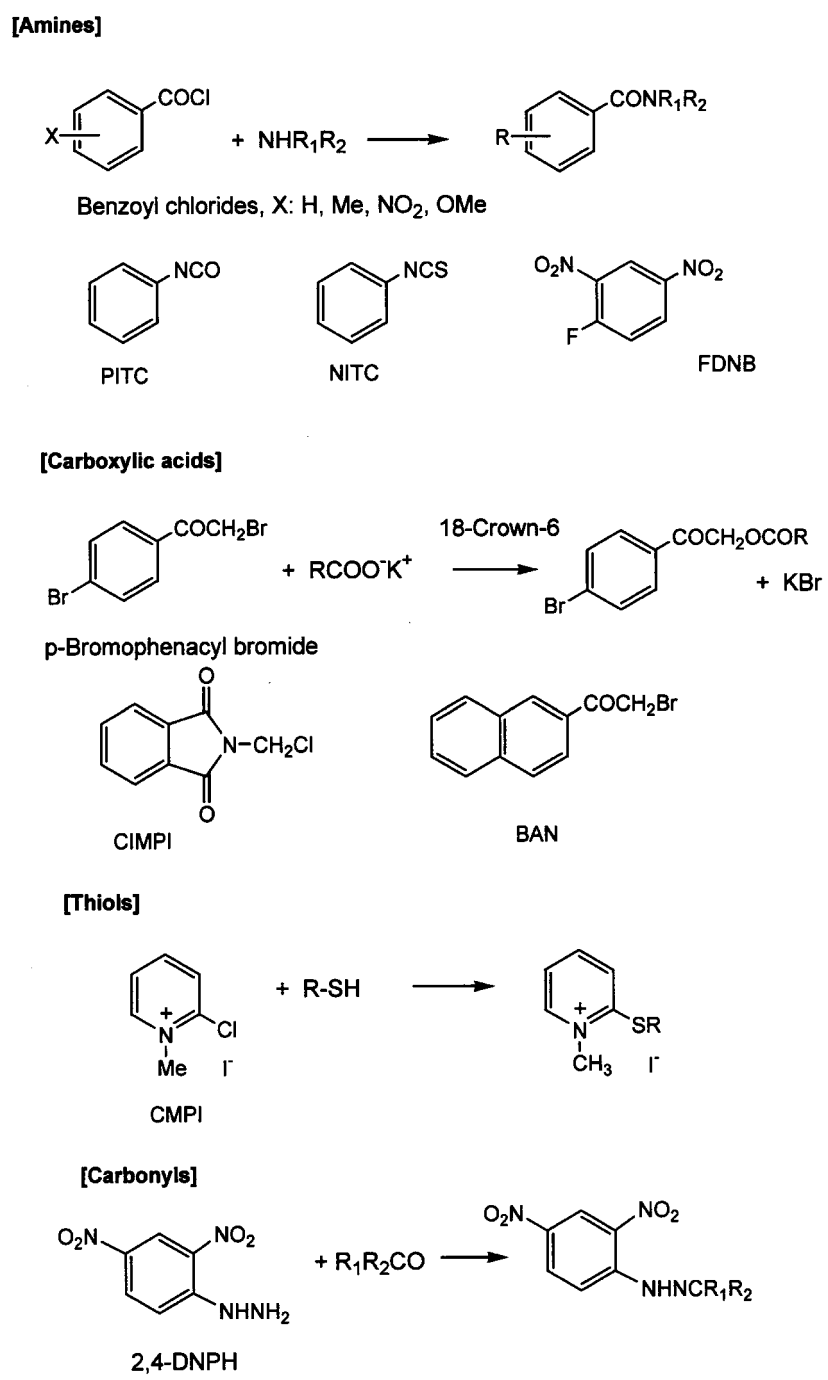

Fig. 2.1. UV-Vis labeling reagents.

[26] in plasma. A Chem Elute CE cartridge packed with celite was used for clobazam in serum [27], whereas an Oasis® HLB cartridge was used for carbamazepine [28] and zidovudine, lamivudine and nevirapine [29] in plasma. In the SPE technique, a vacuum manifold is usually used to obtain rapid and reproducible extraction results.

Ultrafiltration, a straightforward sample preparation method, is also utilized as a convenient and rapid pretreatment of biological fluids to avoid tedious extraction and evaporation techniques. Aliquots of plasma or serum sample are simply filtered by centrifugation in a micron filter, and the ultrafiltrate is injected onto the HPLC system; metformin using Centrifree Micropartition Devices [30], cefepime and cefpirome in serum using a Microcon (10000 Mr cut-off) filter [31], carboplatin [5] and fluconazole using a 5 kDa cut-off filter containing either polysulfone or cellulose membranes [32], were pretreated by this method.

A direct injection method is the preferred technique for the analysis of biological fluids considering time- and cost-consumption, analyte loss, and simplicity. Direct injection is generally combined with column switching. The latter technique allows the adsorption of the analyte on a pre-column and removal of the interfering components such as protein, followed by back-flushing and transfer of the analyte to an analytical column by switching the direction of the effluents. This system makes it possible to develop a fully automated analytical method (Fig. 2.2). To date, many trials using direct injection have been done to develop practical methods for the determination of drugs in body fluids, e.g. voriconazole with a size-exclusion pre-column [33], meropenem and ceftazidime [34], thiacetazone with an internal surface reversed-phase column [35], finasteride [36], propafenone [37], omeprazole and omeprazole sulfone [38], propentfylline [39], carbapenem [40], aceclofenac and diclofenac with a narrow-bore column [41], benzodiazepins [42,43], chlorthalidone [44], amoxicillin with a semi-permeable surface column [45], and methotrexate with ion-pair chromatography using an alkyl-diol silica pre-column [46].

In direct injection systems, for extraction of the analytes from the biological fluids, cartridges packed with several kinds of solid-phases are employed, e.g. a hydrophobic polymer [42], a Capcell Pak MF Ph-1 [41,38], an alkyl-diol-silica C18 or C8 [34,43,46,47], a Capcell Pak CN [32], a pellicular C18 using an in-line-extraction system [33], and a semi-permeable surface support C8 [48], and a biocompatible extraction column (Fig. 2.3) [49].

For separation, a conventional reversed-phase ODS column is the most frequently used: methotrexate [46], lotaradine [18], omeprazole and omeprazole sulfone with a semi-microcolumn [38], atenolol, propranolol and ibuprofen using a biocompatible extraction column [49], propentofyline and its metabolites using two combined columns of a shielded hydrophobic stationary and C18 (chromatogram in Fig. 2.4) [39], gentamicines C, C1a and C2 [3], a carbapenem antibiotic [45], propafenone and 5-hydroxypropafenone [37,47], meropenem and ceftazidime [34], voriconazole [33], benzodiazepins [43], amoxicillin [45], risperidone and its metabolite 9-hydroxyrisperidone by a column packed with 3 μm particles [14], paclitaxel [16], carbiplatin by a column packed with 3 μm particles [5], quinapril and its metabolite quinaprilat [24], omeprazole [20], ketorolac and tolmetin as the IS [25], ampicillin [50], praziquantel [13], ranitidine [12], isoniazide [11], and rifampicin and its metabolite [6]. Besides

ODS, different kinds of packing materials have been developed and utilized: a cation-exchange column for metformin [8,30], a C14 column for carbamazepine [10], a C8 column for amphotericin B [26], indinavir [23], vancomycin [22,27], zidovudine, lamivudine and nevirapine [29], carbamazepine and five of its metabolites [28], benzodiazepins (chromatogram is shown in Fig. 2.5) [42], a NO_2 column for celecoxib

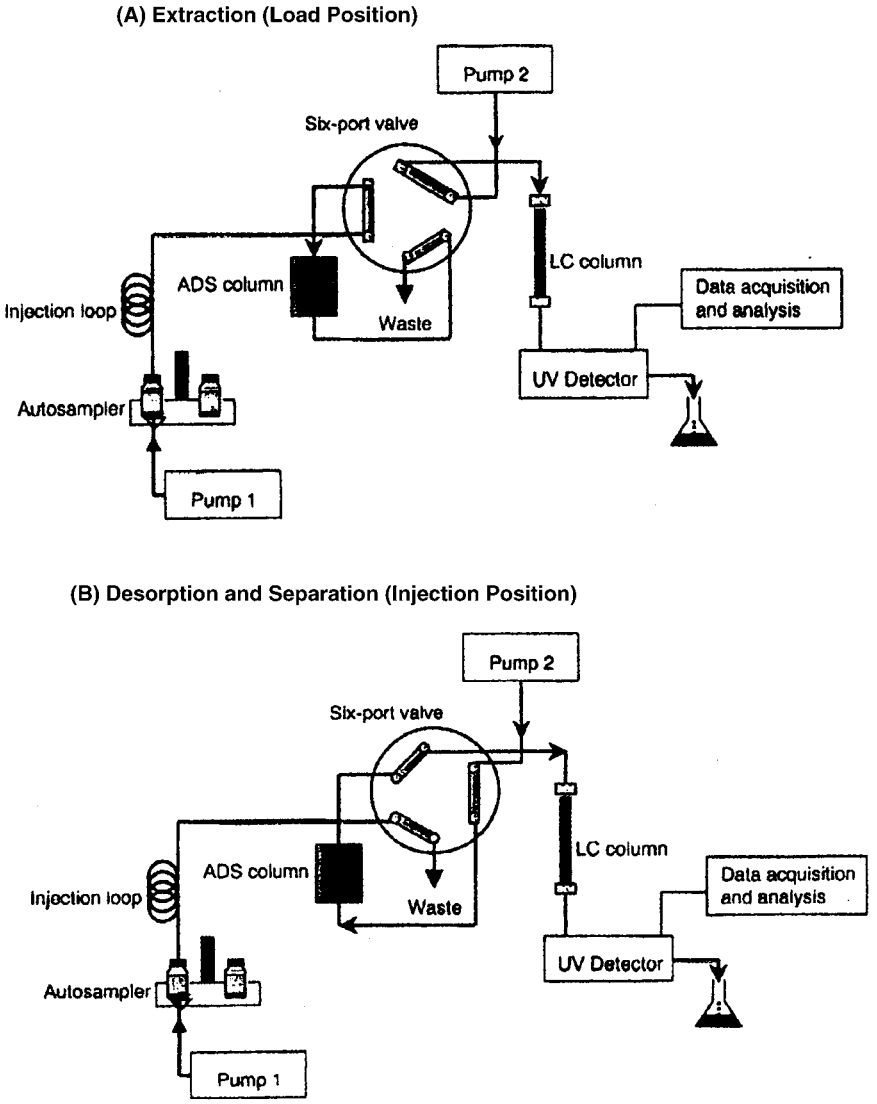

Fig. 2.2. Schematic representation of the automated HPLC method. Printed from ref. 43 with permission from Elsevier Science B.V.

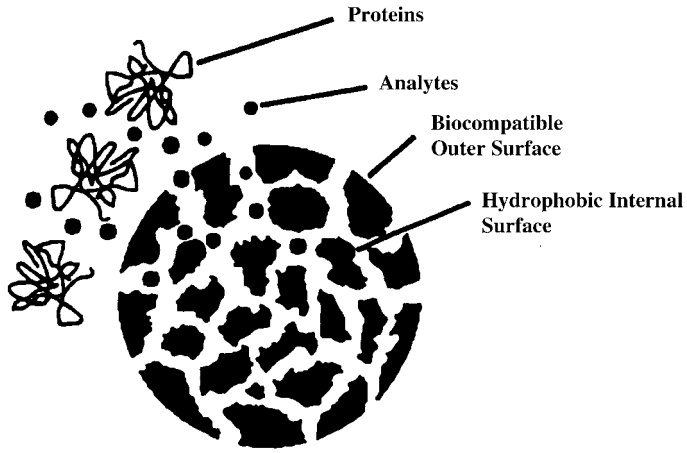

Fig. 2.3. Schematic drawing of a particle for biocompatible extraction. Printed from Ref. 49 with permission from Elsevier Science B.V.

[21], a narrow-bore phenyl-hexyl column for aceclofenac and diclofenac [41], and a phenyl column for thiacetazone [35].

2.2.2 FL detection

Drugs having fluorescence moieties can be detected by monitoring FL at their emission wavelengths. Similar to UV detection, derivatization is also applied to FL detection to increase sensitivity and selectivity for non- or weak-flourescent compounds using a labeling or a conversion reaction. In an HPLC system, two derivatization approaches are used, i.e. pre-column (or pre-label) and post-column (post-label). Representative reactions and labeling reagents are shown in Fig. 2.6. For FL detection, two wavelengths should be selected, i.e. excitation (Ex) and emission (Em); this is the major reason why FL detection is more selective than UV detection. FL detection counts the increase in photon number that occurs relative to a zero background, while UV detection measures the transmitted light that passes out of the sample solution and compares it with the original irradiation, a large background. Generally, FL is more sensitive than UV detection.

Fluorescent drugs have been detected without further derivatization; fluoxetine and norfluoxetine in human plasma [51], ciprofloxacin in serum [52,53], ganciclovir [54], mirtazapine [55], gabapentin in serum samples of 283 patients with partial seizures [56], atenolol [44], designer drugs in whole blood and vitreous humor [57], verapamil and norverapamil in pharmacokinetic and bioavailability studies [58], gemfibrozil and ibuprofen as IS in a pharmacokinetic study [59], fluoroquinolones [60], tramadol and N',N'-dimethylsulfanilamide as IS in plasma [61], flecainnide in serum [62], and itraconazole and hydroxyitoraconazole in plasma samples collected from HIV-infected

patients [63]. The detection without derivatization adds to the convenience of direct injection methods with column switching.

Pre-column derivatization was applied to non-FL drugs after extraction from biological fluids: captopril with monobromobimane [64], fenoterol with *N*-(chloroformyl)-carbazole [65], 5-aminosalicylic acid with acetic acid anhydride [66], and serotonin re-uptake inhibitors with dansyl chloride [67]. An automated pre-column derivatization of methotrexate with cerium(IV) trihydroxyhydroperoxide as a packed oxidant was proposed [68]. Amoxicillin was converted to form a FL compound via an internal rearrangement with mercury (bis)chloride and formaldehyde at pH 6 [69].

For an automated analysis, post-column derivatization is preferable and convenient. A semi-automated determination of a cyclooxygenase II inhibitor was performed by post-column photochemical derivatization with FL detection [70]. Methotrexate and 7-hydroxymethotrexate were converted to FL compounds by a post-column photochemical reaction as shown in Fig. 2.7 [71]. Efavirenz was also converted to a FL

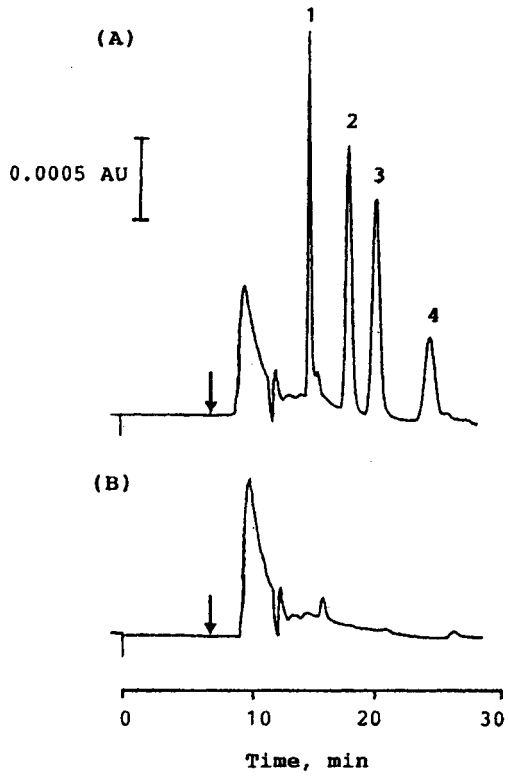

Fig. 2.4. Chromotograms obtained with a normal human serum and a spiked serum. (A) Spiked human serum, (1) A 802751 (30 nmol/ml); (2) A 802831 (30 nmol/ml); (3) propentofylline (30 nmol/ml); (4) A 72087 (30 nmol/ml). (B) Normal human serum. The arrow indicated the time for valve switching. Printed from ref. 39 with permission from Wiley & Sons, Ltd.

product in the same way [72]. Morphine was post-column derivatized to FL pseudomorphine by its dimerization with potassium hexacyanoferrate (III) [73]. Ranitidine and its metabolites were oxidized to primary amines with sodium hypochlorite, and then derivatized with *o*-phtalaldehyde in the presence of 2-mercaptoethanol to fluorescent species [74].

Similar to that in UV detection, pretreatment by deproteinization with organic solvents and acids has been performed: acetonitrile for ciprofloxacin [53] and verapamil [58], cyclohexane for gemfibrozil [59], 70% TCA for captopril [64] and ganciclovir [54], perchloric acid for 5-aminosalicylic acid [66] and gabapentin in serum [56].

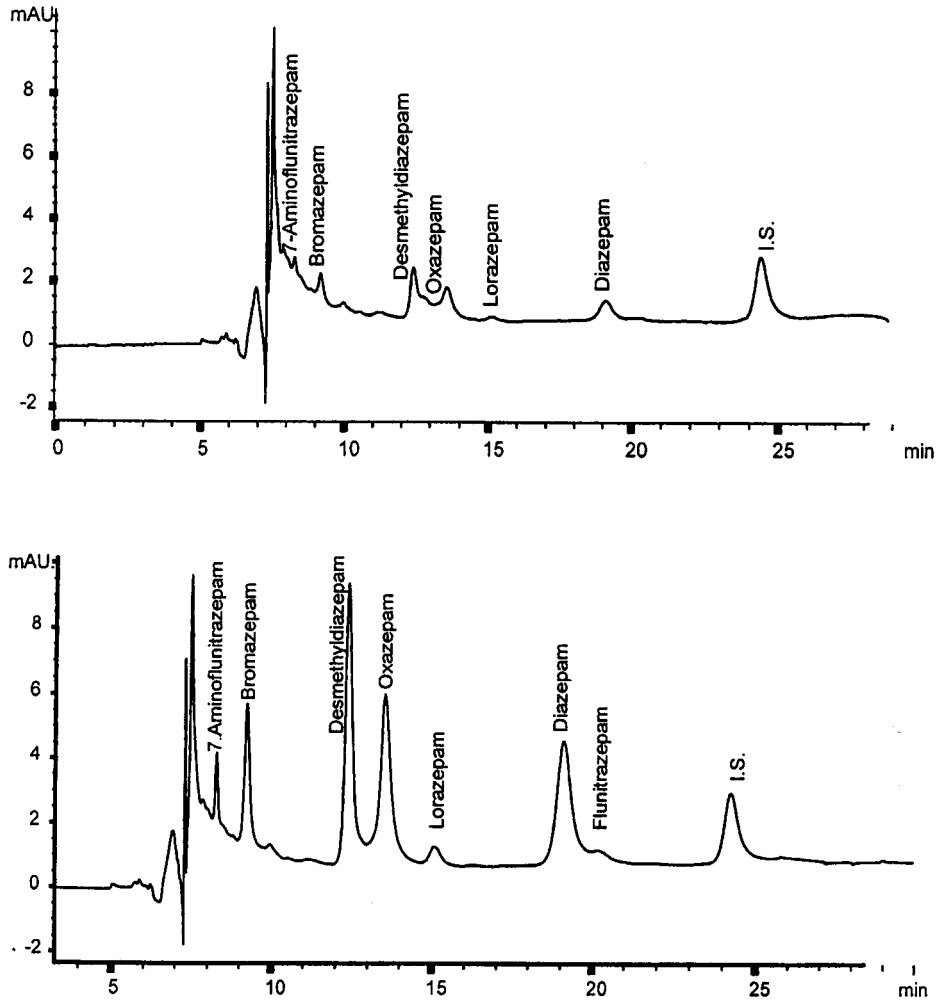

Fig. 2.5. Chromatograms of the certified serum at level 1 (top, 10–100 ng/ml) and level 2 (bottom, 50–600 ng/ml). Printed from Ref. 42 with permission from Elsevier Science B.V.

Liquid-liquid extraction was applied to the pre-treatment of plasma for the determination of efavirenz with a mixture of hexane-methylene chloride (65:35, v/v)[72], ranitidine with methylene chloride [74], mirtazapine with hexane [55],

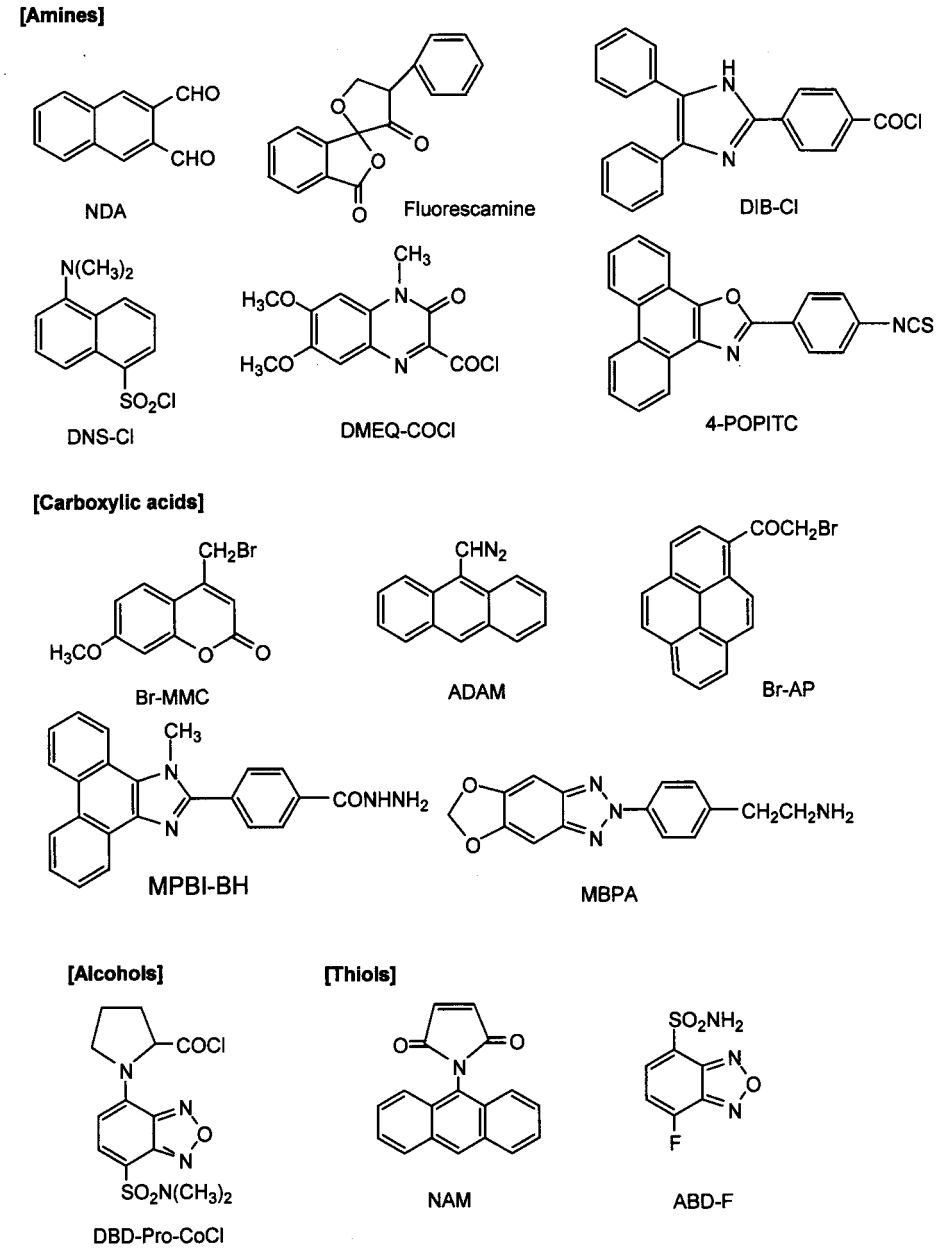

Fig. 2.6. FL labeling reagents.

atenolol with a mixture of dichloromethane-2-propanol (75:25, v/v)[44], designer drugs with a mixture of hexane-ethyl acetate (7:3/v/v) [57], tramadol with *tert*-butylmethyl ether [61], amoxicillin with ethyl acetate [69], selective serotonin reuptake inhibitors with a mixture of heptane-isoamylalcohol (98.5:1.5, v/v)[67], and fenotero with isobutanol [68].

SPE pretreatment was used for cyclooxygenase II inhibitor with a 96-well disk SPE (C8) plate [70], methotrexate with a short protein-coated C18 pre-column [59], fluoxetine and norfluoxetine in plasma using a Bond Elute C8 [51] and flecainide with a C18 cartridge [62], and itraconazole and hydroxyirtaconazole with an ion-exchange LC-SCX cartridge [63].

Ultrafiltration was used to pretreat plasma for the determination of levofloxacin, gatifloxacin, moxifloxacin, trovafloxacin, and cinoxacin by an Amicon Centrifree Micropartition Device [60].

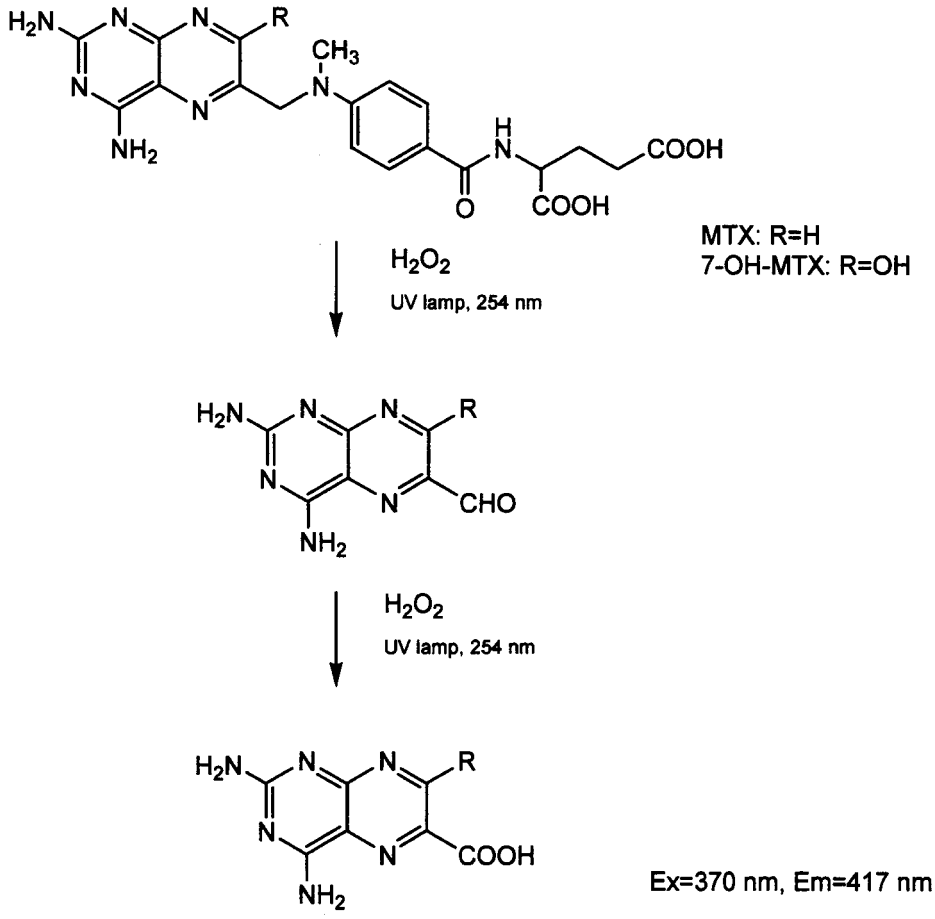

Fig. 2.7. Photo-oxidation of MTX and 7-OH-MTX.

Direct injection combined with column switching is very useful in HPLC-FL methods. Direct injection with column switching using a C18 pre-column was applied to the determination of ciprofloxacin [52], methotrexate and 7-hydroxymethotrexate with a C8 alkyl-diol silica [71], and morphine with a protein-coated column [73].

Analytical columns used for the separation of drugs in an HPLC-FL analytical system are the same as those for HPLC-UV. The most conventional column is C18 with a particle size of 3 or 5 μm. Many drugs were also separated using a C8 column from interfering components: fluoxetine and norfluoxetine [51], efavirenz [72], verapamil and norverapamil [58], and gabapentin [51].

A phenyl column with a particle size of 10 μm and a fluorocarbon-bonded silica gel column were used for mitrazapine [55], and flecainide [62], respectively.

2.3 LC/MS

LC/MS, a powerful analytical technique, has become popular in separation analysis. Although GC/MS is highly sensitive and selective, the sample of interest has to be introduced in the gaseous phase prior to ionization. Its use is, thus, rather limited to the determination of volatile compounds. For non-volatile compounds, HPLC combined with MS detection is preferably used. The development of interfaces between HPLC and MS was very important to build up the valuable LC/MS system. An interface is devised to separate small amounts of analytes from a large volume of solvents and only ionize analytes. As a result of these efforts, practical atmospheric pressure interfaces including ionization process such as thermospray ionization (TSP), atmospheric pressure chemical ionization (APCI), and electrospray ionization (ESI) have been developed (Fig. 2.8). LC-tandem MS (MS/MS) with ESI is well suited for sensitive quantification of medium polar, non-volatile drug compounds and has become a widely applied technique in recent years. In combination with MS detection, capillary LC columns (I.D. < 0.5 mm) offer some advantages over conventional columns; the flow-rate, which is in the range of 1–10 μl/min, is highly compatible with ESI without further splitting, and the detection limit in terms of absolute amount is improved due to less dilution in the column. If the available sample volume is limited, the use of a capillary column is recommended.

Direct injection method was successfully applied to LC/MS/MS with a higher sensitivity and simplicity.

2.3.1 Single MS detection system

Many reports describing LC combined with a single MS as a detector for the determination of drugs in biological fluids have appeared in the literature. Fexofenadine, a non-sedating H1 antihistaminic drug, was determined in human plasma and urine by LC/MS with ESI ionization [75]. Flecainide in serum was also determined with LC/MS using ESI ionization [76]. SPE with a C18 cartridge was used for extraction of both drugs. Haloperidol, an antipsychotic drug, and its metabolites in human plasma and

urine were directly injected and separated on a new polymer column without column switching. The analytes were detected by MS with a sonic spray ionization (SSI) interface [77]. Oxcarbazepine and its active metabolite 10-hydroxycarbazepine in plasma were quantified by LC/MS with APCI. Trimipramine-d3 was used as an IS and extracted with diethyl ether-ethyl acetate (1:1, v/v) [78]. Quantification of busulfan, a bifunctional alkylating agent for chemotherapy regimen of bone marrow transplantation, by LC/MS with an ion spray interface was established [79]. Busulfan was extracted with ethyl acetate from plasma, and after evaporation of the organic phase, the residue was dissolved in the mobile phase and injected on a phenyl column.

2.3.2 Tandem MS detection system

Applying tandem mass spectrometry in LC (LC/MS/MS) has led to the development of rapid and sensitive methods for the determination of many kinds of drugs in body fluids.

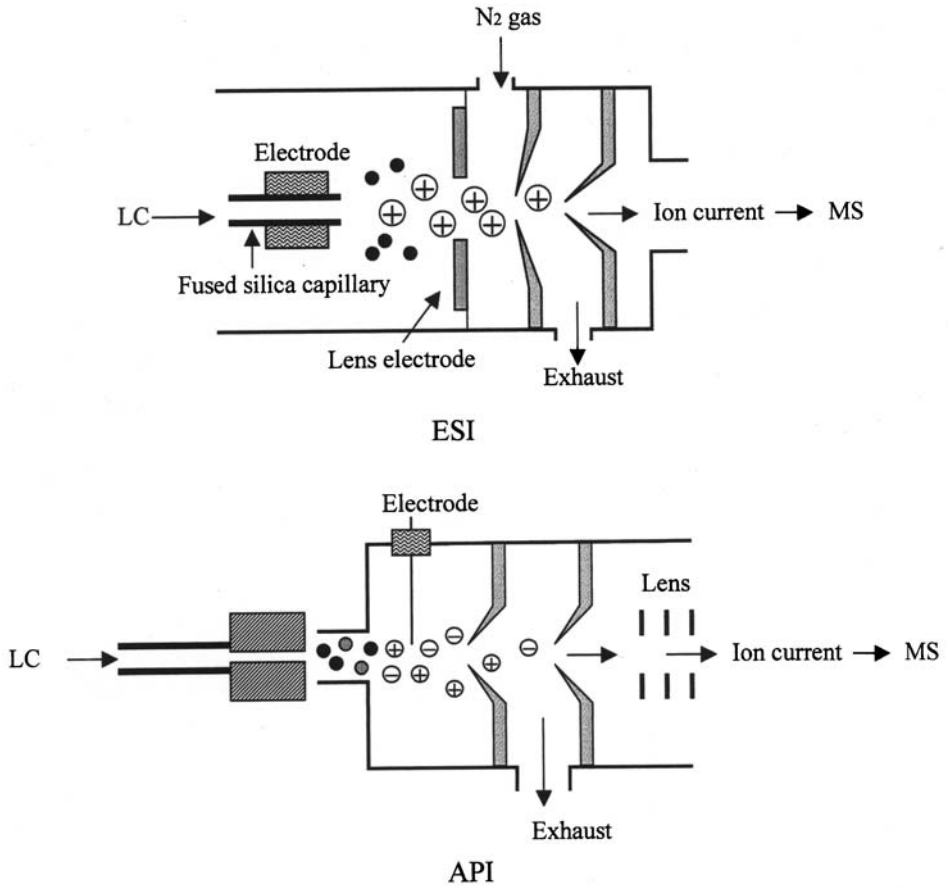

Fig. 2.8. Interfaces for LC/MS.

Compared to LC/MS described above, more sensitive and selective determination could be accomplished with tandem MS/MS. In LC with a tandem MS system, several kinds of interface have been adopted: turbo ionspray ionization (TIS) for indolocarbazole [80], simvastatin [81], and butorphanol [82], ESI for amiodarone and desmethylamiodarone [83], methyldopa [84], olanzapine and clozapine [85], ropivacacine and metabolite [97], tolterodine etc., [86], pravastatin [87], bosentan and its three major metabolites [88], ACPI for olanzapine [89], early drug discovery compounds [90], ketoconazole [91], simvastatin and simvastatin acid [92], and sumatriptan [93], as well as an ionspray ionization for ketoconazole [94]. As a result, in recent drug analyses in biological fluids, ESI and ACPI have been favorably used.

A fast and more efficient sample preparation method is desired for drug analysis in biological fluids. Many analysts have adopted parallel sample preparation methods, such as 96-well SPE in addition to traditional extraction methods.

For the pretreatment of the biological fluids, deproteinization with organic solvents or acids were applied: a mixture of acetonitrile-ethanol (1:1, v/v) for bosentan [88], acetonitrile in a 96-well plate for ketoconazole [91] and drug discovery compounds [90], and perchloric acid for methyldopa [84].

The conventional liquid-liquid extraction method, was also used: a mixture of *n*-butanol-cyclohexane (3:47, v/v) for olanzapine [89], methyl *t*-butyl ether for butorphanol [82], ethyl acetate for busulfan [79], and diethyl ether for ketoconazole [94].

By using a solid phase extraction cartridge (SPE), fast pretreatment with higher recoveries was obtained for biological fluids: flecainide [76] and sumatriptan [93] with a C18 cartridge, vancomycin by on-line extraction with 30 μm particles [95], and famotidine by cation-exchange SPE with a benzenesulfonic acid cartridge [96]. Simvastatine and its hydroxy acid were extracted from plasma by methyl *t*-butyl ether via liquid-liquid cartridge extraction [81]. SPE also allows a fully automatic system with direct injection and on-line extraction.

Direct-injection was adopted for the quantitative determination of olanzapine, clozapine and *N*-desmethylclozapine in plasma [85]: after the addition of IS dibenzepin and dilution with 0.1% formic acid, plasma samples were injected into the LC/MS/MS. The sample clean-up was achieved using an on-line extraction column.

Ultrafiltration was applied to the pretreatment of the free basic drug tolterodine and its active 5-hydroxymethyl metabolite in microliter volumes of plasma [86].

Different types of conventional columns and capillary columns have been used as analytical columns for the separation of drugs in tandem MS: simvastatin and simvastatin acid with a C18 column (50 × 3.9 mm I.D., 5 μm) [92], amiodarone [83] and olanzapine with a column of 150 × 3.0 mm I.D. [85], ketoconazole [94], pravastatin and analogues [87], sumatriptan with a 30 × 4.6 mm I.D. column [93], vancomycin with a C18 cartridge column of 3 μm particle size (40 × 2 mm I.D.), ropivacacine and its metabolite with a packed capillary column (C18, 5 μm, in a fused-silica capillary of 145 × 0.2 mm I.D) [97], tolterodine (250 × 0.2 mm I.D.) [86], methyldopa with a C18 column of 4 μm (100 × 2.1 mm I.D.), a C18 or a silica column (50 × 3 mm I.D.) for clonidine etc. [98], a normal-phase silica column (50x3 mm I.D., 3 μm) for famotidine [96], flecainide with a cyanopropyl bonded-phase column (150 × 4.6 mm. I.D., 5 μm)

[76], butorphanol with a C8 column (100 × 4.6 mm I.D.) [82], ketoconazole with a C30 bonded silica particles column (50 × 4.5 mm I.D.) [91].

2.4 GC

GC has a long history in the analytical separation of many kinds of substances. Although this technique is useful and well-established, its use is limited to volatile compounds. For the determination of non-volatile compounds, a derivatization process to enhance volatility is required. The derivatization serves also to increase the thermal stability of the analytes and prevent their decomposition, and also increase sensitivity detection. For derivatization, methyl esterification with diazomethane, acetylation with acetic anhydride, trifluoroacetylation with trifluoroacetic anhydride and trimethylsilyl etherification have been used extensively. With GC, the most favorable detector can be selected from among many kinds of detection systems such as a thermal conductivity detector (TCD), an electron capture ionization detector (ECD), a flame ionization detector (FID), a nitrogen-phosphorous detector (NPD), a surface ionization detector (SID), and MS to increase the selectivity and sensitivity of the method on the basis of analytes' physicochemical properties. Columns used in GC include packed columns (0.5 ~ 5 m × 2 ~ 6 mm I.D.), packed capillary columns (0.5 ~ 20 m × 0.5–1.0 mm I.D.), and coated capillary columns (film thickness 0.1 ~ 5.0 μm, 10 ~ 50 m × 0.1 ~ 0.8 mm I.D.). Recently, a fused-silica capillary column has become common. Packing materials commonly used are silica gel, activated carbon and alumina, and the coating liquids as a solid phase are polymethylsiloxane, polyethylene glycol, etc.

Clemastine, an ethanolamine-derivative antihistamine, was determined in human plasma by GC-NPD. A capillary column (25 m × 0.22 mm, film thickness 0.33 μm) coated with dimethylpolysiloxane was used. Clemastine was isolated from plasma by liquid-liquid extraction with toluene [99]. A direct injection analysis of an antidepressant drug, fluoxetine, and its metabolite norfluoxetine in plasma was also achieved by GC-NPD detection. An OV-1 fused-silica capillary column (25 m × 0.32 mm I.D. with a film thickness of 0.25 μm) was employed. The analytes were extracted with a mixture of hexane-dichloromethane-isoamyl alcohol (57:42:1, v/v) [96]. A direct-immersion solid-phase microextraction was applied to extract lidocaine, a local anaesthetic, from human plasma. 100-μm polydimethylsiloxane and 65-mm polydimethylsiloxane-divinyl benzene coated fibers were evaluated for SPME with or without deproteinization by TCA. SPEM could be used to obtain a good approximation of the plasma protein binding [101].

2.5 GC/MS

GC combined with MS detection is one of the most widely used analytical techniques. Owing to its intrinsic selectivity and universality, GC/MS has been applied to the analysis of trace amounts of compounds in various samples. A GC-MS system consists of a carrier gas, a sample injector, an interface, an ionization source, a detector, and a

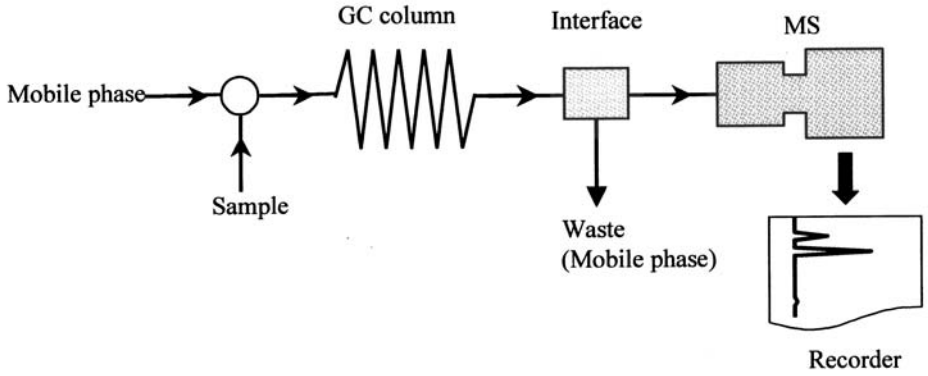

Fig. 2.9. Schematic diagram of GC/MS.

recorder (Fig. 2.9). An injected sample solution is transferred into the column with helium or nitrogen gas, and separated into its components. A splitless or a split injection is used for a capillary GC/MS. An individual component, will then be ionized with electron impact (EI) or chemical ionization (CI), and the resulted ions are detected and recorded. In such a system, similar to LC/MS, the interface is very important. A jet-type separator was developed for the interface, which can remove the mobile phase from the separation column. At present, a fused-silica capillary is often used as a separation column, and the effluent is directly introduced into the ion source without using a separator. Usually, the data obtained from GC/MS are a total ion chromatogram (TIC), a mass spectrum, a selected ion monitoring (SIM) chromatogram and a mass chromatogram. TIC corresponds to the chromatogram obtained by conventional chromatography, in which the separation status of the ions is shown. SIM is used for quantitative analysis. SIM is more selective than TIM; however, the latter is more sensitive. A mass chromatogram is used for qualitative analysis.

2.5.1 Non-derivatization method

Midazolam, a short-acting 1,4-imidazole benzodiazepine, in human plasma was deproteinized with 1.6 *M* perchloric acid, followed by SPME using an 85-μm polyacrylate fiber. GC/MS was carried out in SIM mode after separation using a packed capillary column (30 m × 0.25 mm I.D., film thickness 0.25 μm). The selected ion species were at m/z 280, 307 and 308 for pinazapam as the IS, and m/z 310, 312 and 350 for midazolam. Ion species used for quantitation are m/z 308 ($[M]^+$) and 310 ($[M\text{-}CH_3]^+$ for IS and midazolam, respectively. Typical ion chromatograms are presented in Fig. 2.10 [102]. Ketotifen, an H_1-blocker with antiasthmatic effect, was determined by GC/MS without derivatization. An enzyme hydrolysis of conjugated ketotifen was

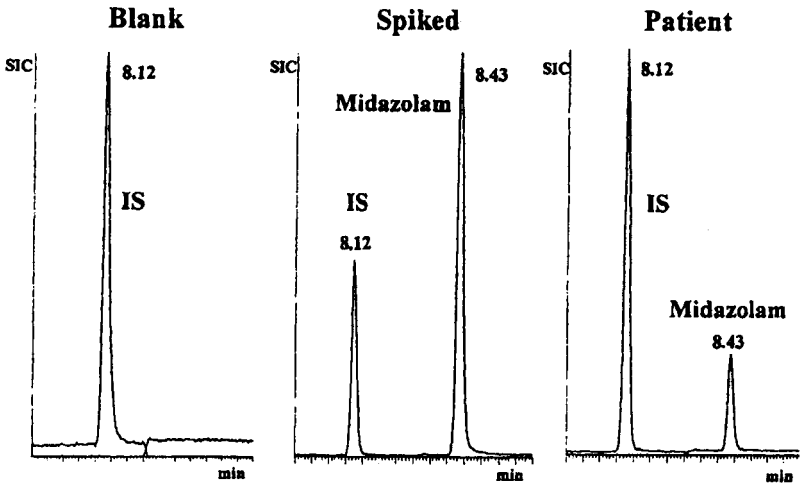

Fig. 2.10. Typical ion chromatograms (ions at m/z 308 and 310) obtained from a blank plasma sample, the same sample spiked with 50 ng/ml of midazolam, and a plasma sample from a subject treated with mizadolam. Printed Ref. 102 with permission from John Wiley & Sons, Ltd.

achieved with combination of β-glucuronidase and arylsulfatase. Ketotifen and pizotifen as the IS were extracted by a SPE-CN-E cartridge and separated on a fused-silica capillary (15 m × 0.25 mm I.D.) with cross-linked 5% PH ME siloxane stationary phase and a film thickness 0.25 μm. MS-EI with SIM mode was used for identification and quantitation [103].

2.5.2 Derivatization method

Naltrexone, a potent competitive antagonist at the opioid μ-receptor, and its major metabolite 6,β-naltrexol were determined by GC/MS after derivatization with acetic anhydride to di- or tri-acetyl derivatives. GC/MS was achieved by EI ionization after SPE extraction of the analytes in plasma and milk, following separation with a capillary column (30 m × 0.25 mm I.D., thickness 0.25 μm) coated with 5% phenyl methyl siloxane. Quantification was achieved by SIM at m/z 411 and 435 for naloxone, at m/z 425 and 467 for naltrexone, and at m/z 427 and 467 for 6,β-naltrexol. The method could be used to quantify both these analytes at relevant in vivo concentrations [104].

A negative ion chemical ionization (NICI) MS method was developed to improve the quantitative measurement of lisinopril, an angiotensin-converting enzyme inhibitor. The method employed SPE of the analyte in human plasma, a successive derivatization with

N-methylbis(trifluoroacetamide) and pentafluorobenzyl bromide, followed by separation on a fused-silica capillary column (15 m × 0.25 mm I.D., film thickness 0.25 μm). The method was applied to evaluate lisinopril pharmacokinetics, and a typical pharmacokinetic-profile was obtained in a healthy volunteer receiving 20 mg of the drug orally [105]. Paroxetine, an antidepressant, was analyzed by a stable isotope dilution NICI MS. After extraction from plasma with hexane-ethyl acetate (1:1, v/v), paroxetine was derivatized with pentafluorobenzoyl alcohol to pentafluorobenzoyl carbamate, which was separated on a fused silica capillary column (15 m × 0.25 mm I.D., film thickness 0.25 μm) and detected by NCIC at m/z 372 using [$^2H_6$]-labeled aroxetine as the IS [106]. A GC-mass fragmentography was developed for routine analysis of busulfan, an alkylating agent for antineoplastic therapy and marrow ablation in preparation for bone marrow transplantation. Busulfan and pusulfan as the IS were extracted from plasma with ethyl acetate and derivatized with sodium iodide to 1,4-diiodobutane and 1,5-diiodopentane, respectively. A methyl silicon capillary fused-silica column (30 m × 0.2 mm I.D., film thickness 0.33 μm) was used for the separation and the split ratio of inlet to column was 15:1. MS detection was carried out in EI ionization mode and monitoring was by both TIM and SIM [107]. TMS derivatization has been applied to tizanidine, a skeletal muscle relaxant, in plasma. *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) reacts with tizanidine to give di-TMS-tizanidine, which was injected via a split injection mode (10:1) and separated with a SE-30 bonded capillary column (17 m × 0.20 mm I.D., 0.33 mm film thickness). The MS detection system was in EI mode with SIM for quantification monitored at m/z 397 for tizanidine and m/z 332 for *N*-acetyltizanidine as the IS [108]. MSTFA was also utilized for the analysis of oxymetholone, a synthetic anabolic steroid, in human plasma. Oxymetholone and methyltestosteron as the IS were extracted from plasma by solvent extraction with *t*-butylmethylether, and the organic phase was evaporated to dryness. The residue was then derivatized with MSTFA-NH_4I-2-mercaptoethanol (100:2:6, v/w/v) at 60 °C for 20 min. and injected into GC-MS system. A fused-silica capillary column (17 m × 0.20 mm I.D.) coated with 0.11 μm film thickness was employed for the separation. The retention times of oxymetholone and IS were 5.56 and 4.57 min, respectively. The carrier gas was helium (1.0 ml/min, split 10:1), and MS was operated with EI in the SIM mode. The method was successfully applied to determine the pharmacokinetic parameters of oxymetholone in healthy volunteers after oral administration of 50 mg of the compound [109]. Nalmefen, an opioid antagonist, and naltrxol-d_7 as an IS were extracted by chloroform-ethanol (80:20) from plasma, and derivatized with pentafluoropropionic anhydride (PFPA). The derivatives were separated on a capillary column (12 m × 0.2 mm I.D., 0.33 μm), and detected by MS operated in NICI mode using methane:ammonia (95:5) as the reagent gas [110]. PFPA was also applied to the derivatization of buprenorphine, a synthetic opioid analgesic, and its metabolite, norbuprenorphine, in human plasma. The analytes were extracted by SPE from plasma, derivatized with PFPA, and separated on a fused-silica capillary column of cross-linked methyl silicon gum (12 m × 0.22 mm I.D., film thickness 0.33 μm). The mass selective detector was operated in SIM mode at m/z 528 and 560 for the IS, m/z 524 and 556 for buprenorphine and m/z 648 and 616 for norbuprenorphine [111]. Memantine, a drug for the treatment of Parkinson's disease and movement disorder, and

adamantine as an IS were extracted from plasma, and derivatized with pentafluorobenzoyl chloride to the corresponding pentafluorobenzoyl derivatives in a one-step procedure, in which extraction and derivatization are performed simultaneously in a small volume of hexane. Separation was achieved on a fused-silica capillary column (15 m × 0.25 mm I.D., film thickness 0.25 μm), and detected using the NICI mode with methane as a moderating gas. During signal ion recording, m/z 353.1 and 325.1 were recorded as the analyte and the IS, respectively. The injection was operated in the splitless mode, and He was used as a carrier gas [112]. Morphine glucuronides in human plasma were extracted by SPE on an C18 cartridge and derivatized to their pentafluorobenzyl ether trimethylether derivatives by using pentafluorobenzyl bromide and bis(*N,O*-trimethylsilyl) trifluoroacetamide. The derivatives were separated on a fused-silica capillary column (15 m × 0.25 mm I.D., film thickness 0.25 μm). The injector was operated in the splitless mode, and He was used as a carrier gas. In the NICI mode with methane as a moderating gas, the ions of m/z 748.3 and 751.3 for the target compound and the IS, respectively, were monitored [113]. An ion-trap GC/MS system was validated for the determination of cocaine, its metabolites and cocaethylene in postmortem whole blood. After an extraction from whole blood by an SPE cartridge, the compounds were derivatized with *N*-methyl-*N*-*t*-butyldimethylsilyltrifluoroacetamide. GC was performed on a capillary column (30 m × 0.25 I.D., 0.25 μm) with a splitless injection followed by an EI ionization [114]. A rapid and sensitive determination of sertraline, a new antidepressant that acts as a selective serotonin re-uptake inhibitor, in human plasma was developed by GC/MS with SIM mode. Using three types of derivatization reagents, i.e. TFAA, PFPA and heptafluorobutyric anhydride (HFBA), GC/MS data in the SIM mode were compared for the derivatives of sertraline and ethylsertraline (IS). They had similar retention times and fragmentation patterns, but showed different characteristic ions and limits of detection. Among these, complete separation from the blank peaks and the largest peak height were obtained by HFBA derivatization as shown in Fig. 2.11 [115]. A direct injection of plasma sample after ultrafiltration into GC using an injector liner (80 mm × 3.2 mm I.D.) packed with a modified Chromosorb-based material was performed for the determination of ropivacaine, its metabolite (PPX) and ropivacaine-d_7 as the IS. The separation column used was a 35% phenyl-(equiv.) polysilphenylenesiloxane column, and detected at m/z 84 for PPX, m/z 126 for ropivacaine, and m/z 133 for IS [116].

GC with tandem MS (GC/MS/MS) was utilized for the determination of lorazapam, an anxiolytic and sedative agent, in human plasma and urine as TMS derivatives. Lorazapam and oxazepam-d_5 as the IS in plasma were extracted by an SPE column with dichloromethane-isopropanol (9:1, v/v), and the organic phase was evaporated to dryness under nitrogen gas. The residue was derivatized with bis(trimethylsilyl)trifluoroacetamide (BSTFA), and the TMS-derivatives were separated on a capillary column. Injection was in the splitless mode. The MS system was operated in the EI mode, and the derivatives were identified and quantitated using characteristic ions of m/z 341, 306 and 267 for lorazepam-TMS and m/z 346, 309 and 271 for oxazepam-d_5-TMS, respectively, formed from the parent ions by collision-induced dissociation in the ion-trap spectrometer [117]. An automated GC/MS/MS for determining tebufelone, an anti-inflamatory candidate and its ^{13}C, ^{18}O labeled analog, was developed. The stable-

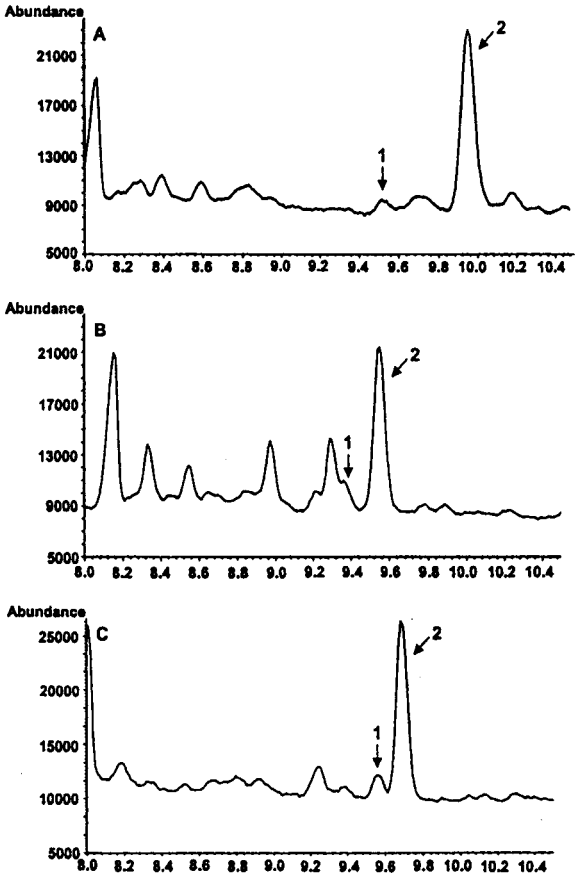

Fig. 2.11. Total ion current chromatograms of sertraline 1 ng/ml (1) and ethylsertraline 40 ng/ml (2) in plasma at selected ion-monitoring (SIM) mode as their TFA- (A), PFP- (B) and HBF-derivatives (C). Printed from Ref. 115 with permission of Elsevier Science B.V.

isotope-labeled tebufelone was used as in vivo IS to determine the oral administration absolute bioavailability. A structural analog, 3-methyl-tebufelone, was used as IS to substitute for deviations due to the analytical procedure [118].

2.6 OTHER METHODS

Micellar electrokinetic capillary chromatography (MECC) was applied to the determination of fluconazole, an antifungal agent, in human plasma. The MECC system was equipped with a 75 μm I.D. fused-silica capillary of 100 cm total length (70 cm to the detector). Injection of sample was performed by vacuum suction for 0.6 s. The analytes

were detected by UV detection at 190 nm. Plasma samples were pretreated with different treatments: centrifugation, deproteinization with acetonitrile, liquid-liquid extraction with dichloromethane and SPE. Clean electropherograms could be obtained for both bovine plasma and healthy volunteer's plasma when samples were applied on SPE using a C18 cartridge. Compared to HPLC, MECC is an attractive and economical analytical method [119]. MECC was also applied to determine naproxen (NAP), naproxen-protein conjugates, and naproxen-lysine (NAPLYS) in plasma. Separation was performed on a fused-silica capillary column (50 μm I.D.) with an effective length of 20 cm (total length of 27 cm). Analyte detection was accomplished by laser-induced fluorescence with an He-Cd laser, which emits at 325 nm. A 366 nm emission filter was used for the detection. MECC with direct plasma injection provided a selective, simple, rapid, and attractive approach for the simultaneous assessment of newly developed NAP-protein drugs used in drug targeting, their primary matabolite NAPLYS, and free NAP [120].

Several chemical reactions that emit light (i.e. chemiluminescence, CL) via electronic excitation have been elucidated and utilized for highly sensitive detection in HPLC. Cyclosporine A, an immunosuppressant, in blood samples of renal transplantation patients was assayed with HPLC-aryloxalate-sulforhodamine CL detection for TDM purposes. Using cyclosporine D as IS and SPE of blood samples, these compounds were well separated within ca. 6 min. The method was 100 times more sensitive than HPLC-UV and comparable to that of fluorescence polarization immunoassay [121]. Propentofylline, a neuronal cell protective agent, in microdialysates obtained from rat hippocampus after single oral administration was determined by HPLC-peroxyoxalate CL. Without further purification of microdialysate, propentfylline was FL-labelled with 4-(*N,N*-dimethylaminosulphonyl)–7-hydrazino-2,1, 3-benzoxadiazole and separated on a C18 column. The method showed 200 times higher sensitivity than HPLC-UV [122].

Simultaneous determination of levodopa, carbidopa and their metabolites in human plasma and urine samples was performed by HPLC-EC. The system involved the use of a second pump, a switching valve, and a pre-column in order to achieve on-line sample clean-up and preconcentration. The analytes were separated on a C8 column followed by amperometric detection using a carbon microelectrode flow cell. The method offers advantages of being simple, less time- and labor-consuming, and thus is suitable for routine analysis and pharmacokinetic purposes [123]. Isoniazide in alveolar cells, bronchoalveolar lavage and plasma in HIV-infected patients was determined by HPLC-EC. Following extraction with a chloroform-butanol mixture, isoniazide was back-extracted, separated on a CN column, and detected with a sealed coulometric detector. Another method using a direct injection with a polymer column was also developed. The method can be used to estimate the very low isoniazide levels in alveolar cells and cell-free lavage recovered from patients undergoing anti-tuberculosis therapy [124]. Buspiron, an anti-anxiety drug, in human plasma was assayed by HPLC-EC using coulometric detection. Buspiron and prazosin as IS were extracted by SPE using a Bond-Elute C18 cartridge. The separation was carried out with a Supelcosil AZP + plus C18 analytical column utilizing a column-switching technique [125]. Manidipine in human serum was also determined with HPLC-EC by using an amperometric detection [126].

The more recent studies on chromatographic analysis of achiral drugs in biological fluids are summarized in this chapter. Many other hyphenated methods with separation techniques to determine biomaterials as well as drugs have been reported elsewhere.

PART II. CHROMATOGRAPHIC METHODS FOR THE ANALYSIS OF CHIRAL DRUGS IN BIOLOGICAL FLUIDS

2.7 INTRODUCTION

Molecular chirality is a fundamental phenomenon that plays an important role in biological processes. Wide ranges of biological and physical functions are generated through precise molecular recognition because enzymes, receptors and other natural binding sites within the biological systems interact with different enantiomers in decisively different ways. As a result of such chiral recognition, drug enantiomers may differ in their pharmacological and/or toxicological profiles [128,129].

For drugs with a single stereogenic center, both enantiomers may be pharmacologically active. However, if the main pharmacological effect is due to only one enantiomer, several possibilities exist for the other enantiomer: inactivity, a qualitatively different effect, an antogonistic effect or severe toxicity [130]. The differences in the biological activities of the enantiomers are due to the differences in protein binding, mechanism of action, rate of metabolism, changes in activities due to metabolism clearance rate and persistence in the environment [131–134]. Because of the different biological activities of enantiomers of active ingredients, the preparation of highly enantio-pure compounds is of utmost importance [135–136].

In view of this, the U.S. Food and Drug Administration has issued certain guidelines for the marketing of racemic drugs [137]. On the other hand, the enantioselective character of pharmacokinetic processes leads to different plasma concentration-time profiles for the constituent enantiomers. For this reason, the evaluation of the disposition of a drug employed as a racemic mixture based on data achieved from the non-selective assay is not only seriously limited, but can also be highly misleading, particularly when attempting to relate plasma concentration to a pharmacological effect or therapeutic benefit [138]. This is true for both newly developed drugs and agents with long-term use in clinical practice, but now subject to re-evaluation.

The investigation of enantioselectivity represents a great challenge for clinical pharmacology in terms of the control of individual variability of clinical responses. The clinical relevance of the phenomenon is particularly important for drugs of low therapeutic index administered as racemates. A prerequisite for progress in acquiring knowledge of stereospecific pharmacodynamics and pharmacokinetics of optical isomers is the development of an enantioselective analytical methodology. When employed in drug studies, this type of analysis plays an important role in the development of new drugs and formulations, and in investigations on bioavailability and metabolism. Furthermore, it has often become the basis of therapeutic drug monitoring and drug management in patients [139].

Enantioselective chiral drug bioanalysis is a complex process that requires taking into consideration numerous factors associated not only with chiral separation techniques,

but also with typical drug analyses of biological samples. Here one should bear in mind the isolation of the enantiomers and/or of the metabolites from the biomatrix, such as blood, plasma, urine, hair, etc., via extraction, then the detection method, as well as validation, which has become an essential element of the entire analytical method [140].

TABLE 2.2-1
ANALYSIS OF CHIRAL DRUGS IN BIOLOGICAL FLUIDS BY HPLC USING PIRKLE CONCEPT CHIRAL COLUMN

Column	Chiral Drug	Mobile Phase	Detection	Biological Sample	LOQ	Ref.
Covalent (R,R)Whelk-O	warfarin	acetonitrile/0.5% acetic acid (2:3, v/v), 1 ml/min	UV 313 nm	plasma	–	141
Sumichiral OA-4500	nicardipine	hexane/ 1,2-dichloroethane /methanol/tri-fluoroacetic acid	UV 254 nm	plasma	2.5 ng/ml	142
(3S,4S)-1-(3,5-dinitrobenz-amidol 1,2,3,4-tetrahydro-phenanthene (Whelk- O-(SS)	ibuprofen	hexane/propan-2-ol/ glacial acetic acid (980:12.5:0.5, v/v/v), 1 ml/min	UV 254 nm	serum	1μg/ml	143, 144
(3S,4S)-1-(3,5-dinitrobenz-amidol 1,2,3,4-tetrahydro-phenanthene (Whelk- O-(SS)	PNU-103017 HIV aspartyl protease inhibitor*	absolute ethanol/ 0.1% acetic acid in hexane (30:70, v/v), 1 ml/min	UV 295 nm	plasma	0.1–0.2 μM	145
(S)-N-(3,5-dinitrobenzoyl) leucine	glutethimide	hexane/propan-2-ol/ acetonitrile (965 25:1), 1 ml/min	UV 220 nm	serum & urine	0.5 μg/ml	146
(R)-N-(3,5-dintirobenzoyl) phenylglycine	propranolol	hexane/propan-2-ol/ acetonitrile (97:3:1)	SF290/350 nm	serum	–	147
(R)-N-3,5-dinitrobenzoyl-phenylglycine	debrisoquine	hexane/ethanol/-t-butyl methyl ether (98:1:1), 1 ml/min	SF 265/380 nm	plasma	3.5 nM	148
Sumichiral OA-series	amino acids	citric acid/methanol	SF 470/530 nm	plasma	0.48–3.1 μM	149
Dinitrobenzoyl Leucine (Pirkle DNBL)	cholecystokinin β-antagonists**	25 mM ammonium acetate buffer pH7.0 /40% acetonitrile, 0.8 nl/min	MS-MS	Liver microsomes	–	150

* PNU-1-3017 is 4-cyano-N-[3-{cyclopropyl (5,6,7,8,9,10 - hexahydro-4-hydroxy-2-oxo-2H-cycloocta (6) pyran-3-yl) methyl} phenyl]- benzene sulfonamide. ** Cholecystokinin β-antagonists are racemic benzodiazineamidines.

TABLE 2.2-2
ANALYSIS OF CHIRAL DRUGS IN BIOLOGICAL FLUIDS BY HPLC USING CELLULOSE DERIVATIVES CHIRAL COLUMN

Column	Chiral Drug	Mobile Phase	Detection	Biological Sample	LOQ	Ref.
Chiralcel OD	SCH 56592 triazole antifungal*	hexane/ethanol/ diethylamine (30:70:0.2, v/v/v)	SF 270/ 320 nm	serum	0.1–2 μg/ml	153
	ibuprofen	hexane/propan-2-ol/ triethylamine (100:1:0.1,v/v/v), 1 ml/min	UV 230 nm	plasma	–	154
	ketamine and norketamine	hexane/propan-2-ol (98:2, v/v/), 0.8 ml/ min, 35°C	UV 215 nm	plasma	5–10 ng/ ml	155
	10-hydroxy carbazepine	hexane/propan-2-ol/ ethanol (70:20: 10, v/ v/v), 1 ml/min	UV 215 nm	plasma	–	156
	bisoprolol	hexane/propan-2-ol (1:1,v/v)	SF 228/ 298 nm	plasma & urine	2 ng/ml	157
	lorazepam	hexane/propan-2-ol/ ethanol (5:5:1, v/v/ v), 1 ml/min	UV 254 nm	plasma	3 ng/ml	158
	propranolol	hexane/propan-2-ol-N,N-dimethyl-octamine (98:8:0.01, v/v/v), 1.5 ml/min	UV 254 nm	plasma	10 ng/ml	159
	R,S-MTPPA** anti-inflammatory agent	hexane/propan-2-ol-acetic acid (96:4:0.5, v/v/v), 1 ml/min, 30°C	UV 272 nm	urine	0.2 ng/ ml	160, 161
	praziquantel	hexane/propan-2-ol/ (1:1, v/v)	UV 212 nm	serum	5 ng/ml	162
	propafenone	hexane/n-propanol/ diethylamine (9:1: 0.4, v/v/v), 1 ml/min	UV 247 nm	serum	–	163
	metoprolol	hexane/propan-2-ol/ octylamine (9:1:0.1, v/v/v) 1 ml/min	SF 275/ 315	serum	–	164
Chiralcel OD-R	pyridoglutethim-ide	acetonitrile/0.3 M $NaClO_4$ (25:75) (pH 6.2 with $HClO_4$), 0.8 ml/min	UV 257 nm	serum	50 ng/ml	165
	fluoxetine and norfluoxetine	acetonitrile/ potassium hexafluorophosphate	UV 227 nm	plasma	10 ng/ml	166
	lansoprazole and 5-hyroxy-lansoprazole and lansoprazole sulfone	methanol/water (75:25, v/v), 0.5 ml/ min.	UV 285 nm	Liver microsomes	0.25 μM/ml	167

TABLE 2.2-2
CONTINUED

Column	Chiral Drug	Mobile Phase	Detection	Biological Sample	LOQ	Ref.
	propranolol	acetonitrile/1M $NaClO_4$ (40:60, v/v), 0.5 ml/min	UV 289 nm	plasma & serum	–	168
	4-hydroxy-debrisoquine	methanol/ acetonitrile/ 0.125 M $NaClO_4$, pH5.0 (3:12:85, v/v/v), 0.8 ml/min, 30°C	SF 210/ 290 nm	urine	3.75 ng/ml	169
	amphetamine methamphetamine p-hydroxy-methamphetamine	propan-2-ol/ acetonitrile/ phosphate-citrate buffer (pH 4.5) containing 50 mM $NaPF_6$ (1:59:40, v/v/v), 35°C	SF 330/ 440 nm	urine & hair	1 μM/ml	170, 171
	selfotel	acetonitrile/0.1M phosphate buffer (35:65,v/v), 0.5 ml/min, 30°C	SF 262/ 314 nm	urine	0.25 μg/ml	172
	tramadol	acetonitrile/50 mM phosphate buffer containing 0.2 M $NaClO_4$, pH 6.0 (1:3)	UV 220 nm	plasma	0.5 ng/ml	173
	ondansetron	acetonitrile/0.7 M $NaClO_4$ (25:75, v/v), 0.5 ml/min	UV 210 nm	serum	7 ng/ml	174
	dizepam and nordiazepam	0.3M $NaClO_4$/ acetonitrile (57:43), 0.5 ml/min	UV 210 nm	serum	25 ng/ml	175
	apomorphine	35% acetonitrile/0.05 M $NaClO_4$, pH2.0, 0.5 ml/min	UV 273 nm	serum	5 ng/ml	176
	Pirlindole	acetonitrile/ phsophate buffer (pH 5.0) contaning 50 mM $NaClO_4$ (2:3), 0.6 ml/min	SF 295/340 nm	plasma	10 ng/ml	177
Chiralcel OD-RH	amphetamine and methamphetamine	acetonitrile/ phosphate citrate buffer, pH4.0 containing $NaPF_6$ (0.3 M) 57:43, v/v), 1 ml/min	SF 330/440 nm	hair	4.7 F mol.5μL	178
	verapamil and norverapamil	acetonitrile/30 mM $NaPF_6$ (34:66, v/v, pH 4.6), 1 ml/min	SF 280/315 nm	plasma	10 ng/ml	179

TABLE 2.2-2
CONTINUED

Column	Chiral Drug	Mobile Phase	Detection	Biological Sample	LOQ	Ref.
Chiralcel OJ	cisapride	ethanol/hexane/DEA (35:64.5:0.5, v/v/v), 2 ml/min	UV 275 nm	plasma	5 ng/ml	180
	isradipine	9.5% propan-2-ol/ hexane, 1 ml/min, 39°C	UV 240 nm	serum	0.26 ng/ ml	181
	metyrapone	hexane/ethanol/DEA (92:8:0.1, v/v/v), 1 ml/min	UV 261 nm	plasma & urine	0.03 μg/ ml	182
	manidpine	hexane/ethanol/ methanol (80:15:5), 1 nl/min	UV 230 nm	serum	0.2 ng/ ml	183
Chiralcel OJ-R	rogletimide	0.25 M NaClO4/ acetonitrile (pH5.6 with $HClO_4$) (80:20, v/v), 0.5 ml/min	UV 257 nm	serum	50 ng/ml	184
	antoprazole	acetonitrile/50 mM $NaClO_4$ (multisteps linear gradient), 0.5 ml/min	UV 290 nm	serum	0.25 μg/ ml	185
	ketorolac	45% acetonitrile in 3 mM $HClO_4$ (pH2.0), 1.2 ml/min	UV 313 nm	plasma & urine	5 ng/ml	186
	praziquantel	0.1 M $NaCl0_4$/ acetonitrile (33:17), 0.5 ml/min	UV 210 nm	serum	5 ng/ml	187
	promethazine	0.5 M $NaClO_4$/ acetonitrile (63:37, v/ v), 0.5 ml/min	UV 249 nm	serum	2 ng/ml	188
Chiral Tribencel	thalidomide	methanol with 10–25% of 99.5% ethanol, 0.5 ml/min	UV 220 nm	plasma	–	189, 190

* SCH 56592 is (-)-[4-[4-[4[(2R-cis)-5- (2,4-difluorophenyl)-tetrahydro-5-(H)-(1,2,4-triazol-1-phenymethyl)-3-furanyl) methoxy] phenyl-1-piperazinyl-2-[(S)-1-ethyl-2-(S)-hydroxy propyl]-2-4-dihydro-3H- 1,2,4-triazol-3-one.
** MTPPA is R,S.-2-[4-methyl-2-thienyl) phenyl] propionic acid.

Chiral separation techniques employed both in liquid and gas chromatography can be classified as direct and indirect separation techniques. The most commonly employed method of direct enantiomer separation is based on the formation of the so-called reversible diastereomeric complexes of the stereoselective analyte and stereoselective host molecule (selector) representing the chiral stationary phase (CSP). The chemical nature of CSP spans from small molecules to stereoselective macromolecules, including biopolymers, such as cellulose, proteins or cyclodextrins (CDs) as well as macrocyclic antibiotics.

TABLE 2.2-3
ANALYSIS OF CHIRAL DRUGS IN BIOLOGICAL FLUIDS BY HPLC USING AMYLOSE DERIVATIVE – CHIRALPAX AD AS A CHIRAL SELECTOR

Column	Chiral Drug	Mobile Phase	Detection	Biological Sample	LOQ	Ref.
	mirtazapine	hexane/ethanol/ propan-2-ol (98:1:1 v/v/v), 1.5 mnl/min	UV 290 nm	plasma	–	191
	albendazole sulfoxide	hexane/propan-2-ol/ ethanol (81:4.25:4.75, v/v/v), 1.2 ml/min	SF 280/320 nm	plasma	–	192
	metoprolol	hexane/ethanol/DEA (88:12:0.2, v/v/v)	SF 229/298 nm	urine	–	193
	disopyramide mono-N-dealkyl /disopyramide	hexane/ethanol (91:9, v/v), 0.1% DEA, 1.2 ml/min, 22°C	UV 270 nm	plasma & urine	12.5 ng/ ml	194
	tramadol o-desmethyl-tramadol	isohexane/ethanol/ DEA (97.3, v/v/v), 1 ml/min	MS-MS	plasma	0.5 ng/ ml	195
	chlorpheni-ramine, monodesmethyl chlorpheni-ramine	hexane/ethanol/DEA (96:4:0.05), 0.5 ml/ min	UV 262 nm	plasma & urine	35 ng/ml	196
	propafenon 5-hydroxypropa-fenone	hexane/ethanol/DEA (88:12: 0.1), 1.3 ml/ min	UV 315 nm	plasma	–	197
	omeprazole	0.004 % acetic acid/ 30% ethanol/1% acetonitrile in isohexane (by volume), 1 ml/min	MS	plasma	10 n mol/L	198
	acebutolol	hexane/ethanol/DEA (85:15: 0.1, v/v/v), 0.8 ml/min	UV 240 nm	serum	10 ng/ml	199
	ketoprofen	hexane/propan-2-ol/ TFA (80:19:1, v/v/v) 1 ml/min	UV 254 nm	plasma	–	200
	halofantrine	hexane/ethanol/ butan-2-ol /DEA(93:4.5:2.5:0.1, v/v/v/v), 03 ml/min	SF 300/ 380 nm	plasma	6 ng.ml	20
	antifungal agent SCH 56592	hexane/ethanol/DEA (30:70:0.2, v/v/v)	SF 270/320 nm	serum	–	202
	anti-tumor agent SCH 66336	hexane/ethanol/DEA (30:70: 0.2 v/v/v), 0.8 ml/min, 39°C	UV 280 nm	plasma	0.25 μg/ ml	203

TABLE 2.2-4
ANALYSIS OF CHIRAL DRUGS IN BIOLOGICAL FLUIDS BY HPLC USING CYCLODEXTRIN DERIVATIVE AS CHIRAL STATIONARY PHASE

Column	Chiral Drug	Mobile Phase	Detection	Biological Sample	LOQ	Ref.
β-CD (Cyclobond)	chlorpheniramine and metabolite	DEA (0.25%, pH 4.4)/ methanol/ acetonitrile (85:7.5:7.5 v/v/v), 0.5 ml/min	MS	plasma	125 pg/ ml	205
	methadone and metabolite	0.1% TEA, 0.6 % glacial acetic acid adj. pH 5.0 with 2 M NaOH/40% acetonitrile, 0.3 ml/ min	UV 220 nm	plasma & urine	2.5 ng/ ml 5.0 ng/ ml	206
	PNU-83894* PNU-83892** (anticonvulsant agent and metabolite)	acetonitrile/water/ 0.2% TEA adj. to pH 6.0 with acetic acid (20:80, v/v), 0.7 ml/ min	UV 230 nm	plasma	25 ng/ml	207
	mianserin and desmethyl-mianserin	6% acetonitrile/94% TEA buffer (1% TEA to pH 3 with ortho-phosphoric acid), 1 ml/min	SF 270/430 nm	plasma	4 ng/ml (S)-,2.5 ng/ml (R)-	208
	warfarin	acetonitrile/glacia acetic acid/TEA (100:0.3:0.25, v/v/v)	UV 320 nm	plasma	–	209
	Simendan	0.5% TEAA (pH 6.0) containing 24–33% methanol	UV 380 nm	plasma	5 ng/ml	210
	Sch-39304 triazole antifungal agent	water/methanol/ acetonitrile (80:15:7, v/v/v), 0.71 ml/min	UV 205 nm	plasma	–	211
	propranolol	acetonitrile/ethanol/ glacial acetic acid/ TEA (96:4:0.4:0.3, v/ v/v/v), 1 ml/min	SF 222/340 nm	plasma	1.5 ng/ ml	212
	ibuprofen	acetonitrile/0.02% TEA (pH 4.0), 3:2, v/v), 1 ml/min	UV 220 nm	plasma	1μg/ml	213
Acetylated β-CD	fluoxetine & norfluoxetine	methanol/0.3% TEA (pH 5.0), (25:75, v/ v), 1 ml/min, 30°C	UV 214	plasma & urine	8 μg/ml	214
	warfarin	acetonitrile/acetic acid/ TEA (1000:3:2.5, v/v/v), 1 ml/min	MS-MS	plasma	1.0 ng/ ml	215

TABLE 2.2-4
CONTINUED

Column	Chiral Drug	Mobile Phase	Detection	Biological Sample	LOQ	Ref.
β-CD (Chiradex)	mephenytoin	acetonitrile/water/ glacial acetic acid/ TEA (14:86:0.1:0.2, v/v/v/v), 0.9 ml/min	UV 207 nm	urine	12.5 μg/ ml	216
	modafinil	20 mM phosphate buffer (pH 3.0)/acetonitrile (21:4, v/v), 0.6 ml/ min	UV 225 nm	plasma	–	217

* PNU-83894 is 3.4-dichloro-N-[(1R-2S)-2-(methylamino) cyclohexyl] benzamide.
** PNU-83892 is N-[(1R-2S)-2-aminocyclohexyl]-3,4-dichloro benzamide.

2.8 PIRKLE CONCEPT CHIRAL COLUMNS

CSPs based on small chiral selector molecules include "Pirkle-type" CSPs. Appropriate selectors of this type, which are presented in Table 2.2-1, have been employed in studies performed in animal and human models on the enantioselective pharmacokinetics of a preparation, warfarin sodium, anti-coagulant agent to be used in patient treatment [141]. A column-switching technique has been used for determination of nicardipine in human plasma [142], as well as in studies of the well-known preparation of ibuprofen to assess its pharmacokinetic profile in rabbits that have been administered the drug in the form of suppositories [143,144].

Pirkle CSP operating in the normal-phase mode has been used to investigate the separation of enantiomers of PNU 103017, a potential agent for AIDS treatment, glutethimide and propranolol [145–147]. The enantiomers of debrisoquine and amino acids have been successively determined in human urine and plasma using Pirkle CSP operating in the reversed phase mode, respectively. [148,149]. Pirkle DNBL chiral stationary phase operating in the reversed-phase mode has been used to investigate the metabolism of racemic cholecystokinin-β-antagonists [150].

Occasionally, other chiral stationary phases have been used such as crown ether (Crownpak CR (+)), which has been employed for analysis of clinafloxacin in human plasma [151] or Chiralpak WE, which operates by the ligand exchange principle, used in studies on the stereoselective pharmacokinetics of RS-8359, a monoaminoxidase A inhibitor, chemically known as (±)–4-(4-cyanoanilino-5,6-di-hydro-7-hydroxy-7H-cyclopenta [d] pyrimidine [152].

2.9 CELLULOSE-DERIVATIVE CHIRAL COLUMNS

Among CSPs that have found widespread application in the bioanalysis of numerous drugs and their metabolites are CSPs based on cellulose or amylose derivatives adsorbed

on macroporous silica gel. Table 2.2-2 presents drugs for which cellulose derivatives have been used as a chiral selector for the enantioselective analysis of their concentration. Depending on the column type, normal or reversed phase conditions have been employed. The cellulose type CSPs (Chiralcel OD) have been used in separating the enantiomers of triazole antifungal [153], ibuprofen [154], ketamine and its active metabolite norketamine [155], the antiepileptic 10-hydroxycarbazepine [156], bisoprolol [157], lorazepam [158], propranolol [159], anti-inflammatory agent (MTPPA) [160,161], praziquantel [162], propafenone [163] and metoprolol [164]. For all these drugs the developed methods have been sensitive and sufficiently reproducible for stereoselective monitoring of these agents in human plasma (serum and urine) during pharmacokinetic studies.

TABLE 2.2-5
ANALYSIS OF CHIRAL DRUGS IN BIOLOGICAL FLUIDS BY HPLC USING CYCLODEXTRIN DERIVATIVE AS CHIRAL MOBILE PHASE ADDITIVES

Column	Chiral Drug	Mobile Phase	Detection	Biological Sample	LOQ	Ref.
C_{18} Lichrospher	Chlorthalidone	methanol/0.1 M phosphate buffer pH 4.0) containing 2% TEA and 12.5 mM-β-CD (25:75, v/v), 0.8 ml/min	UV 230 nm	plasma	50 ng/ml	218
Non-porous octadecyl-silane	ketoprofen	0.1% TEA (pH 4.0)/ acetonitrile containing 10 mM-HP-β-CD (49:1, v/v), 0.8 ml/min	UV 220 nm	serum	10 ng/ml	219
Prodigy-ODS	pentazocine	10 mM KH_2PO_4 (pH 5.8)/ethanol containing 20 mM sulphated-β-CD (4:1, v/v), 0.7 ml/min	UV 220 nm	serum	15 ng/ml	220
Prodigy-ODS	trimipramine	10 mM ammonium acetate (pH 4.0)/ethanol containing 20 mM β-CD (4:1, v/v), 0.7 ml/min	UV 220	serum	10 ng/ml	221
TSK-gel ODS	thiamylal	17 mM β-CD in 150 ml ethanol and adding 10 mM KH_2PO_4 to litre, 0.9 ml/min	UV 288 nm	serum	50 ng/ml	222
Suplecosil LC-Si	azepinoindole	10 mM Na_3PO_4 (pH 7.0) /15 mM-β-CD/ 2 mM TEA (1:1:1:1, v/v/v/v), 1 ml/min	UV 231 nm	plasma	–	223

TABLE 2.2-6
ANALYSIS OF CHIRAL DRUGS IN BIOLOGICAL FLUIDS BY HPLC USING PROTEINS AS CHIRAL SELECTOR

Column	Chiral Drug	Mobile Phase	Detection	Biological Sample	LOQ	Ref.
α_1-acid glycoprotein (Chiral-AGP)	methadone	10 mM phosphate buffer containing 0.1% of N,N-dimethyloctylamine (pH 6.6)/propan-2-ol (90:10, v/v), 0.6 ml/min	UV 205 nm	serum & blood	25 ng/ml	224
	methadone	10 mM ammonium acetate buffer, with 0.05% of N,N-dimethyloctylamine (ph 6.6)/propan-2-ol (85:15, v/v)	MS-ESI	serum & saliva	5 ng/ml	225
	methadone and metabolite	20 mM NaH_2PO_4 containing 2 mM dimethyloctylamine and 9% acetonitrile (pH 5.5 with H_3PO_4), 0.4 ml/ml	UV 210 nm DAD	urine	0.125 μM	226
	methadone	Propan-2-ol/2mM ammonium acetate pH 5.8 buffer using gradient 500 μL/min	MS	sweat	2ng/patch	227
	propafenone and 5-hydroxy-propafenone	10 mM ammonium acetate buffer (pH 5.95)/propan-2-ol (100:9, v/v), 0.5 ml/min	MS-SRM via ESI	plasma & urine	20 ng/ml	228, 229
	ketamine norketamine	10 mM KH2PO4 (pH 7.0) containing 16% methanol, 1 ml/min, 40°C	UV 220 nm	plasma	2 ng/ml	230
	mainserin & desmethyl mainserin	6% acetonitrile in 20 mM K H2PO4 (pH 5.3), 0.9 ml/min	UV 214 nm	plasma	10 nM	231
	verapamil norverapamil	0.01 M phosphate buffer (pH 6.65)/acetonitrile (91:9), 0.9 ml/min	SF 227/308 nm	plasma	–	232
	bupivacaine and metabolite	8 mM NaH2PO4 and 0.1 M NaCl/propan-2-ol/DEA (96:4:3:0.6, v/v/v) (pH 7.04) with 50% H3PO4), 0.9 ml/min	UV 210 nm	urine	5 ng/ml	233

TABLE 2.2-6
CONTINUED

Column	Chiral Drug	Mobile Phase	Detection	Biological Sample	LOQ	Ref.
	labetalol	0.02 NaH_2PO_4– buffer, 0.1 M tetrabutylammonium hydrogen bromide, (pH 7.1 with H_3PO_4), 0.7 ml/min	SF 330/418 nm	plasma	–	234
	propanolol	20 mM ammonium acetate buffer (pH 4.1)/acetonitrile (98:2), 1.0 ml/min	SF 290/340 nm	tissue homo-genates	–	235
	ketoprofen	5 mM phosphate buffer (pH 7.0) with 1 mM N,N-dimethyloctylamine, 1 ml/min	UV 225 nm	water	–	236
	tiaprofenic acid	2% propan-2-ol in 0.01 M phosphate buffer pH 6.5, 0.5 ml/min, 17°C	UV 317 nm	plasma	–	237
	cetirizine	10 mM phosphate buffer (pH 7.0)/acetonitrile (95:5, v/v), 0.4 ml/min	UV 230 nm	plasma	25 ng/ml	238
	epibatidine	10 mM NH4COOH (pH 7.4 with NH4OH), 0.7 ml/min	MS	liver microsomes	–	239
	amlodipine	10 mM acetate buffer (pH 4.5)/propan-2-ol (99:1, v,v)	MS	plasma	0.1 ng/ml	240
	ibuprofen	0.4% (v/v) propan-2-ol in 0.1 M phosphate buffer pH 7.0, 0.9 ml/min	UV 220 nm	plasma	1.16 μg/ml (R)–(–) & 1.7 μg/ml (S)–(+)	241
	thiamylal	Propan-2-ol/20 mM KH2PO4 (3:97, v/v), (pH 4.7), 0.9 ml/min	UV 288 nm	serum	–	242
	albendazole	propan-2-ol/8 mM phosphate buffer (pH 7) (1:49, v/v), 0.9 ml/min	UV 290 nm	plasma	–	243
	lansoprazole	propan-2-ol/0.1 M NaH_2PO_4 (pH 7.0 with acetic acid) (1:1 v/v)	UV 283 nm	serum	–	244

TABLE 2.2-6
CONTINUED

Column	Chiral Drug	Mobile Phase	Detection	Biological Sample	LOQ	Ref.
	ketorolac	propan-2-ol/90 mM NaH_2PO4 /10 mM Na2HPO4/2 mM dimethyloctylamine (pH 5.5) (17:183), 0.5 ml/min	UV 320 nm	plasma	5 ng/ml	245
	masapride	0.02 M Na_2HPO_4/ 0.02 M citric acid/ methanol (gradient elution)	SF 314/354 nm	plasma	–	246
	citalopram	10 mM hexanoic acid in H_3PO_4/NaoH buffer (pH 6.5), 0.9 ml/min	UV 240 nm	plasma	–	247
	thiopentone	4.5% propan-2-ol in 0.1 M phosphate buffer (pH 6.2), 0.9 ml/min	UV 220 nm (6 min) then 287 mm	plasma	–	248
	vamicamide	20 mM phosphate buffer (pH 6.3)/ acetonitrile (20:1)	UV 260 nm	serum & urine	10 ng/ml	249
Human serum albumin (Chiral HAS)	ketoprofen	94% (v,v) 0.01 M phosphate buffer 6% (v/v) propan-2-ol and 5 mM octanoic acid at pH 5.5, 0.6 ml/min	UV 260 nm	plasma	16 ng/ml (S)– (+)– 18 ng/ml (R)–(–)	250
Ovomucoid (Ultron ES-OVM)	nimodipine	23.5% ethanol in 20 mM phosphate buffer, 1 ml/min	UV 230 nm	plasma	1 ng/ml	251
	propranolol	20 mM phosphate buffer (pH 6.9)/acetonitrile	SF 290/340 nm	micro-dialysis probes	25 ng/ml	252

The Chiralcel OD-R column has been used for the separation of the enantiomers of pyridoglutethimide in serum using glutethimide as internal standard [165]. Also, the column has been used in the separation of the enantiomers of fluoxetine and its active metabolite norfluoxetine [166], lansoprazole and its metabolites 5-hydroxylansoprazole and lansoprazole sulfone [167]. With the help of the above-mentioned selector, a method has been developed to separate the enantiomers of propranolol in investigations of their stereoselective permeation across human skin in vitro, where the permeation of (S)-propranolol has been demonstrated to be slowed in the presence of an enantioselective excipient [168]. In addition, the selector has been employed in a method that is suitable for studies of cytochrome P450 isozyme (CYP2D6) activity

utilizing debrisoquine as a marker drug of the oxidative genetic polymorphism [169]. Also Chiralcel OD-R column has been utilized successfully in developing a method to determine the enantiomers of methamphetamine and its metabolites in the hair and urine of abusers. To improve the sensitivity of the fluorescence detection, which constitutes an element of the method, the compounds under investigation have been derivatized with 4-(4,5-diphenyl-1H-imidazol-2-yl)-benzoylchloride as a fluorescence-labeling reagent [170,171]. As well as selfotel [172], tramadol [173], ondansetron [174], diazepam and its metabolite nordiazepam [175], apomorphine [176] and pirlindole [177] have been enantioselectively resolved with the help of the above-mentioed selector. Chiralcel OD-RH has been used for the separation of amphetamine and its metabolite in hair [178] and verapamil and its metabolite norverapamil [179]. The cellulose-type CSP (Chiralcel OJ) has been used in the separation of the enantiomers of cisapride [180], isradipine 181], metyrapone [182] and manidipine [183].

TABLE 2.2-7
ANALYSIS OF CHIRAL DRUGS IN BIOLOGICAL FLUIDS BY HPLC USING ANTIBIOTICS AS CHIRAL SELECTOR

Column	Chiral Drug	Mobile Phase	Detection	Biological Sample	LOQ	Ref.
Vancomycin (Chirobiotic V)	fluoxetine	Methanol containing 0.075% (by weight) ammonium trifluoroacetate	MS-MS	plasma	1 ng.ml	256
	citalopram and metabolites	Methanol/acetic acid/ TEA (400:02:0.25, v/ v/v) 1 ml/min	UV 240 nm	plasma	5 ng/ml	257
Teicoplanin (Chirobiotic T)	arotinolol	Methanol/acetic acid/ TEA (100:0.1:0.1, v/ v/v), 0.8 ml/min	UV 317 nm	plasma	50 ng/ml	258
	clenbuterol	methanol/ acetonitrile/ acetic acid/TEA (70:30:0.3:0.2, v,v,v,v), 1 ml/min, 25°C	UV 254 nm	tissue homo-genates	–	259, 260
	atenolol	acetonitrile/ methanol/acetic acid/ TEA (55:45:0.3:0.2, v/v/v/v)	SF 274/325 nm	urine	–	261
	albuterol	methanol/ acetonitrile/acetic acid/TEA (50:50:0.3:0.2, v/v/v/ v), 1 ml/min	SF 230/310 nm	plasma	125 pg/ ml	262
Ristocetin A (Chirobiotic R)	2-hydroxy-glutaric acid	5 mM triethylamine pH 7.0 with acetic acid/methanol (9:1), 0.5 ml/min	MS-MS ESI	urine	–	263

Rogletimide has been determined in human serum by utilizing the Chiralcel OJ-R column [184]. With the help of a multistep linear gradient system, the OJ-R column has been used to separate the enantiomers of pantoprazole [185]. Chiralcel OJ-R selector has also been used for the separation of the enantiomers of ketorolac administered to

TABLE 2.2-8
ANALYSIS OF CHIRAL DRUGS IN BIOLOGICAL FLUIDS BY GC USING CYCLODEXTRIN DERIVATIVES AS CHIRAL SELECTOR

Column	Chiral Drug	Gas carrier/ column temp.	Detection	Biological Sample	LOQ	Ref.
octakis-(3-O-butanoyl-2,6-di-O-pentyl)-γ-CD (dissolved in polysiloxane SE-54)	valnoctamide	Nitrogen/112°C isothermally	FID	plasma	0.2 ng/L	264
heptakis(2,3-di-O-methyl-6-O-ter-butyldimethyl silyl-β-CD (dissolved in polysiloxane SE-52)	isoflurane	Hydrogen/27°C isothermally	MS-SIM	blood	–	265
heptakis-(6-O-hexydimeth-ylsilyl-2,3-di-O-methyl)-β-CD	cyclophospha-mide and dechloro-ethylated metabolite	hellium/temp. program from 100°C to 180°C	MS-SIM	plasma & urine	0.4 nmol/L	266, 267
heptakis (2,3,-di-O-methyl-6-O-tert-butyldimethy-lsilyl-βCD (dissolved in polysiloxane SE-52)	3-hydroxy-2-methyl-butanoic acid	Hydrogen/temp. program from 60°C to 200°C	MS-ITD	urine	–	268
heptakis (2,3,-di-O-methyl-6-O-tert-butyldimethy-lsilyl-βCD (dissolved in polysiloxane SE-52)	3-hydroxy and 3-amino isobutyric acid	Hydrogen/temp. program from 60°C to 210°C	MS-ITD	urine	–	269

children for postsurgical analgesia. The investigators demonstrated the stereoselective character of the binding process occurring between the drug and plasma proteins, as well as its metabolism occurring via glucuronidation [186]. Praziquantel and promethazine have been quantitated in human serum using the Chiralcel OJ-R column [187,188]. The chiral tribencel selector has been used to separate the enantiomers of thalidomide in human plasma with UV detection [189,190].

2.10 AMYLOSE-DERIVATIVE CHIRAL COLUMNS

Amylose-derivative CSPs have been used for the determination of the enantiomers of several drugs and their metabolites (Table 2.2-3). Chiralpak AD has been employed in the enantioselective bioanalyis of the concentration of mirtazapine, a novel anti-depressant, albendazole sulfoxide, an anthelmintic agent and metaprolol, a β-adrenergic antagonist in patients with depression, neuorocysticerosis and hypertension, respectively. The results of these studies point to the enantioselectivity and kinetic disposition of these drugs in the investigated groups [191–193]. In the case of metoprolol [193], these investigations have revealed the accumulation of the S-(-)-enantiomer in hypertensive patients phenotyped as extensive metabolizers of debrisoquine. Amylose AD column has also been employed in the enantioselective analysis of the concentration

TABLE 2.2-9
ANALYSIS OF CHIRAL DRUGS IN BIOLOGICAL FLUIDS BY HPLC OR GC METHOD USING CHIRAL DERIVATIZING AGENTS

Chrial Drug	Chiral derivatizing agent	Detection	Biological Samples	LOQ	Ref.
mexiletine and N-hydroxy-mexiletine glucuronide	o-phthaladehyde N-acetyl-L-cysteine	SF 350/455 nm	plasma	1 ng.ml	271, 272
ibuprofen	S-(-)-1-(1-naphtyl) ethylamine in the medium of ECF and TEA	SF 280/320 nm	plasma	0.1 μg/ml	273
ibuprofen	L-Leucinamide in the medium of ECF and TEA	UV 220 nm	plasma	0.1 μg/ml	274
indobufen	L-Leucinamide in the medium of ECF and TEA	UV 275 nm	serum	0.25 μg/ml	275, 276
3,4-methylene-dioxymeth-amphetamine	R-(-)-α-methoxy-α-trifluoro-methyl-phenylacetyl chloride	MS	plasma	0.1 μg/ml	277
clopidogrel	S-(-)-1-(1-naphthyl) ethylamine in the presence of HOBT and EDAC	SF 280/330 nm	plasma	0.1 μg/ml	278

of disopyramide, tramadol, chlorpheniramine, propafenone and their corresponding active metabolites [194–197]. The methods developed for each drug and its metabolites have been validated and their clinical relevance has been confirmed in pharmacokinetic studies performed in healthy volunteers.

Furthermore, an amylose AD column has been used as a CSP for enantioseparation of omeprazole, acebutolol, ketoprofen, as well as halofantrine and their active metabolites in humans [198–201]. The validation of these methods has demonstrated their effectiveness in clinical pharmacokinetic studies. With the help of the above-mentioned selector two methods have been developed, which have been successfully applied to determine the enantiomers of antifungal agent SCH 56592 and anti-tumor agent SCH 66336 in plasma and evaluation of its chiral inversion in animals [202, 203].

2.11 CYCLODEXTRIN-DERIVATIVE CHIRAL COLUMNS

Cyclodextrins (CDs) are extensively used in HPLC as stationary phases bonded to a solid support or as mobile phase additives. CDs, like other biopolymers, are CSPs based on inclusion complexation. In other words, the solute molecule may enter completely or in part to chiral cavities or chiral bay areas, thus leading to different diastereomeric inclusion complexes with different association and/or dissociation constants. Detailed information on the basic principles of chiral separation and application of CDs can be found in a review [204]. Table 2.2-4 illustrates examples of drugs for which CD derivatives have been used for enantiomeric separation by HPLC. CD derivatives have been employed as chiral selectors in the bioanalysis of enantiomer concentration of chlorpheniramine [205] methadone [206], novel anti-convulsant agent, PNU 83892 [207] and mainserin [208]. The validation parameters for these methods have been determined in the presence of their metabolites and can be applied to the analysis of clinical samples. With the help of the above-mentioned column, a method has been developed which has been successfully applied to determine the enantiomers of warfarin [209], simendan [210] triazole antifungal agent SCH 39304 [211], propanolol [212] and ibuprofen [213].

Chiral separation on an acetylated-β-CD column has been employed in studies of the pharmacokinetic profiles of the enantiomers of fluoxetine and norfluoxetine [214], as well as warfarin [215]. In the case of mephenytoin, this is used for routine determination of the catalytic activity of CYP2C19 in humans based on the measurements of its S/R enantiomer ratio [216]. A β-CD (Chiraldex) selector has been used for quantitation of the enantiomers of modafinil in human plasma [217].

2.12 CYCLODEXTRIN-DERIVATIVE CHIRAL MOBILE PHASE ADDITIVES

As previously mentioned, CDs can also be used successfully as chiral mobile phase additives leading to diastereomeric inclusion complexes of the analyte in the chiral cavity of the host polysaccharide. Table 2.2-5 shows examples of drugs for which CD

derivatives have been added to the mobile phase. This type of chiral separation has been employed in the enantiospecific analysis of chlorthalidone [218], ketoprofen [219], pentazocine [220], trimipramine [221], thiamylal [222] and azepinoindole [223].

2.13 PROTEIN CHIRAL COLUMNS

CSPs that are commonly used include α-acidglycoprotein (AGP), human serum albumin (HSA) as well as ovomucoid (OVM). One of the advantages of these protein-based chiral phases is that separation is performed in the reversed-phase mode. Examples employing proteins in enantioselective drug analysis are listed in Table 2.2-6.

2.13.1 AGP column

The chiral-AGP column belongs to the second generation of this type of CSP, and has been used in the enantioselective analysis of methadone in various biological samples collected from drug abusers undergoing methadone detoxification. These methods have been successfully employed to quantitate methadone enantiomers in serum as well as in other biological fluids collected noninvasively, such as saliva, sweat and urine [224–227]. In view of the preferential metabolism of the active (R)-methadone and of important interindividual variability in the enantiomeric ratio, the stereoselective determination of methadone is necessary to improve the quality of treatment of narcotic addiction. Chiral-AGP has also been employed in investigations of other drugs: for example, to assess the stereoselective pharmacokinetics of propafenone and its major active metabolite 5-hydroxypropafenone in healthy volunteers [228,229]. Other examples of the enantioselective analysis of drugs such as ketamine [230], mainserine [231] and verapamil [232] and their metabolite in plasma were also reported. The enantioselectivity of urinary excretion of bupivacaine and its metabolite during long-term continuous postoperative epidural infusion of the racemic drug has been determined by this selector [233].

In an investigation of the role of gender differences in labetalol kinetics in humans, the chiral-AGP analytical column has been used to determine the concentration levels of four stereoisomers of this drug [234]. Three of the four stereoisomers of labetalol have been demonstrated to be higher in women than in men. However, the concentrations of the β-blocking stereoisomer (R,R-labetalol) were similar between sexes, possibly explaining the similarity between sexes in antihypertensive response. HPLC using the chiral-AGP column has also been employed in investigations of the stereoselective enzymatic hydrolysis and skin permeation of a diastereomeric propranolol ester product [235] and in studies on skin permeability of ketoprofen enantiomers [236]. This type of chiral separation has also been employed in studies of the stereoselective disposition of tiaprofenic acid enantiomers [237] and citrizine [238] in rats. While using the chiral-AGP column in investigations of the metabolism of the enantiomers of epibatidine, a natural product active at nicotinic acetylcholine receptors,

heptafluorobutyric acid has been employed as an ion-pair reagent, in view of the hydrophilicity of this compound [239]. With the help of the above-mentioned selector, methods have been developed which have been successfully applied to determine the enantiomers of a mlodipin [240], ibuprofen [241], thiamylal [242], albendazole [243], lansoprazole [244], ketorolac [245], mosapride [246], citalopram [247], thiopenton [248] and vamicamide [249].

2.13.2 HSA column

Human serum albumin (HAS) has been used as a chiral selector in a method for determining the concentration of ketoprofen enantiomers in horse plasma and, in order to avoid time-consuming manipulations, the column-switching system has been applied [250].

2.13.3 Ovomucoid column

Ovomucoid-CSP (OVM) has been used in investigations of the concentration-time profiles of nimodipine enantiomers in patients with subarachnoid bleeding after IV and oral administration of nimodipine [251], as well as in the analysis of the free fraction concentration of propranolol enantiomers obtained by microdialysis in rats [252].

2.14 MACROCYLIC ANTIBIOTIC CHIRAL COLUMNS

Among the newest CSP types are macrocylic glycopeptide antibiotics, which are covalently bound and perhaps constitute the most varied class of chiral selectors. They include a variety of functional groups, which are ideal for providing multiple stereoselective interactions [253–255]. Table 2.2-7 lists examples of compounds whose enantiomeric separation has been performed using these antibiotics as chiral selector.

2.14.1 Vancomycin column

Vancomycin has been employed as a chiral selector in a stereoselective HPLC assay for fluoxetine in plasma of depressed patient [256]. Also, this chiral selector has been employed to the assay of citalopram and its metabolite in the plasma of depressed elderly patients [257]. The method described is rapid, sensitive and reliable and allows assessment of pharmacokinetic parameters and their putative relationship to clinical (side) effects.

2.14.2 Teicoplanin column

Teicoplanin is a macrocyclic antibiotic which has been recently employed as a chiral selector for the determination of arotinolol enantiomers in human plasma [258]. This

chiral selector has also been used for the determination of clenbuterol enantiomers in human [259] and rat plasma [260] by HPLC. A teicoplanin analytical column has also been applied in the enantioselective assay of atenolol [261] and albuterol enantiomers [262]. The analytical method for the enantioselective determination of atenolol enantiomers in human urine involved a combination with on-line column switching.

2.14.3 Ristocetin A column

Ristocetin A, a glycopeptide antibiotic, has been applied in the determination of the configuration of 2-hydroxyglutaric acid in urine. This method can be used in diagnosing patients with two metabolic disorders, D-2-hydroxyglutaric aciduria and L-2-hydroxyglutaric aciduria [263].

2.15 GC

Gas chromatography (GC) has found uses in the enantioselective analysis of chiral drugs in biological samples utilizing CDs. Examples of such applications are shown in Table 2.2–8. Stereoselective GC has been used in investigations aiming at comparing the interspecies pharmacokinetics of the enantiomers of valnoctamide, a new anticonvulsant agent [264], as well as in the pharmacokinetic profiles of isoflurane enantiomers during and after anaesthesia [265]. Stereoselective GC methods have been employed in studies of the enantioselective pharmacokinetics of cyclophosphamide and its dechloroethylated metabolites in cancer patients. These investigations have been very helpful in evaluating the proportion of a cyclophosphamide dose that is transformed to a toxic or active metabolite [266,267]. CD derivative CSPs have also found use in the analysis of three biologically-active endogenous chiral compounds, 3-hydroxy-2-methylbutanoic acid (HMBA), 3-hydroxyisobutyric acid (HIBA) and 3-aminoisobutyric acid (AIBA), which can play important roles in the diagnosis of β-ketothiolase deficiency of [HMBA] or 3-hydroxyisobutyric aciduria, respectively [268,269]. These investigations made use of the enantioselective multi-dimensional gas chromatography – mass spectrometry, which has proven to be a valuable tool for the discrimination of enantiomers from complex matrices when present in trace amounts.

2.16 INDIRECT CHIRAL CHROMATOGRAPHIC METHODS

As previously mentioned, another type of chiral separation technique employed both in liquid and gas chromatography is indirect separation. Table 2.2-9 presents examples of employing the indirect method for determining the concentration of enantiomers of selected drugs and endogenous substances in various biological matrices using HPLC or GC. Under indirect separation conditions, the enantiomeric analytes react with a chiral derivatizing agent (CDA) to form a mixture of diastereomers, which posses different physicochemical properties and thus are separated by conventional achiral chromatography. This technique puts high demands on the enantiomeric purity and stability of the

CDA. Moreover, the process of derivatization may often be time-consuming [270] and frequently requires strict control of temperature and time. Despite such hindrances, as is shown in Table 2.2-9 indirect methods employing various CDAs, such as derivatives of L-cysteine, isocyanates, ethylamine, acid chlorides or L-leucinamide, have been used in the enantioselective determination of concentrations of numerous drugs in various biological matrices by HPLC or GC. The technique has also been employed in studies on the enantioselective mexiletine glucuronidation [271,272]. It has also found an application in investigations of the pharmacokinetics of the enantiomers of ibuprofen [273, 274] and indobufen [275, 276], as well as 3,4-methylenedioxy methamphetamine [277] in humans. The techniques has been employed in studies of stereoselective inversion of clopidogrel [278] using liver microsomes and hepatocyte suspension, respectively.

2.17 OTHER METHODS

As has been mentioned, the quality of the analytical method employed for the determination of xenobiotics in biological materials is also determined by isolation of the analyte from endogenous substances. A technique suggested in this area is coupled achiral/chiral chromatography, where the interferences are separated from the target compound on an achiral column and the eluents containing the compound of interest are selectively switched to the CSP for stereochemical analysis. This switching system, which minimizes the loss of compounds during sample preparation, shortens the process and allows for its automation. This has been employed in the enantioselective analysis of serum phenylcarbarmic acid derivatives [279] and urine terbutaline [280].

In addition to the chromatographic system, the method of detection also contributes to assay specificity and sensitivity. Commonly used detection methods such as UV absorbance, fluorescence and electrochemical detection are increasingly replaced by mass spectrometry. GC-MS and LC-MS have become the methods of choice in the analysis of xenobiotics in biological media due to their enhanced sensitivity and selectivity in comparison to the traditional technology. This allows an assay to be conducted at more physiologically relevant concentrations and less stringent sample preparation methods are required. In the past few years, increasingly perfected techniques have been employed in bioanalysis, allowing for shorter analysis times and higher resolution capacities, coupled with lower consumption of solvents, which is environmentally more friendly compared to normal phase liquid chromatography. An example of this kind of technique is packed-column supercritical fluid chromatography (pcSFC). In SFC the mobile phase consists of a mixture of carbon dioxide as the main component and polar organic modifiers such as methanol are added. This technique has been applied in the study of the pharmacokinetics of ketoprofen enantiomers in humans [281].

2.18 LIST OF ABBREVIATIONS

Chiralpak AD	Amylose tros (3,5-dimethylphenylcarbamate)
AGP	α_1-Acid glycoprotein

C_{18}	Octadecylsilane
CD	Cyclodextrin
DEA	Diethylamine
DNBL	Dinitrobenzyl leucine
GC	Gas chromotography
HSA	Human serum albumin
HPLC	High performance liquid chromotography
LOQ	Limit of quantification
MS	Mass spectrometry
MS-MS	Tandem mass spectrometry
Chiralcel OD & OD-R	Cellulose tris(3,5-dimethylphenylcarbamate)
Chiralcel OJ & OJ-R	Cellulose tris(4-methylbenzoate)
OVM	Ovomucoid
SF	Spectrofluorometry detector
TEA	Triethylamine
UV	Ultraviolet spectrophotometry

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CHAPTER 3

Capillary electrophoresis for the determination of drugs in biological fluids

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In the last few years, steady progress has been achieved in the application of capillary electrophoresis (CE) to drug analysis in biological fluids. As the environmental protection and safety rules are getting tighter in regard to organic solvents disposal, the merits of CE become more evident. Continued interest is observed in the application of stereoselective analysis by CE especially. The CE instruments do not consume too much of these expensive additives and the separation is much faster compared to that by HPLC. These stereoselective methods are being applied to the analysis of drugs not only in tablet form but to those in serum samples too. Various agents are explored for stereoselectivity such as different proteins and polysaccharides.

Since a main problem in CE is poor detection limits, several strategies to concentrate the drugs from the biological samples by stacking and by extraction are described. Many new extraction methods are emerging as more suitable for CE, such as solid-phase microextraction compared to the traditional method of liquid-liquid extraction. Many of these methods do not require sample evaporation. Several methods for stacking as a simple means for sample concentration on the capillary are described.

Non-aqueous CE (NACE) is found to have several advantages for the analysis of drugs. Since many drugs are not that water soluble, NACE offers better solubility, different selectivity, and the ability to use higher voltages. Several clever ideas are described to obtain basic studies on drugs by CE; e.g. rapid methods to speed up determination of the pKa or drug binding. Commercial CE-MS instruments are now available for drug metabolism and confirmation. As far as therapeutic drug monitoring (TDM) is concerned, only new drugs are analyzed by CE. The routine drugs are more conveniently analyzed by immunoassays. Both practical and recent developments in the analysis of drugs by CE are discussed.

References pp. 92–94

3.1 INTRODUCTION

CE is a very flexible technique which is useful for the analysis of a wide variety of drugs. It has great potential for the separation and analysis of samples which contain numerous compounds because of the high plate number generated in the capillary, due to the flat profile which does not disperse the migrated band compared to the parabolic profile in high-performance liquid chromatography (HPLC). This potential has been captured elegantly in the area of DNA analysis, which has resulted in speeding up the mapping of the human genome. However, in drug analysis less impressive but steady progress has been achieved, especially in the pharmaceutical field. Compared to HPLC, CE offers several distinct advantages for therapeutic drug monitoring (TDM), e.g. speed, ease of analysis, low cost of operation, and especially the high plate number for a very good resolution. The problem of using and disposing of large amounts of organic solvents for column elution is almost absent in CE. In addition, CE offers basic information on the physico-chemical properties of the drug, such as binding and dissociation constants, especially in the early stages of drug development where only small amounts may be present.

On the other hand, CE suffers from a few problems. Understanding these problems leads to better strategies when encountering them. Unlike HPLC, CE is greatly affected by the sample matrix; i.e. salts and proteins [1], which can be both disadvantageous as well as advantageous, as will be evident later. Another major problem in drug analysis by CE is the sub-optimal detection limits, especially for analysis of new drugs in serum. In order to utilize CE successfully for the practical separation of drugs in biological samples, it is important to understand the limitations of this technique and the means to overcome them. Here we discuss both the practical aspects and the recent advances in drug analysis by CE. We stress in this section three important aspects in the analysis of drugs by CE: (1) Sample concentration, including stacking and extraction, in order to analyze the new drugs which tend to be present in low concentration. (2) Improving precision in order to get reproducible data. (3) Description of how CE can be used to expand the horizon of therapeutic drug monitoring such as stereoisomer analysis in serum, drug protein binding, etc.

3.2 CE, HPLC AND IMMUNOASSAYS IN DRUG ANALYSIS

The majority of drugs can be analyzed successfully by any of several techniques including immunoassay, CE, GC, and HPLC. Because of their ease, convenience, and automation, immunoassays remain the favorite choice in routine laboratories provided a commercial automated system is available. However, new drugs do not have commercial immunoassays available and the real choice for these drugs is between HPLC and CE. Several papers have compared CE and HPLC [2]. As an example we will discuss later the analysis of caffeine by both HPLC and CE. It is now accepted that these two methods are complementary to each other. Some drugs tend to be more suited for HPLC, e.g. hydrophobic compunds, and those present in low concentration while ionized and large molecules and those present in high concentrations tend to be

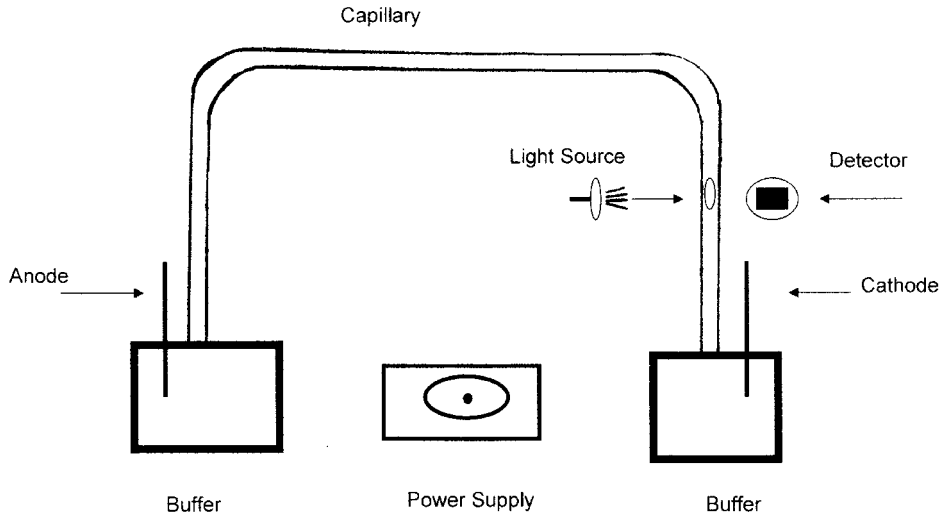

Fig. 3.1. Components of the capillary electrophoresis instrument.

analyzed more easily using CE. Method development in general is faster by CE. Several studies have shown that CE, when compared to HPLC for TDM, is faster and easier with better resolution, especially for the polar compounds, with less operating cost. The capillary is less expensive than the HPLC column and can tolerate extreme pH and direct protein injection. The sample size is very small in CE (~ 10 nl) with the whole volume of the capillary only 1–3 μl. The use of large volumes of expensive organic solvents in HPLC is not needed in CE.

3.3 INSTRUMENTATION AND DETECTION

The CE instrument is composed mainly of a capillary 10–60 cm long with 20–100 μm (i.d.) filled with a buffer and its ends are dipped into two buffer reservoirs. A 5–30 kV voltage is passed through the two electrodes dipped into the two buffer reservoirs, Fig 3.1. The sample is injected usually on the anodic side of the capillary. The silanol groups of the capillary wall impart a negative charge to the surface of the capillary, which under the applied electric field leads to net movement of fluid towards the cathode (electroosmotic flow). As the pH increases, the electroosmotic flow increases too. The sample analytes migrate based own their own charge/mass ratios in addition to that by the electroosmotic flow. Thus the sample components pass through the detector window located at the cathode end of the capillary with positively charged compounds arriving first followed by the neutral, and finally the negatively charged ones. Because the capillary requires thorough washings and filling with fresh buffer for each sample, a fully automated instrumentation is necessary in CE analysis. Most of the commercial instruments in drug analysis use a single capillary while in DNA analysis many

instruments use multi-capillaries to analyze several samples at the same time. Temperature control for the capillary improves the reproducibility. The ultraviolet detection is very common in CE. Laser induced fluorescence, electrochemical detection, and mass spectrometry are used less frequently.

3.4 MODES OF CE IN DRUG ANALYSIS AND DETECTION

In CE, there are several general methods or modes of analysis which separate the analytes on the basis of different principles. These different modes can be used to separate and also to confirm an unknown drug. However, in drug analysis few of these modes, as described here, are commonly utilized:

3.4.1 Capillary zone electrophoresis (CZE) (Free solution CE)

This is the simplest and most commonly used type of CE. The capillary is filled with a buffer, which provides separation based on the differences in the velocity of the molecules, i.e. charge/mass ratio differences directly without major additives.

3.4.2 Micellar electrokinetic capillary chromatography (MECC)

This method is useful for separating neutral (or uncharged) compounds. Here, the buffer contains a surfactant, such as sodium dodecyl sulfate in the form of micelles. The interior region of the micelle is hydrophobic while the outside is hydrophilic (ionic). Most of the micelles carry a charge and thus migrate under the influence of the electrical field. The sample components partition between the buffer and the micelles in MECC similar to that in chromatography. Neutral and hydrophobic compounds spend more time bound to the micelle migrating with it relative to a hydrophilic species.

3.4.3 Chiral separation

Here the buffer contains special chiral additives such as the cyclodextrins (CD) and some macrocyclic antibiotics that can preferentially interact with one isomer versus another and affect its migration. This mode of CE is used for the separation of optical isomers, especially in the pharmaceutical industry.

3.4.4 Non-aqueous CE (NACE)

This method is becoming popular in drug analysis. It is similar to CZE in principle but the separation is performed in organic solvents, mostly in the presence of buffers. This technique is suitable for compounds which are water-insoluble.

3.5 BUFFERS

Buffers with low absorbance and with matched ion mobility are chosen in CE to give the proper ionization to the analyte, with the pH approximating the average pK of the solute mixture. The buffer is used preferably within 1 pH unit from its pKa. Buffers have been traditionally used at very low concentration in CE to yield rapid analyses while keeping the current and the generated heat very low. These low concentrations are very suitable for analyzing drugs in pure solvents but not suitable for stacking or for dealing with samples with high salt content. A high ionic strength buffer, which tends to generate more heat and eventually slow the separation, is needed for biological samples and for stacking. It has the advantage of also shielding the analytes from interaction with silanol groups on the capillary walls. Based on trial and error, some buffers separate the compound from the interferences better than others. Organic buffers which contain amine or zwitterions give a better stacking for cationic compounds and favor transient isotachophoresis too. Borate is used often in CE because it does not generate too much heat so high voltages can be used. Phosphate buffers have a low ultraviolet absorbance. Uncharged compounds require the addition of a charged surfactant such as SDS to the buffer to separate them by MECC.

3.6 SAMPLE SIZE AND INJECTION

Analysis in CE can be performed using continuous buffer conditions, i.e. the buffers at the anode, at the cathode, in the capillary, and in the sample are all of the same composition and same ionic strength or also by using discontinuous buffers. The sample size in CE, based on continuous buffers, should be kept below 1%. However, if a discontinuous buffer is used or the sample is of low ionic strength, a larger volume can be injected. In practice, the sample size is plotted against the peak height. As long the peak height increases (linearly) the sample size can be increased, indicating there is stacking. However, when this relationship stops it indicates there is sample overloading. Also, in biological samples the interfering peak becomes a limiting factor to sample size. Samples can be introduced into the capillary hydrodynamically, e.g. by pressure or sample elevation and also by electromigration (by voltage), provided the sample is low in ions. Both of these methods have their own merits.

3.7 SAMPLE PREPARATION IN CE

Sample preparation from biological fluids can be both simple as well as difficult, depending on the drug concentration and presence of interfering substances. Drugs relatively present in high concentration in serum (~ 10 mg/L) can be injected directly without any sample treatment or after a simple deproteinization step [1]. Acetonitrile is a much more preferred method than acid for deproteinization in CZE. Salts formed in the process of neutralization of the acid filtrate tend to deteriorate the separation. On the other hand acetonitrile (one volume of serum sample to two volumes of acetonitrile)

removes the protein and causes sample concentration (stacking) as will be described later.

The surfactants such as sodium dodecyl sulfate (SDS) used in the micellar electrokinetic capillary chromatography (MECC) methods solubilize the proteins, allowing injection of serum directly on the capillary. However, the majority of new drugs are present in concentrations < 1 mg/L, far below the detection limit of CE by direct serum injection, necessitating sample concentration and also clean up.

In practice, sample preparation remains the major obstacle and the most time-consuming step in CE analysis, especially for new drugs, which are more potent compared to the older generation of drugs, and thus are present in very low concentration. Sample clean up offers another advantage of removing the sample matrix, salts and proteins, which can interfere in the analysis in CE. General strategies for improving the detection limits in CE have been reviewed by Hempel [3], including stacking, extraction, and the use of special flow cells (bubble flow cells). Here are in brief some of the methods used for sample cleanup and concentration in CE.

3.7.1 Off-column concentration

Traditional liquid-liquid extraction (LLE) and solid phase extraction are used often for sample preparation in CE. However, in the last few years there has been interest in new methods which are more suitable for CE since this technique does not require large volumes of sample.

3.7.1.1 Solid-phase microextraction (SPME)

SPME is a solvent-free sample preparation technique that is commercially available and utilizes a thin coating attached to the surface of a fused fiber (e.g. silica) as the extraction medium. The coating is a material like polymethylsiloxane, polyacrylate, or carbowax of different thickness. The fiber is dipped first into the sample for a certain period of time then dipped into the eluting solvent, e.g. organic solvent, and injected directly [4]. Extraction yield depends on optimizing the pH, salt, temperature and time. The method has been described by Pawliszyn [5,6] and initially was applied for the analysis of organic compounds in the field of environmental sciences, especially by GC. However, it has been successfully extended to the analysis of a wide variety of compounds by CE, LC, and GC [4,7]. For example, acidic drugs in urine such as naproxen [8] and the tricyclic drugs (9) were extracted from urine and analyzed by CE using this method. The application of this technique has been reviewed recently [4].

3.7.1.2 Molecular imprints

These compounds select or interact with certain compounds based on steric and chemical memory for the template. They are analogous to antibodies in immunoassays where they can also be used in competitive assays. They have been used in LC and CE [10,11].

3.7.1.3 Membrane based techniques

Different membrane types such as microporus, homogeneous, ion exchange and asymmetric have been employed for sample preparation for GC, HPLC and CE. The extraction in this technique can be further automated [12,13].

3.7.1.4 Immunoaffinity solid-phase extraction

The optimization of the binding and elution and the miniaturization for this type of sample cleaning has been reviewed [14,15] and has been applied in CE and LC.

3.7.1.5 Homogeneous liquid-liquid extraction

This uses the phase separation phenomenon from homogeneous solution as salting effect or cloud point extraction with the target solute being extracted into a small volume of separate phase. This method can give high concentration and is suited for CE [16].

Sometimes the extract after solvent evaporation is not concentrated enough to yield the desired sensitivity. Thus, it is recommended to dissolve the sample in an appropriate solvent, which can give stacking as will be described later. The importance of sample extraction and concentration together with stacking for CE has been emphasized by dedicating a special issue of The *Journal of Chromatography A* to this subject [17].

3.7.2 Online concentration

3.7.2.1 Solid-phase extraction

Sample clean up and concentration can be performed directly on the capillary by inserting a small piece of a capillary packed with different solid packing materials as described [18,19].

3.7.2.2 Stacking

The electric current can be used to achieve three distinct goals: to separate the sample, move fluids and the analytes and also to concentrate the sample. Two or three of the above goals can be achieved at the same time, such as sample concentration and separation. Stacking is a general term to indicate sample concentration under the influence of the current. Originally it was used in ITP to indicate the movement of the sample components adjacent to each other as a stack of coins [20]. Stacking methods are not general enough, thus some of them work only under certain conditions. However, they can be performed conveniently on the capillary with the separation step.

In stacking, a large sample of about 5–50% of the capillary volume usually is injected; however, the sample band is markedly sharpened before it enters the separation buffer. In order to obtain sample concentration or stacking in CE, the same analytes at the different areas of the sample plug have to migrate at different velocities in such a way that the band sharpens. The major factor is the difference in the field strength or charge at different areas of the sample plug which arises from the use of discontinuous buffers.

3.7.2.2.1 Methods for stacking for capillary zone electrophoresis
Salts present in the sample can ruin the separation [1]. However by choosing the proper stacking method the salts can be beneficial for the separation and for the concentration. It is easier to stack in the capillary zone electrophoresis (CZE) compared to the other modes.

3.7.2.2.1.1 Isotachophoresis (ITP) and Transient-ITP. Isotachophoresis is based on the use of discontinuous buffers (in respect to ions with different mobilities) which produces different field strength. All the ions and analytes have to migrate at the same velocity. An ion with fast mobility and in high concentration is used as a leading ion while a slow mobility ion is used as a terminating ion. The sample ions should have an intermediate mobility. At equilibrium the sample ions adjust their concentration in order to move at the same velocity as the leading ion. The electric field strength is inversely proportional to the ion mobility in that region.

The method can concentrate and semi-purify the samples 10–1000 fold for both small and large molecules. The concentrated segments can be coupled to the CZE for further separation and quantification using a single or separate capillary [21]. ITP is difficult to perform in practice because of the rigid requirements. A simpler form of the ITP is the transient ITP, which is easier to couple to CZE in the same capillary [22,23]. In this method, the ITP conditions are met briefly just before the sample enters the separation buffer [24]. It is suited for samples which contain salts since they can act as leading ions.

3.7.2.2.1.2 (Pseudo-transient-ITP). Mixing organic solvents such as acetonitrile acetone and small alcohols with the sample (~66%) removes proteins and in the presence of salts, either from the sample or added, leads to stacking with ~20 fold increased sensitivity. The salts, present in the samples act briefly as leading ions, while the organic solvents can function as a terminating ion in what is called "pseudo-isotachophoresis" (pseudo-ITP). Because of their low conductivity, these water-miscible organic solvents provide the high field strength necessary for band sharpening similar to that provided by the terminating ion [25,26]. Thus sodium chloride (present in serum at ~150 mmol/l), together with the organic solvents, enhances the stacking process. Organic buffers containing amine or zwitterionic groups stack better for cationic compounds [27]. In practice this type of sample treatment is well suited for practical work, especially for samples which contain salts such as serum, food, or of industrial origin.

3.7.2.2.1.3 High-Field Stacking. A Low ionic strength buffer in the sample (dilution) cause the sample resistance and the field strength in the sample plug to increase. In turn, this causes the ions to migrate rapidly and stack as a sharp band at the boundary between the sample plug and the electrophoresis buffer, with the positive ions lining up in front of the negative ones [27,28]. Once in the electrophoresis buffer, the components of the sample migrate in different zones according to their charge/mass characteristics. The sample can be injected hydrodynamically or by electromigration. The sample usually is dissolved in a lower ionic strength buffer (~10 times less) relative to the separation

buffer. In practice, this is a very simple method to perform. In this technique the amount of sample concentration is limited to about 10 times and often does not exceed about 5 fold. Buffer removal is important when injecting very large sample volumes to avoid the miss-match of EOF. After hydrodynamically injecting the sample, the polarity is reversed [28] to improve the stacking. Furthermore in the electromigration a plug of water can be injected before the separation buffer, which can enhance this technique further. The analytes (cations) experience high field strength, move rapidly, and concentrate at the tip of the capillary at the boundary between the sample and the separation buffers. Several hundred-fold enhancements in concentration have been shown to be possible by various forms of this technique for some drugs [28].

Other methods to concentrate for CZE which utilize pH changes or isoelectric focusing have been described [20].

3.7.2.2.2 Stacking in MECC

Stacking in MECC is more difficult than CZE. However several strategies have been devised by Terabe and coworkers for stacking in this mode based on low ionic strength in the sample, polarity reversal to remove the sample buffer and sweeping the analyte (interaction) with the micelles [29–32].

3.8 PRECISION

Precision is very important, especially in routine analysis. Early work indicated that the precision of CE is less than that of HPLC. However, more recent work showed improvement in the precision through better washing steps (1M NaOH, or 100 mM phosphoric acid), using internal standards, fresh buffers, constant temperature and the effective mobility. Mayer [33] and Altria and Bestford [34] have reviewed and discussed in detail the conditions which lead to a better precision in CE [33]. The quality control for a few antiepileptic drugs by CE has been assessed by Thormann et al. [35]. The intra-day RSD was less than 3% and the inter-day RSD was less than 8% [35]. The effective mobility is more reproducible than migration time giving a RSD of mobility of 0.01–0.03% [36]. The corrected effective mobility, based on standards, was applied by different labs analyzing a set of 20 acidic compounds. It decreased the CV for MECC into half without effect on that for CZE [37]. Stacking improves the precision for both peak height and area.

3.9 CE AND BASIC INFORMATION FOR DRUGS

CE offers several advantages which are not well appreciated in the routine therapeutic drug monitoring area. For example, it offers the ability to analyze simultaneously the main drug and its metabolites or several related drugs in the same run and to study the bound and the free drug levels. Many of these studies are important in assessing the therapeutic level of the drug and hopefully will find more application in clinical labs. In this respect the CE offers several features to these basic studies such as small sample

size, speed, assessing protein binding, and the prediction of drug interaction with cell membranes.

3.9.1 Chiral separation

About 40% of drugs contain at least one chiral center and mostly are sold now in one enantiomeric form. Isomers have very close chemical structures; however, in many cases they exhibit different biological effects, bind to proteins, or are metabolized differently. The principles of this technique depend on the selective interaction of one isomer with the cavity of special additive compounds such as cyclodextrins (CD), heparin, Chaps, or Big Chaps during electrophoresis.

CE and capillary electrochromatography (CEC) are becoming more utilized for this purpose. Because the amount used in CE is very small, the expensive chiral additives are usually added to the buffer and the separation is performed by MECC. In CEC the chiral separations can be performed by columns similar to those for the HPLC or by adding the chiral selector directly to the mobile phase.

A rapid method for chiral separation for chiral molecules has been described [38] based on using the short end of the capillary and with a highly sulfated cyclodextrins (CD). The enantiomers of 12 chiral amino-containing pharmaceutical basic compounds belonging to various therapeutic categories were analyzed by CE using carboxymethyl-CD [39]. The chiral separation of drugs has been reviewed recently [40].

3.9.2 Acid dissociation constant (pKa)

The pKa is an index of the ionization of a drug at different pH values and is an important indicator of solubility and interaction with membranes. In CE, the pKa can be determined by measuring the mobility of the drug as a function of pH similar to that by HPLC. Schmutz and Thormann [41] determined how the physical and chemical properties of 25 drugs would affect their analysis by MECC. They found that compounds which did not bind tightly to proteins, in addition to those with a low pKa, dissociated easily from the bound proteins and migrated as sharp peaks. A rapid screening method for the determination of pK(a) of candidate drugs by pressure-assisted capillary electrophoresis (CE) coupled with a photodiode array allowed very rapid analysis (< 1 min) based on pH mobility-shifts as well as their uv spectra [42]. The difference between pKa values by this method and by other conventional methods is within 0.25 units for 82 ionic functional groups of drugs [42]. Another fast screening method for the determination of the dissociation constants of acidic, basic, and multivalent compounds was developed by using also pressure-assisted capillary electrophoresis [43]. The method was evaluated in terms of accuracy, precision, and ruggedness by using a set of 48 compounds with pKa values ranging from 2 to 10 [43]. The advantage of the CE in pKa is the small amount of sample which is needed for the measurement, which makes this method valuable at the early stages of drug discovery [44,45].

3.9.3 Predicting drug-membrane interactions

Hydrophobicity is important in drug interactions with biological membranes, bioavailability distribution, and blood-brain barrier permeability. Often it is estimated in vitro by octanol-water partition coefficient or capacity factors by HPLC. However, CE offers some advantages such as the small sample size, low operating costs and speed [46,47]. Microemulsion electrokinetic chromatography, a technique similar to MECC [47] was used to estimate octanol-water capacity factors for some drugs and the values were very close to those in the literature. Using oppositely-charged surfactant vesicles as a buffer modifier to estimate hydrophobicity, there was a linear relationship between the log of capacity factor and the octanol-water partition coefficient for both neutral and basic species. Vesicular electrokinetic chromatography using surfactant vesicles as buffer modifiers was used for the estimation of hydrophobicity [48]. The chiral surfactant dodecoxycarbonylvaline has been used as a pseudo-stationary phase for the separation of many enantiomeric pharmaceutical compounds [49]. Its hydrophobicity was a good predictor for n-octanol-water partition coefficients for 15 beta amino alcohols [49]. A correlation was obtained for capacity factors determined by MECC, involving the use of phosphatidylcholine-bile acid mixed micelles in the separation buffer, by HPLC and log P of octanol-water [50]. The combination of MECC and HPLC data yielded a better predictive model for hydrophobicity [50]. The concentration of apolipoprotein H, a plasma glycoprotein, was performed by CE. Based on this determination, an interaction model of apoliporotein H and lipid monolayer was constructed [51].

Liposomes can be used as delivery vehicles for peptide and oligonucleotide drugs, influencing drug tissue distribution and protection. A simple method has been described for free and encapsulated oligonucleotide drugs in liposomes by CE in entangled polyacrylamide solution [52].

3.9.4 Free and bound drugs

Drugs bind to various serum proteins with different affinities with the free fraction of the drug considered as the active. In routine drug monitoring, the total of a drug (bound + free) is assayed. Drug–protein interaction becomes important in some disorders such as malnutrition and renal failure where the amount of the free drug changes due to changes in protein concentration. The free, bound, percentage of binding and the binding constant can be determined based on several well-established techniques such as dialysis, filtration, and size exclusion, including CE. All have some advantages and some disadvantages such as time, membrane leakage, or low concentration. CE required 200 times less serum compared to the filtration methods.

In CE, drug binding is followed based on changes in the electrophoretic mobility [53–55], especially by frontal analysis, which can be performed by CE and HPLC. In CE (55), the capillary is filled with the buffer and the different drug concentrations, in the presence and absence of a fixed binding protein concentration, are incubated at 25°C followed by injection of a large amount of sample (5–7% of the capillary volume). The

free drug, the complex and the protein yield each a frontal, plateau shaped peak. The free drug concentration can be calculated from the height of the frontal peak [56,57].

Kraak et al. [54] described three different methods for measuring protein-drug binding by CE. The first is based on the Hummel-Dryer method in which the capillary is filled with a buffer containing the drug and giving a large background signal. The sample, which contains the drug, protein, and the buffer, is injected. The bound drug migrates differently from the free drug producing a negative peak, which is a measure of the bound drug. The second method is based on the vacancy method where the capillary is filled with a mixture of the buffer, the drug and the protein. The sample, which contains only the buffer, is injected. Both the free and the bound drug migrate separately and each gives a negative peak. The third method depends on frontal analysis. The percentage and the binding association constant can be also calculated from a Scatchard plot of the data at the different drug concentrations [56].

Drug binding for anionic drugs which had similar mobility to plasma proteins were studied by frontal analysis in the presence of cyclodextrins [58]. The binding values were comparable to those by ultra filtration [58]. Rundlett and Armstrong described and reviewed six different CE-based techniques for evaluating binding constants with examples [59]. Also,the estimation of binding constants based on affinity CE has been reviewed with a description of different methodologies by Tanaka and Terabe [60].

3.10 SELECTED APPLICATIONS OF DRUGS ANALYZED BY CE

Many drugs can be analyzed for therapeutic use as well as for abuse. Thus the boundary between the two areas is not that sharp. CE has great potential in drug screening and confirmation because of its high plate number [61–63]. Furthermore, the same CE instrument can be applied to separations in various modes, which is useful in confirmation. Each mode offers a distinct separation mechanism and has distinct merits. In this regard, a test mixture of six basic and six acidic compounds was used to study the separation behavior of five CE methods by different modes. The results showed that three methods (CZE, MECC, and NACE) were suitable for the analysis of basic compounds while three methods (based on CZE and MECC) were suited for the analysis of the acidic compounds. Solid-phase extraction (SPE) and LLE methods provided clean extract from serum and urine, with the SPE method producing cleaner extracts and higher peaks, and were able to elute basic and acidic compounds in one fraction. The potentials and pitfalls of the various methods were discussed [64].

Basic drugs tend to interact with the capillary wall, giving poor separations. Several strategies are used to overcome this problem such as high buffer concentration, coated capillaries, different pH, including dynamic coatings. A commercial dynamic coating was used to test the separation of some drugs such as methamphetamine, amphetamine, and cocaine. The separation using phosphate buffer with polyanion was complete in 5 minutes. The relatively high EOF for the dynamically coated capillary system allowed for the rapid screening of basic, acidic, and neutral drugs [65]. The use of CE–MS in the analysis of some enantiomeric drugs and their metabolites has been described [66].

Morphine (MOR) and related opioids (free and glucuronidated) in human urine, after liquid-liquid extraction or solid-phase extraction, were analyzed by CE–MS [67]. Wey

and Thormann [68] described a CE method, based on binary phosphate buffer containing ethylene glycol and UV detection, for identification of codeine and its major metabolite in urine. Tagliaro et al. [69] described heroin and cocaine analysis in hair by immunoassays and CE. The complete separation of amphetamine, methamphetamine, 3,4-methylene-dioxy-amphetamine, 3,4-methylene dioxy methamphetamine, mescaline, cocaine and benzoylecgonine was obtained using an acetonitrile-based buffer [70]. Electrochemical detection using a platinum micro-disk electrode set was found to be selective for MDA, MDMA and mescaline [70].

Drug analysis by CE has been described and reviewed [71–76]. However, in this section we describe a few selected drugs which have been analyzed recently by CE. The application of MS, which is very important in drug confirmation and metabolism, to CE represents a very specialized area and has been reviewed recently [77].

We start here by giving examples for the analysis of serum caffeine used for treatment of apnea in the neonatal by both HPLC and CE. The details are given in the legend of Fig 3.2. The sample is introduced directly on the CE without treatment but it was deproteinated with methanol for HPLC. The speed of the CE method is ~4 min. vs. ~8 min. for HPLC with no interferences. The analysis was linear by the two methods from 2–50 mg/l. The CV of the CE was slightly better because of less sample handling (2.4 vs. 3.5%, $n = 12$). The regression analysis between the two methods CE = HPLC X 1.01 + 0.01, $r = 0.98$.

Analgetics: The release of *R* and *S* isomers of ibuprofen, a non steroidal anti-inflammatory drug, was monitored *in vitro* in buffered solutions and in rat plasma and separated by CE [78]. Also, several acidic non-steroid anti-inflammatory drugs were determined by CE after liquid-phase microextraction (8). An enantioselective method for analysis of ibuprofen in human plasma in phosphoric acid/triethanolamine buffer containing sulfated β-CD after liquid extraction was described [79]. A special automated continuous flow system operating simultaneously with the CE has been also described to determine the anti-inflammatory drugs [80].

Amphetamines: Amphetamines are powerful stimulants of the central nervous system. Because they are often abused they are routinely analyzed or screened for in most labs. For CE, whole blood was extracted by acetonitrile, evaporated and injected into the CE instrument and separated in a phosphate buffer, pH 2.5. The analysis was accomplished in 7 min. [81]. Another derivative, 3,4-methylene-dioxymethamphetamine, has also been determined in urine by non-aqueous CE and fluorescence detection [82]. A nonaqueous CE method, coupled to UV and electrospray mass spectrometry, was described for the simultaneous analysis of ecstasy (amphetamine-like) and other related derivatives in urine [83].

Antibiotics: Several antibiotics such as cephalosporins, piperacillin, imipenem, amoxicillin and clavulanic acid have been determined by CE [84] and compared to HPLC [85]. The results were similar but CE did not require organic solvents [85]. Addition of pentanesulfonic acid to the running buffer at pH 6.5 improved the separation. Quinolones such as enrofloxacin, ciprofloxacin, danofloxacin, difloxacin, marbofloxacin, flumequine, and oxolinic acid belong to a group of synthetic antimicrobial drugs. They have been analyzed in pig kidney samples after solid phase

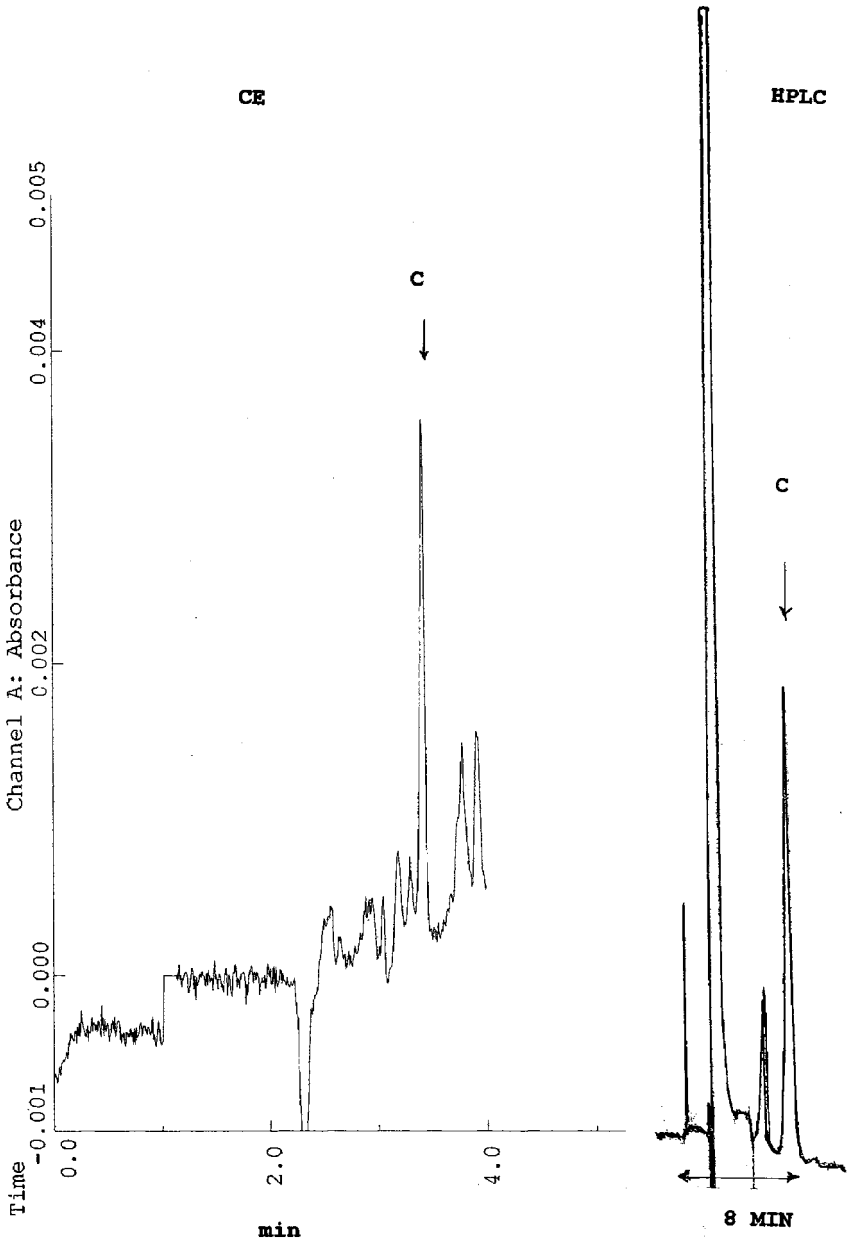

Fig. 3.2. Comparison of analysis of caffeine (C)(30 mg/L) by CE and HPLC: CE Conditions: Serum was injected directly for 15 s on a capillary (50um × 32 cm). The sample was electrophoresed for 5 min. at 10 kV, 280 nm in a buffer composed of 60 mmol/L triethanolamine, 70 mmol/L boric acid, pH 9.1, and 30g SDS /L. HPLC Conditions: Serum 20 μL was deproteinized in 200 μL methanol. A 20 μl aliquot of the supernatant was injected on CN 150 mm × 4.6 Microsorb MB, 5 μm column which was eluted by acetate buffer mmol/L pH 3.7 at flow rate of 1.0 ml/min with detection at 280 nm.

extraction by MECC [86] and by NACE [87]. The analysis of aminoglycosides by CE has been reviewed [88].

Antidepressants: The tricyclic antidepressants amitriptyline, imipramine, nortriptyline, and desipramine have been extracted from urine by solid-phase microextraction, using a thin coating attached to the surface of a fused silica-fiber and determined by CE with β-CD [9,89]. The antidepressant drug citalopram and its main metabolite *N*-desmethylcitalopram in human plasma were determined by CE after liquid-phase microextraction [90]. Fluoxetine, an antidepressant drug and its metabolite, norfluoxetine, in serum samples were analyzed using a CD-phosphate buffer at pH 2.5. The method was optimized for stereoselectivity. The combination of a neutral and a negatively charged CD (dimethylated-β- and phosphated-γ) provided the baseline enantiomeric separation of the two compounds. In all of the analyzed samples the enantiomeric forms of fluoxetine and norfluoxetine were easily identified. The fluoxetine and its metabolite enantiomeric ratio confirmed the stereoselectivity of the metabolic process of this drug [91].

Acyclovir: Acyclovir is a guanine derivative nucleoside analog with strong antiviral activity against herpes simplex and varicella zoster viruses. It was separated from plasma components using solid phase extraction. Separation was performed by MECC (SDS and hydroxypropyl-β-CD) in borate buffer (pH 8.8) containing 0.2% NaCl. High sensitivity was achieved by large volume sample introduction and stacking [92]. The analysis of this drug and its analogs has been reviewed [93]

Antiepileptic Carbamazepine: A MECC method for the analysis of this drug and its five major metabolites in plasma has been described [94]. The method involved using a phosphate buffer, pH 8.0 with SDS. The sample was extracted by solid phase extraction with detection at 214 nm [94].

Antineoplastics: *Doxorubicin*: is an anticancer drug for treatment of lymphoblastic leukemia in children. Hempel and coworkers [95] described an assay for this compound and its kinetics in pediatrics. To monitor the peak plasma levels, samples were deproteinated with acetonitrile. After centrifugation, the supernatant was applied directly to the capillary by hydrodynamic injection. For the determination of lower amounts of doxorubicin and its main metabolite, doxorubicinol, plasma was extracted by liquid-liquid extraction with chloroform. After evaporation of the organic phase, the sample was reconstituted in acetonitrile/water and injected into the capillary by electrokinetic injection [95]. Peak plasma concentrations of this drug were studied by CE [96]. Hempel and coworkers also described a similar strategy for analyzing methotrexate, leucovorin and their metabolites in serum by CE with UV detection [97]. A CE method with LIF was also described for the analysis of plasma daunorubicin and daunorubicinol, another anticancer drug [98]. Tomoxafin, an antiestrogenic drug, used by women at risk for developing hormone-dependent breast cancer, and its two major metabolites, *N*-desmethyltamoxifen and 4-hydroxytamoxifen, were extracted from mouse fetal tissues and analyzed by NACE with UV detection [99]. The uptake of vincristine, another anticancer drug, was studied by CE using electrochemical detection

with a carbon a microdisk bundle fiber electrode. A phosphate buffer pH 7.5 was used for the separation [100].

Furosemide: This diuretic was analyzed by CE with direct injection or after alkaline extraction using CZE and MECC with different detection schemes UV, LIF and MS [101]. Another diuretic, Torasemide, and three of its metabolites were also separated by CZE with UV detection [102].

Pilsicainide: This anti-arrhythmic drug was analyzed by CE. Serum was alkalinized and extracted with diethyl ether. Procainamide was used as an internal standard and the drug was separated in phosphate buffer at pH 2.3 [103].

Sulpiride: This selective dopamine D2 antagonist with antipsychotic and antidepressant activity is effective in the treatment of mental disorders. Plasma and urine samples were extracted by a double-step liquid-liquid extraction procedure and separated by CE in phosphate buffer (pH 8.0) with electro-generated chemiluminescence detection using tris(2,2′-bipyridyl)ruthenium(II) with platinum disc electrode as a working electrode [104].

Thalidomide: This sedative-hypnotic drug was introduced in the late 1950s but was discontinued because it caused birth defects through oxidative damage to DNA. However, the drug and its derivatives have a new therapeutic use due to the anti-inflammatory and immunomodulating properties in such diseases as leprosy, graft-versus-host disease following bone marrow transplantation, rheumatoid arthritis and several dermatological diseases. The isomers have important but different activity and toxicity, e.g. the R isomer is more tetragenic while the S is more sedative. This drug has been studied by CE in liver homogenates and its isomers were separated by sulfobutyl-β-CD [105].

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CHAPTER 4

Immunoassays for therapeutic drug monitoring and clinical toxicology

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4.1 INTRODUCTION

Immunoassays are quantitative analytical methods that make use of antibodies as reagents. The highly specific binding and large association constants (K_a) of antibodies make them useful for detection and quantitation of analytes in complex sample matrices such as body fluids including blood, urine, saliva [1], sweat [2], or vitreous humor [3]. Methods based on immunochemical reactions are some of the most sensitive and specific assays available to laboratorians. Many formats of immunoassays have been automated and are widely used in the clinical laboratory for therapeutic drug monitoring [4,5].

Antibodies belong to a group of glycoproteins called immunoglobulins that specifically bind to both natural and synthetic analytes, also called *antigens*. The site on the antigen where antibody binds is known as the *epitope*; each antigen may have multiple epitopes depending on its size and shape. Antibodies can be classified as either polyclonal or monoclonal. *Polyclonal antibodies* are a group of antibodies derived from multiple cell lines in an animal that are all specific for the same antigen, but bind to different epitopes on that antigen. *Monoclonal antibodies* are derived from an immortalized single cell line and are more specific in that they are not only directed toward a certain antigen, but also a specific epitope on that antigen.

There are five different classes of immunoglobulins based on their function and are designated as A, D, E, G and M. These proteins are represented by the abbreviations IgA, IgD, IgE, IgG, and IgM respectively; however, only IgG is generally used for an immunoassay reagent. Immunoglobulin G (IgG) is a glycoprotein composed of multiple subunits with a molecular weight of approximately 160 kD. It consists of two identical heavy chains and two identical light chains that are joined by disulfide bonds (see Fig. 4.1). The carboxy-terminal amino acid sequence is conserved regardless of antibody specificity and is known as the constant region of the antibody. However, the amino-terminal sequence is highly variable and is the region that confers antigenic specificity

to the antibody. The shape of the antibody is roughly similar to the letter "Y", giving it a single constant region with two variable regions that allow one antibody to bind two antigens, barring steric interference.

Regions of the antibody can be further characterized with respect to how it is cleaved by proteolytic enzymes. The enzyme papain cleaves IgG in the hinge region and divides the antibody into three sections as shown in Fig. 4.1. The constant region, or "stem" of the Y, is one fragment and is called the Fc fragment; it has no antigen binding properties. The remaining two fragments are the "branches" of the Y, and are identical antigen-binding fragments called Fab fragments; each branch contains one antigen-binding site. Alternatively, pepsin can be used to digest antibodies yielding a different fragmentation pattern. No Fc fragment is produced in this digestion because it is digested completely by pepsin. The fragment that remains after digestion contains both binding sites and is called the $F(ab)_2$ fragment, shown in Fig. 4.1.

Antibodies are produced by the immune system in response to an *immunogen* that is introduced into a foreign host. The immunogen can be a protein or in some cases an analyte of interest coupled to a large carrier molecule. For a substance to display immunogenicity, it must possess randomness of structure while maintaining some areas of structural stability. In addition, it must have a molecular weight of at least 4 to 5 kD and be recognized as foreign by the host animal. An analyte or antigen that is unable to

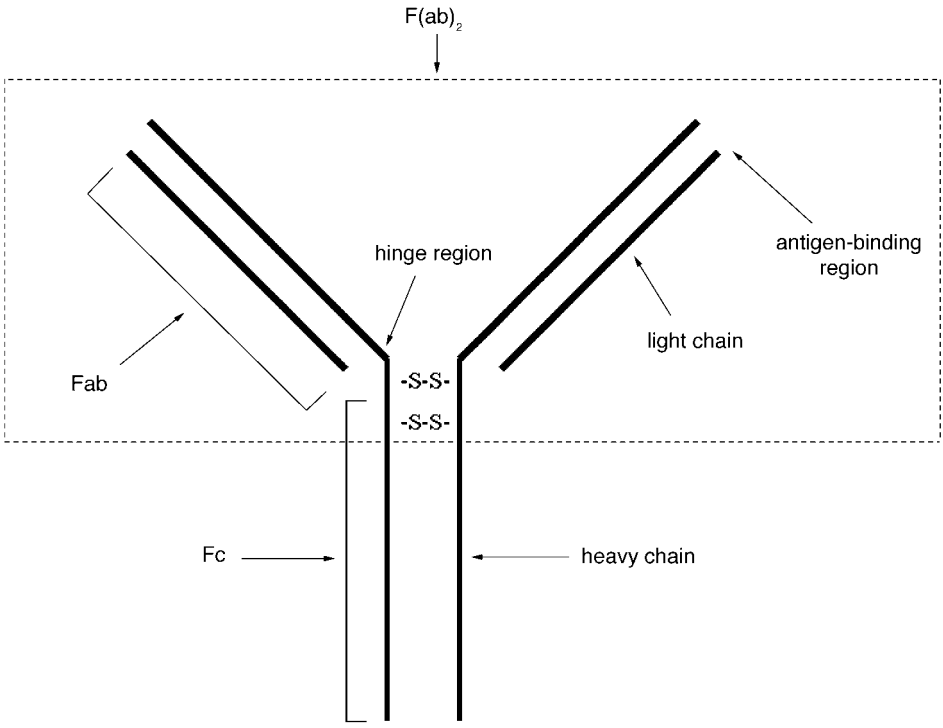

Fig. 4.1. Basic antibody structure.

induce an immunogenic response on its own is called a *hapten*, and must be coupled with a large carrier molecule to stimulate a response.

Antiserum is the product of immunogenic stimulation that contains antibodies directed toward the antigen of interest. *Polyclonal antibodies* are obtained by introduction of an immunogen to a normal animal host. This process produces a heterogeneous mixture of antibodies from many cell clones directed against a wide variety of epitopes present in the antigen of interest. These antibodies vary both in their specificity of binding and the affinity with which they bind. The antibodies can be isolated by affinity chromatography, which uses a chromatographic support containing immobilized antigen, or immobilized protein A or protein G. Protein A and protein G are bacterial cell wall proteins that display a site-specific affinity for the Fc region of IgG and isolate all IgG molecules regardless of specificity. Alternatively, the use of immobilized antigen allows the isolation of only IgG that is specific for the analyte of interest.

Monoclonal antibodies are the product of a single clone or plasma cell line; so all the antibodies produced are identical and bind to the same epitope with the same affinity. The process for producing monoclonal antibodies begins with immunization of a host animal (usually mice) just as in polyclonal antibody production. Then, sensitized lymphocytes from the spleen of the immunized host are fused with an isolated murine myeloma cell line, which consists of immortalized B-cells. The myeloma cell line is deficient in the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT), meaning they cannot synthesize purine bases from thymidine and hypoxanthine in the presence of aminopterin. The spleen cells, however, are not deficient in the enzyme so when the fusion mixture is cultured in a selective medium containing hypoxanthine, aminopterin, and thymidine (HAT medium), only the fused cells survive. The surviving cell lines are screened for binding to the antigen of interest, with the end product being immortal cell lines that produce antibodies of a single, known binding specificity.

Antibody-antigen binding can be described in terms of both binding affinity and avidity. *Affinity* describes the strength of binding for one site and can be defined in terms of the energy of interaction for a single antibody binding site and its corresponding epitope. *Avidity* describes the overall binding of one antibody molecule to antigen; defined as the sum of binding sites for the whole molecule (e.g. IgG has 2 binding sites while the IgM pentamer has 10 binding sites). For example, when comparing an IgG molecule versus an Fab obtained from the same clonal population; they would have identical affinities for the antigen of interest, but the IgG would have a higher avidity because it could bind two antigens and the Fab could only bind one. Generally speaking, affinity is characterized as a property of the antigen, while avidity is a property of the antibody.

Binding between and antibody and antigen is best described as an equilibrium process:

$$Ag + Ab <=======> AgAb$$

where the equilibrium constant for association of the complex (K_a) is much greater than the equilibrium constant for dissociation of the complex (K_d); so depending on the time frame of the experiment, the binding can seem to be irreversible. These interactions are

based on electrostatic (Van Der Waals) forces, hydrophobic forces, coulombic (ionic) forces, or a combination of the three. Antibody-antigen binding is pH and temperature dependent and can also be affected by electrolyte concentration as well, depending on which types of interactions are prominent in binding.

If there is significant cross-linking of antigens during antigen-antibody binding, then precipitation can occur, but this is highly concentration dependent. When the concentration of antibody binding sites is much higher than the concentration of epitopes for binding, then the epitopes are quickly saturated and 1:1 complexes of antibody and antigen predominate. Alternatively, if the concentration of antibody binding sites is limited in comparison with excess of epitopes for binding, then the antibody sites are quickly saturated, 2:1 complexes of antigen to antibody predominate, and cross-linking does not occur. However, if the antibody is in moderate excess (2- to 3-fold), then cross-linkage is more likely and large complexes can be formed that will precipitate; this is called the *precipitin reaction*. Addition of a linear polymer such as dextran, polyvinyl alcohol (PVA), or polyethylene glycol (PEG) will increase the rate of immune complex growth and enhance precipitation of the immune complex. Polymers used for this purpose must be high molecular weight, have minimal branching, and a high aqueous solubility.

4.2 IMMUNOASSAYS

Immunoassays comprise the group of immunochemical methods that are most widely used in clinical diagnostics. The use of chemical labels coupled to antibodies or antigens, allows much lower concentrations of analyte to be detected in samples than with more traditional immunoprecipitation methods. These assays can be performed in many different formats with a wide variety of detection methods for both large and small analytes. Immunoassays can be characterized in a number of ways: whether a separation step is necessary, on the basis of whether the antigen or antibody is labeled, or by the type of label that is used for sample detection.

4.2.1 Homogeneous vs. heterogeneous immunoassays

Heterogeneous immunoassays are assays that require physical separation of bound and free labels for analysis [6]. One approach to separation is precipitation of labeled antigen-antibody complex from solution using either a precipitating chemical or cross-linking with other antibodies, and reconstituting the precipitate to quantify the amount of bound label. Alternatively, adsorbents such as activated charcoal or other small particles can be added to solution to bind free antigen, followed by centrifugation to isolate the particles from solution. Another approach is to attach the antibodies to a solid support such as the interior of a microwell plate or magnetic beads, and then remove the unbound molecules through various wash steps. Traditional separation techniques such as size-exclusion or affinity chromatography, as well as electrophoresis can also be used for heterogeneous immunoassays.

Homogeneous immunoassays are those that can distinguish between the free and bound label for detection without a physical separation step [6]. In this format, the label is modified by antibody binding, resulting in either an increase or decrease of signal. For this format to be useful, the change in signal due to antibody binding must be proportional to the concentration of analyte under investigation. Homogenous immunoassays allow a combination of the label incubation and detection of analyte into one simple step. This approach is useful because it eliminates the wash step and is compatible with most automated chemistry analyzers. However, the major disadvantage to homogenous immunoassays is that they are less sensitive than heterogeneous immunoassays, making it difficult to measure analytes that occur in very low concentrations during therapy, such as digoxin.

4.2.2 Competitive binding immunoassays

Competitive binding immunoassays are based on antibody-antigen interactions in which the number of antigen binding sites on the antibody is limited. The antigen of interest and a labeled analog are incubated with a fixed concentration of the antibody, and the signal produced is based on competition between the analyte and corresponding labeled analog for antibody binding sites [7]. The amount of labeled antigen bound to the antibody is inversely proportional to the amount of analyte in the sample, producing a calibration curve as seen in Fig. 4.2a.

The two approaches to a competitive binding immunoassay are the simultaneous or sequential addition techniques. In *simultaneous addition*, both the label and sample (or calibrator) are added to the reaction mixture at the same time. After a fixed period of time, the amount of bound label is determined and used to construct a calibration curve

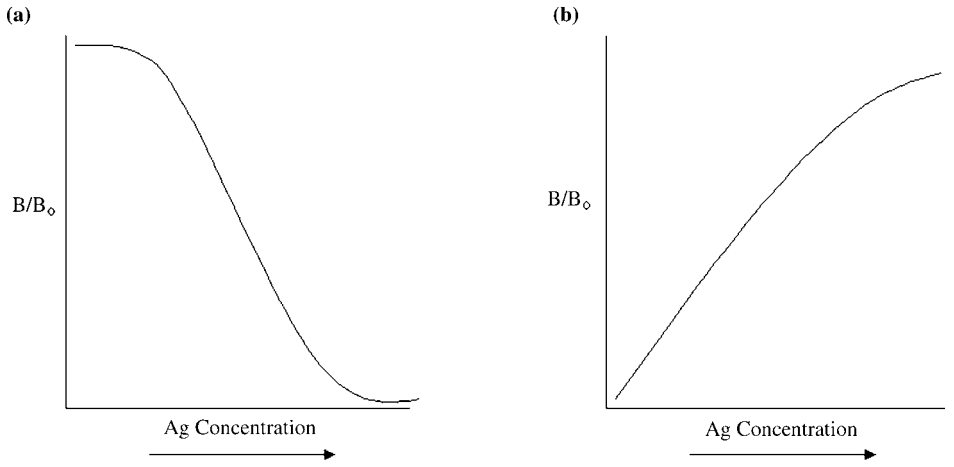

Fig. 4.2. Immunoassay calibration curves: (a) Competitive binding assay; (b) Non-competitive binding assay.

or determine the concentration of the unknown. For this method to be useful, the antibody should demonstrate the same or similar binding affinity for both the sample antigen and the calibrator antigen. In addition, it is important for this approach that the probability of binding for both the labeled antigen and the sample antigen are roughly equal. *Sequential addition* is a technique that is useful when a lower limit of detection is desired. First the sample is incubated with the antibody to equilibrium, followed by incubation with label to equilibrium, finishing with detection of the bound label. This sequential approach can lower the limit of detection up to 5-fold, and is also useful if there are slight differences in avidity for the analyte antigen and the labeled analog.

An alternative approach to the competitive binding immunoassay is the *displacement immunoassay*. The displacement immunoassay format has been used in the development of dry chemistry thin film immunoassays [8]. Dry film immunoassays have been developed for drugs such as carbamazepine, phenytoin, and valproic acid [9]. In this technique, the antibody is saturated with a labeled analog, followed by addition of sample. Analyte in the sample displaces the label and the displaced label is measured rather than bound label. This type of assay is dependent on local equilibria, so if the K_a is much greater than the K_d, then less label is displaced in contrast to the situation where K_a is slightly greater than or equal to K_d. The signal for displacement immunoassays is directly proportional to the analyte concentration as shown in Fig. 4.2b, in contrast to the inversely proportional signals seen in the simultaneous and sequential addition immunoassays.

4.2.3 Non-competitive (immunometric) immunoassays

Non-competitive binding immunoassays, or immunometric assays, are based on an antibody-antigen reaction where there are an excess of antigen binding sites [7]. In non-competitive immunoassays, the signal is directly proportional to the amount of analyte in the sample, producing a similar curve to that obtained with a displacement immunoassay (Fig. 4.2b). The most common non-competitive immunoassay format is the *sandwich immunoassay*, where two antibodies are used for analyte detection. Both polyclonal and monoclonal antibodies are suitable for use in sandwich immunoassays; however, in order to use this format the antigen of interest must be large enough to simultaneously bind two separate antibodies. The first antibody in the reaction is called the *capture antibody*, which is either adsorbed or covalently linked to a solid support and is used to extract the analyte from the sample matrix. A second labeled antibody, known as the *tracer* or *conjugate antibody*, reacts with the captured antigen at a separate and distinct epitope from the capture antibody and is used to detect the analyte when all other components have been washed away. This type of assay can be performed either in a sequential or simultaneous incubation format, although for simultaneous incubation two monoclonal antibodies specific for distinct and distant epitopes must be used to prevent competition.

Another approach to non-competitive binding immunoassays is demonstrated in the *one-site immunometric assay*. The one-site immunometric assay begins with incubation of an excess of labeled Fab fragments (for 1:1 binding) or antibodies (2:1 binding) with

a sample containing the antigen of interest. The incubation mixture is then introduced to a solid support containing immobilized antigen of interest to remove unbound antibodies or Fab fragments from solution; the amount of label left in solution is directly proportional to the concentration of analyte in the sample. This format is particularly useful for automated flow based or chromatographic immunoassays. Fab fragments are preferable to whole antibodies in this approach because an antibody can be bound to both the support and analyte, which would yield a falsely low signal.

4.2.4 Immunoassay labels

There are many different types of labels, for both antigen and antibody, that can be used in immunoassays. The choice of label is dependent on the analytical sensitivity desired for the measurement as well as the immunoassay format. Early immunoassays used isotopic labels such as ^{125}I, ^{131}I, ^{3}H, ^{57}Co, and ^{32}P for antigens in competitive binding immunoassays, and for antibodies in sandwich immunoassays. The utility of radioactive labels is based on low limits of detection due to very low background interference. Radioactive labels were also used to develop an early homogeneous immunoassay [10] based on modulation of ^{3}H or ^{125}I labels by scintillant-laden microparticles. Early radioimmunoassay were also developed for the detection of drugs of abuse in body fluids [11]; more recently, a radioimmunoassay for the antiviral drug, zidovudine [12]. Immunoassays using radiolabels are currently falling out of favor in clinical laboratories due to concerns regarding safe handling and disposal of radioactive materials as well as the inherent instability of radiolabels.

Recently, there has been an emphasis on the development of non-isotopic labels in light of the drawbacks that come with handling radioactive labels. As early as the 1970s, enzyme labels for immunoassay were in development, and since then a wide variety of non-isotopic labels have been established. Non-isotopic labels include enzyme-based, fluorescent, chemiluminescent, and even electrochemical labels. Some of these labels are able to achieve detection limits well below those possible with radiolabels, and none of them pose the problems of handling and disposal that occur when working with radioactive materials. Increased specificity can be gained in an immunoassay by incorporating a biotin-avidin interaction with the antibody-antigen interaction. This is accomplished using a biotin-conjugated antibody or antigen and then introducing labeled avidin to the reaction mixture. In some instances, signal is increased using multiple labels by taking advantage of the multiplicity of avidin-biotin binding. One avidin molecule can bind four biotin molecules so this complex scheme would involve a biotin conjugate, followed by addition of avidin, which could then bind three more labeled biotin molecules.

4.2.4.1 Fluorescence

Chemical labels that exhibit fluorescence are commonly used for competitive binding and sandwich immunoassays in the same manner as radioactive labels. The most commonly used label is fluorescein isothiocyanate (FITC), but dyes such as Lucifer Yellow or Rhodamine B can also be used as fluorescent labels. This type of label is

sensitive enough to use for low concentration analytes, but avoids the handling and disposal issues that come with using radioactivity. However, a major problem with fluorescence detection is that many substances found in clinical samples such as drugs and drug metabolites, proteins, and bilirubin exhibit fluorescence that can cause a high background and interference with the assay.

One way to avoid interference from fluorescent background is the utilization of *time-resolved fluorescence* (TRF) with labels derived from lanthanide elements (i.e. europium, terbium, and samarium). The fluorescence lifetime for these lanthanide chelates is much longer than typical fluorescent molecules. To take advantage of this extended lifetime, after excitation a delay time (400–800 ms) is introduced to let background fluorescence decay, and then the signal is measured [6]. Immunoassays with TRF detection have been used to test for analytes in applications such as detection of testosterone in saliva [13] or development of a dry chemistry immunoassay for zeranol [14].

Fluorescence detection can be used for homogenous assays by employing a *fluorescence polarization immunoassay* (FPIA) format. This technique is a competitive binding assay using a fluorescein labeled antigen to compete with antigen in the sample for antibody binding sites [6]. An FPIA uses the polarization of fluorescence due to molecular rotation in solution to determine the extent of label-antibody binding. When the labeled antigen is unbound, it rotates very rapidly giving it a high degree of anisotropy. As a result of the anisotropy, the label complex has a low degree of polarization. However, when the label is bound to an antibody molecule that rotates at a much lower rate, anisotropy is decreased and the degree of polarization is increased (see Fig. 4.3). The change in polarization is proportional to the concentration of antigen in the sample, and because the label behavior is modified by antibody binding, no separation step is needed. FPIA is used clinically to measure drugs such as amikacin, gentamicin, quinidine, and theophylline [15] as well as the immunosuppressant cyclosporine A (Neoral® and Sandimmune®) [16]. Fluorescence polarization assays can also used to detect drugs of abuse such as cocaine [17,18], opiates, marijuana, and barbiturates [18].

Another homogeneous fluorescence-based immunoassay is based on *fluorescence energy transfer* [19]. This approach uses a dual label system, where the analog is linked to a donor fluorophore, and the antibody is linked to an acceptor molecule. When the donor is in close proximity to the acceptor – as in the case of antibody-antigen binding – then fluorescence of the labeled analog is quenched by energy transfer to the acceptor molecule. In this manner, the signal from the label is modified by antibody binding, making it a suitable format of homogeneous immunoassay. This approach has been used to develop assays for analytes such as phenytoin [20] and digoxin [21] in body fluids, but is not widely used in the clinical laboratory.

4.2.4.2 Enzymes

Enzyme-based labels such as alkaline phosphatase, horseradish peroxidase, g-6-dehydrogenase and β-galactosidase are also frequently used in quantitative immunoassays [6,22]. A significant advantage for enzyme labels is the amplification aspect; one enzyme label can produce many reporter molecules, for up to 100-fold

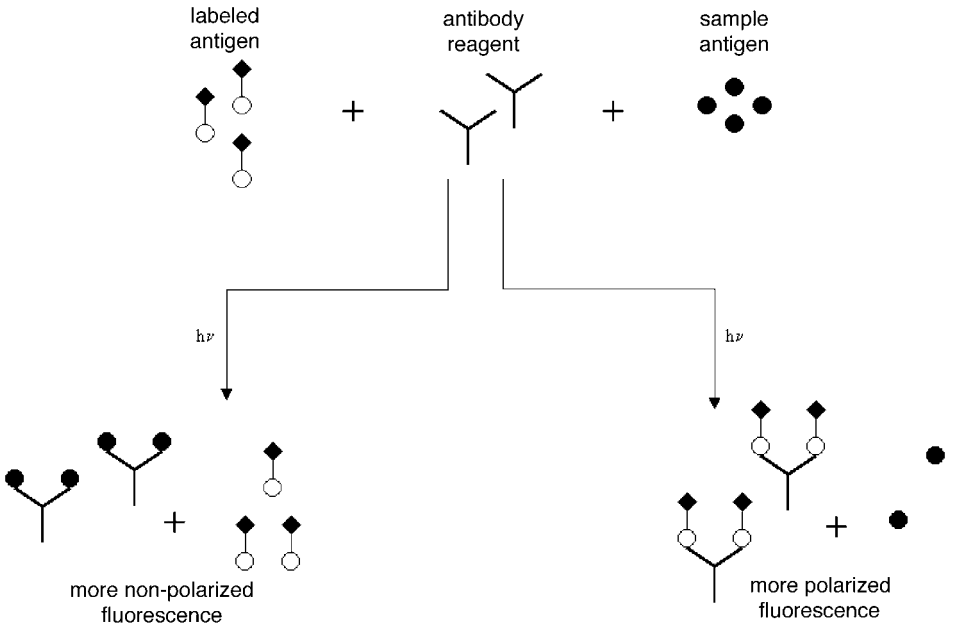

Fig. 4.3. Fluorescence polarization immunoassay (FPIA).

signal amplification (using a fluorescent substrate can produce amplification up to 100,000-fold). The substrate for detection can be chosen to produce a colored product for photometric monitoring, a fluorescent product such as umbilliferone, or even to produce an intermediate substrate for a second enzymatic reaction to produce further amplification and specificity.

The most common heterogeneous enzyme immunoassay is the *enzyme-linked immunosorbent assay* (ELISA), which is a sandwich immunoassay where the capture antibody is bound to a solid support and the tracer antibody is enzyme-labeled [6]. After the capture:antigen:tracer complex is formed, all other sample components are washed away and substrate is added. The amount of product formed by the enzyme label is proportional to the concentration of antigen in the sample (Fig. 4.4). The ELISA can be modified to detect antibodies of a given specificity by using antigen linked to the support, adding sample and using labeled antibodies to IgG as the tracer (e.g. detection of anti-HCV antibodies). Since two epitopes are necessary for the sandwich ELISA, this format is more suited for peptide or protein therapeutics, such as B-type natriuretic peptide (BNP) used in patients with congestive heart failure. Other examples include the use of ELISA for determination of amlodipine in plasma [23] or determination of antivenin levels in snakebite victims [24].

Another approach to heterogeneous enzyme immunoassay is the *microparticle enzyme immunoassay* (MEIA). In this assay, antibodies directed toward the analyte of interest are immobilized to the surface of microparticles. The sample is then incubated with the antibody-coated particles in the presence of enzyme labeled analog. After a

fixed period of time, the particles with bound sample analyte and/or label are separated from the rest of the sample by centrifugation or binding to a glass fiber matrix. The particles are washed to remove any non-specifically binding components, followed by addition of enzyme substrate. The signal produced is inversely proportional to the amount of drug present in the original sample. This type of assay is commonly used to monitor ProGraf® (tacrolimus) or rapamycin in transplant patients [16].

As previously discussed, homogeneous immunoassays require a label that will be modified by antibody binding and enzyme labels are particularly well suited for this role. There are two commonly used types of homogeneous enzyme immunoassays: the *enzyme-multiplied immunoassay technique* (EMIT) and the *cloned enzyme donor*

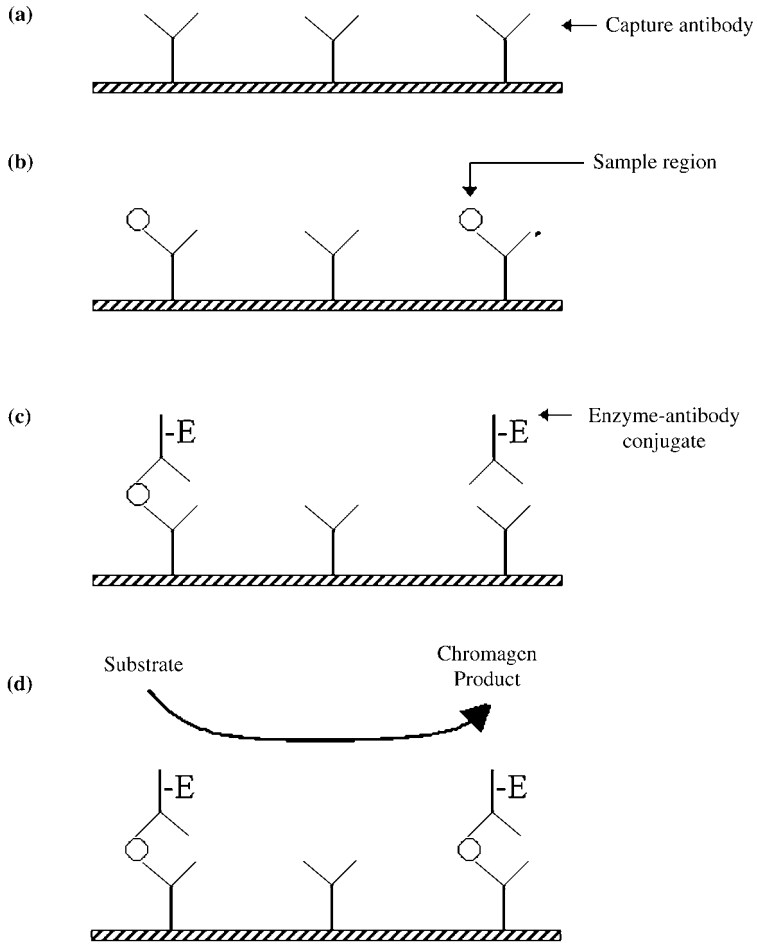

Fig. 4.4. Enzyme-linked immunosorbent assay (ELISA): (a) couple capture antibody to well surface; (b) add patient sample or calibrator to well; wash; (c) add tracer antibody reagent to well; wash; (d) add enzyme substrate to well for detection.

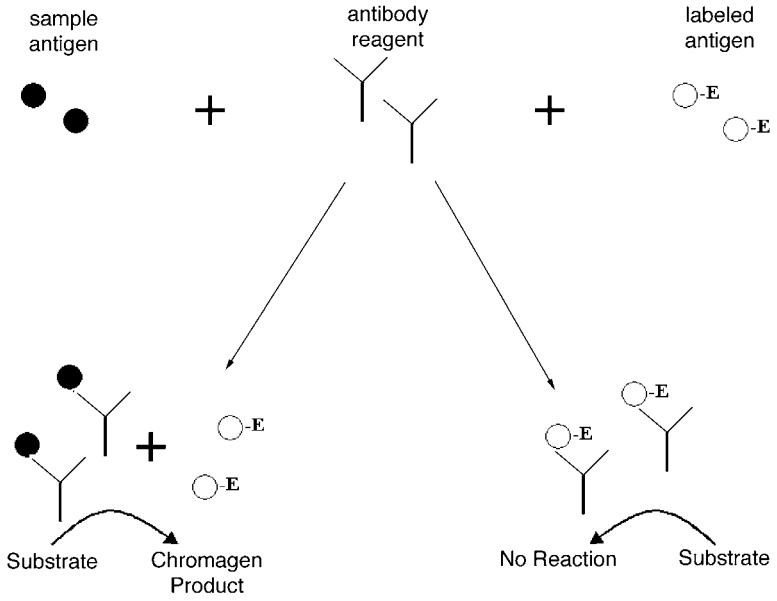

Fig. 4.5. Enzyme-multiplied immunoassay technique (EMIT).

immunoassay (CEDIA) [6,25]. In the EMIT assay, an aliquot of the patient sample or calibrator is incubated with an enzyme-labeled antigen and a fixed amount of antibody specific for the antigen of interest as well as enzyme substrate (Fig. 4.5) [6,25]. The antigen in the sample and the labeled antigen compete for antibody binding sites; when antibody is bound to the labeled antigen, the enzyme is inhibited because it is blocked from interaction with the substrate. The change in enzymatic activity is proportional to the concentration of antigen in the patient sample or calibrator (e.g. as the concentration increases, so does the enzyme activity). EMIT assays are widely used in the clinical laboratory for TDM including valproic acid [26], lidocaine [27], CellCept® (mycophenolic acid) [28], and phenytoin [29]. They are also used for toxicological analyses in the clinical laboratory including screening for marijuana, cocaine, and opiates [18], as well as for amphetamines [30], benzodiazepines and barbiturates [31].

The CEDIA assay is also a competitive binding immunoassay [25,32], but it is a little more complex than the EMIT assay in its execution. Using genetic engineering techniques, two inactive fragments of β-galactosidase are produced by manipulating the Z gene of the *lac* operon of *E. coli*. These two fragments are able to reassemble to form an active enzyme, even if one of the fragments is covalently linked to another molecule. The competing antigen for the assay is labeled with one fragment of the cloned enzyme and is incubated with a mixture of the patient sample, the second enzyme fragment, enzyme substrate, and a fixed amount of antibody directed toward the antigen of interest (Fig. 4.6). Antibody that binds to the labeled antigen prevents reassembly of the enzyme and no reporter molecule is produced; as the sample antigen concentration is increased, the enzyme activity is also increased. As with the EMIT assay, the change in enzymatic

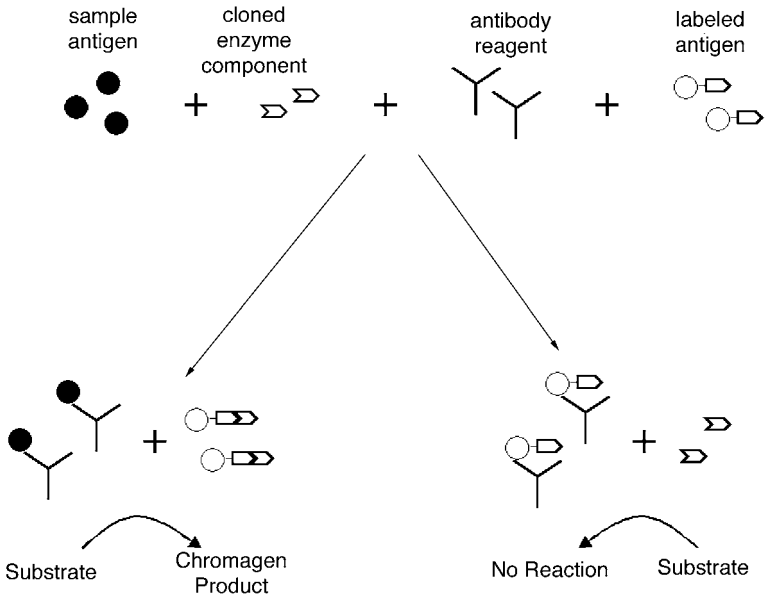

Fig. 4.6. Cloned enzyme donor immunoassay (CEDIA).

activity is directly proportional to the concentration of antigen in the sample. CEDIA assays are also commonly used in today's clinical laboratory. Cyclosporine [33] and phenobarbital [34], theophylline, and phenytoin [35] can all be monitored with this method. Assays for drugs of abuse also commonly use the CEDIA format including screening assays for all the major drugs of abuse including benzodiazepines [36], methamphetamine, barbiturates, phencyclidine, cocaine, and opiates [37].

4.2.4.3 Chemiluminescence

A chemiluminescent label is based on the emission of light produced during a chemical reaction. These labels are very useful because they offer very low limits of detection with little to no background interference, however, the complexity of the immunoassay is increased because the reaction conditions must be carefully controlled. In addition, the label is not modified by antibody binding, so chemiluminescence detection would not be suitable for homogeneous immunoassays. Chemiluminescent labels can be found as both biological and chemically manufactured compounds. Biologically derived chemiluminescence labels include firefly luciferase [38] and apoaequorin, a protein derived from the bioluminescent jellyfish *Aequorin* [39]. Firefly luciferase activates D-luciferin substrate by oxidation in the presence of ATP, molecular oxygen and magnesium ions to emit light at 560 nm. Apoaequorin is activated by reaction with coelenterazin and calcium ions to emit light at 469 nm.

Other non-biologic compounds can be used as chemiluminescent labels, including isoluminol and acridinium esters [40]. Isoluminol is oxidized in the presence of a catalyst such as microperoxidase, producing a relatively long emission at 425 nm.

Acridinium esters are commonly used, highly sensitive labels that are activated by oxidations using hydrogen peroxide in alkaline solution to give a brief flash of light at 429 nm. Drugs that can be monitored by chemiluminescent assays include, carbamazepine, phenytoin, phenobarbital, and valproic acid [41]. Chemiluminescence can be made even more sensitive when combined with other detection methods. Electrochemiluminescence utilizes a molecule such as ruthenium (II) tris(bipyridyl) that undergoes chemiluminescence at a given redox potential, producing mass detection limits as low as 20×10^{-21} moles [39,42]. In addition, chemiluminescent substrates can be combined with enzymatic labels to take advantage of enzyme amplification effects along with the inherent sensitivity of chemiluminescence using a substrate known as AMPPD (disodium 3-(4-methylspiro-[1,2-dioxetane-3,2′-tricyclo-[3.3.1.1]decan]–4-yl) phenyl phosphate) to obtain mass detection limits as low as 1×10^{-21} moles [43,44]. Current applications of this technology have been developed for cardiac markers and endocrine testing, but as of yet there are no commercially available drug assays using this assay technique.

4.2.5 Immunoassay interferences

Although immunoassays provide an easily automated, sensitive method of detection, there are some important factors to consider that may interfere with the assay. These factors include interfering antibodies from the patient's serum, assay artifacts caused by large analyte concentrations (the *hook effect*), and standardization between immunoassays for the same analyte that use different epitopes for binding. In addition, certain substances may cross-react with the antibody and interfere with the assay. Each of these interferences has a characteristic effect that influences both immunoassay performance and utility.

One particular problem encountered when using immunoassays is interference from circulating heterophilic human antibodies specific for animal immunoglobulins that are used as reagents. The most common type of heterophilic antibodies are human anti-mouse antibodies (HAMA) often present in the blood of patients that have been given mouse monoclonal antibody imaging or therapeutic agents, such as Atgam® or Digibind®, or have been exposed to other mouse antigens [45]. Heterophile antibodies can cause false positive results by cross-linking the capture and label antibodies in a sandwich immunoassay, as if the analyte of interest were present, or they can cause false negative results by blocking analyte binding to the capture or label antibody (Fig. 4.7). The presence of HAMA or other heterophile antibodies can be confirmed through dilution experiments that do not give proportional results, or retesting of a sample after incubation with a blocking agent such as lyophilized animal serum (e.g. mouse antisera for HAMA). Non-immune animal serum is sometimes included in immunoassay kits using animal antibodies as reagents to minimize the effects of heterophile antibodies.

Another problem that can arise, specifically in simultaneous addition sandwich assays, to give a falsely low result is known as the "hook effect" [46,47]. This effect occurs when the concentration of the analyte is so high that both the capture and label antibodies are saturated. In response, the assay signal will decrease at high antigen

concentrations, which can be demonstrated by a calibration curve including these concentrations. The response drops off after a maximum response giving the curve a characteristic "hook" shape (see Fig. 4.8). This situation can lead to falsely low patient

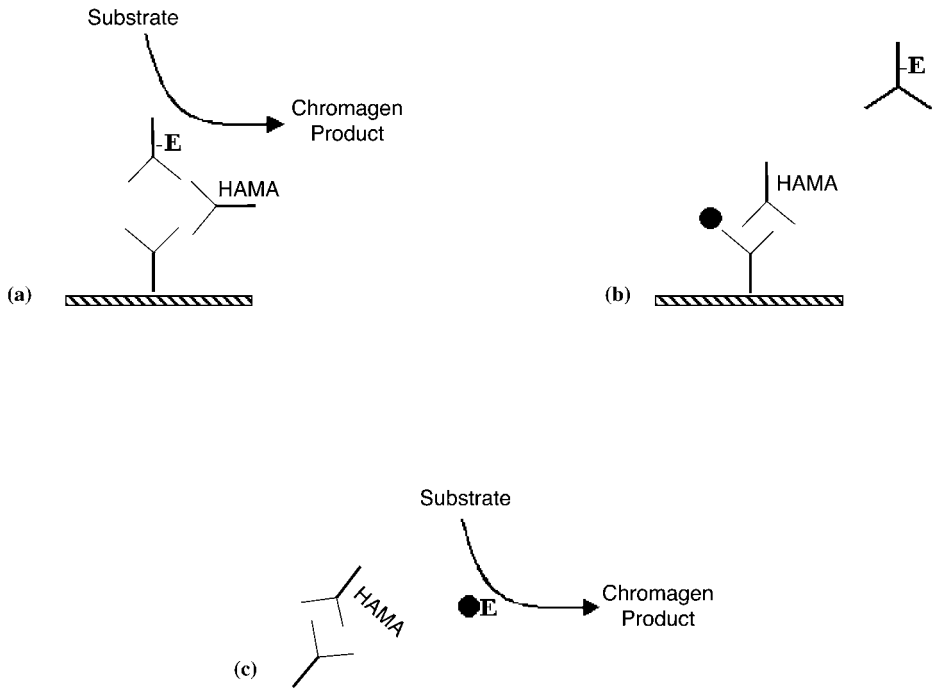

Fig. 4.7. Heterophilic antibody interference (HAMA): (a) positive interference for sandwich immunoassay; (b) negative interference for sandwich immunoassay; (c) positive interference for homogeneous immunoassay.

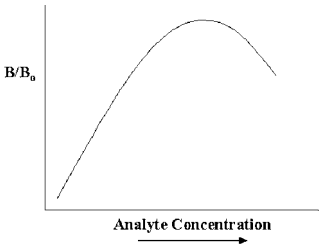

Fig. 4.8. Calibration curve demonstrating the hook effect.

results when the concentration of an analyte is elevated far above the analytical range of the assay and can be discovered by dilution followed by sample retesting. Generally, this occurs with endogenous substances such as tumor markers that are overproduced in response to disease, but the scenario could occur in cases of therapeutic overdose. This type of error is avoided by using a large excess of capture and label antibodies with respect to the analytical range of the assay.

A different sort of problem arises when immunoassays are developed for the same analyte by different companies or researchers. Each developer uses a different antibody directed to a unique epitope that may exhibit varying binding properties leading to different responses for the same concentration of analyte. In an isolated environment this is not a problem so long as the institution uses only one type of assay; however, the establishment of a standard reference interval for the analyte becomes much more difficult because various institutions often use different assays for the same analyte. A prime example of this challenge is demonstrated in the development of a cardiac troponin I assay. Cardiac troponin I (cTnI) is present in the circulation in three forms: as free cTnI, a two-unit complex with cardiac troponin C (cTnI-cTnC), and a three-unit complex with cTnC and cardiac troponin T (cTnI-cTnC-cTnT). The various assays fail to agree with each other because the reagent antibodies for each assay recognize different epitopes, making it difficult to develop a primary reference material [48,49] for standardization of the method. Lack of standardization is a problem for drug assays in TDM in cases where a patient is followed long term by different physicians or institutions. If the values given between two different assays do not correspond, therapy for the patient may be modified unnecessarily.

Antibody cross-reactivity of metabolites or unrelated substances that have structurally similar epitopes can lead to inaccurate results when samples are analyzed by immunoassay. For instance, phenytoin metabolites such as 5-(*p*-hydroxyphenyl)–5-phenylhydantoin glucuronide (HPPH-G) and phenytoin-N-glucuronide that accumulate in patients with uremia can cross-react in certain phenytoin immunoassays and lead to erroneously high results [29]. In patients with decreased renal function receiving vancomycin, the accumulation of vancomycin crystalline degradation products (CDP-1) that cross-react in FPIA assays has been shown to induce overestimation of vancomycin concentrations by 40–53% [50]. Cross-reactivity can lead to either falsely elevated or decreased results, depending on the assay format. A good example of this is in the case of digoxin monitoring where *digoxin-like* (DLIS) and *digitoxin-like* (DTLIS) *immunoreactive substances* have been shown to produce falsely elevated results with FPIA [51]; but caronene and spironolactone have demonstrated the ability to produce falsely low results for digoxin when using an MEIA format [52].

4.2.6 Point-of-care immunoassays

A recent development is the adaptation of immunoassay formats to develop testing outside the clinical laboratory, specifically for *point-of-care testing* (POCT). The most widely used POCT immunoassay is used for the detection of human chorionic gonadotropin (hCG), more commonly known as a “home pregnancy test”. This type of

home testing has also been developed for testing drugs outside the laboratory as well [53]. The general format is essentially the same, regardless of the analyte of interest. A urine specimen is applied to a porous membrane that draws the liquid from the application point to immobilized antigen positioned further up the membrane. If antigen is present in the sample, it will be bound to the immobilized antibodies, followed by a detection step. Detection can be accomplished in a number of ways, depending on the manufacturer. One way is to add a second labeled antibody solution that will bind to the captured analyte, followed by a color development step [54]. Another approach is to use colored microparticles linked to the antibody for detection. In this approach the initial capture antibody is immobilized to colored beads, which are located at the point of application. As the sample and microbeads migrate up the membrane, they encounter a second capture antibody that will serve as a "detection zone"; if there is analyte present in a sample, a sandwich complex will be formed and visible positive signal will occur.

One clinical application for POCT immunoassays is in the hospital emergency department (ED) and other locations where testing for drugs of abuse is necessary. In addition, POCT devices based on immunoassays are being developed to screen for drugs of abuse in oral fluids [1]. In order to be useful, each POCT device must not only produce a clear signal that makes it easy to distinguish between positive and negative, it must also contain a control mechanism to ensure that the device is working properly (e.g. the specimen moves up the membrane, all reagents are included in the reaction, detect the presence of interfering heterophilic antibodies, etc.). This type of testing would most likely not be useful for therapeutic drug monitoring because more than just a positive/negative result is needed. However, if a system that could deliver quantitative data for POCT immunoassays were developed, then this approach to TDM would be feasible.

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CHAPTER 5

Validation of bioanalytical methods

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5.1 INTRODUCTION

The validation of bioanalytical methods has been the topic of many discussions over the past decades. Although some degree of consensus has been reached concerning the requirements for acceptable method validation [1], the procedures were significantly different between laboratories. Since the release of the "Guidance for Industry in Bioanalytical Method Validation" on May 2001 [2], it is much clearer what is requested regarding method validation, especially regarding physical techniques (HPLC, LC/MS/MS, . . .). This guidance provides general recommendations for bioanalytical method validation. The recommendations can be modified depending on the analytical method and based on scientific knowledge.

Based on this guidance, this chapter describes both aspects of the validation. First of all, the validation prior to starting the sample analysis (pre-study validation) and secondly, the within-study validation.

5.2 REQUIREMENTS FOR BIOANALYTICAL METHODS

Prior to starting any validation, some requirements need to be fulfilled. A non-exhaustive list of actions is listed below.

- The good laboratory practices [3] must be implemented in the laboratory willing to validate a bioanalytical method.
- Solutions, solvents, chemicals, reagents, analytes and internal standards used in the laboratory should be labeled in such a way that their identity, concentration (if applicable), date of preparation (if applicable) and expiry date (if applicable), initials of person who prepared a solution or first user, as well as storage instructions (e.g. temperature, dark) appear clearly. Outdated materials should not be used.
- The operation, maintenance and qualification (IQ, OQ, cFT, PQ, cPT) of all devices, instruments, hardware and software (e.g. centrifuge, pipette, balance, evaporation

References p. 128

device, immunoassay equipment, LC-MS system, . . .) used in the bioanalytical method must be properly performed and documented.
- All laboratory staff members must receive adequate training in the use of the particular apparatus and in the follow-up of all the laboratory procedures.
- The procedures for the validation of bioanalytical method must be documented prior to performing the validation in a "Standard Operating Procedure".

5.3 PRE-STUDY VALIDATION

5.3.1 Scope

A new analytical method or an existing analytical method, whether transferred between laboratories or between instruments or applied to different species or matrices, should be properly validated prior to use.

Formal validation of a new analytical method includes the analysis of sufficient numbers of quality control samples and calibration standards to adequately describe the calibration model, accuracy, precision (intra- and inter-day), sensitivity, selectivity, and limit of quantitation of the method, as well as the stability of the tested component(s). When applied to the development and validation of a method for stereoisomers, each isomer (enantiomer, diastereomer) must be treated as an individual chemical entity/component, if deemed necessary. The pre-study validation must be performed in the matrices foreseen in future studies. However, the pre-study validation could be extended to other matrices by doing additional experiments.

The objectives of the pre-study validation are to:

- to establish standards (within the limits described in the following procedure) and/or criteria sufficient to validate a new chromatographic or binding assay-based analytical method intended for use in clinical drug disposition, pharmacokinetics, bioavailability/bioequivalency or toxicology studies.
- to describe standards and/or criteria sufficient to qualify a fully validated analytical method that is transferable between laboratories or between instruments or that is to be applied to a different species or sample matrix.

5.3.2 Validation procedure for a new analytical method

5.3.2.1 General information

An analytical method has to be validated for the intended use prior to the time of its application. The anticipated and/or necessary dynamic concentration range is to be estimated based on available preliminary data.

Validation of an analytical method in a matrix of human origin should be carried out using at least three batches of matrix for QC samples, and six for zero and matrix blank samples, where each batch is collected from a different source. Each run should contain a calibration curve, QC samples, a zero sample (matrix containing only internal

standard), a blank sample (no internal standard), and a reference standard (sample containing only the analyte of interest). The QC samples in each run should be prepared from a different batch of matrix; however, the calibration curves for all validation runs should be prepared from the same batch of matrix. In animals, when obtaining large volumes of matrix from individual animals is difficult, all validation runs may be performed using a single pooled batch of matrix.

5.3.2.2 Reference standard(s)

If possible, the reference standard should be identical to the analyte. When this is not possible, an established chemical form (free base or acid, salt or ester) of known purity can be used as a surrogate. The reference standard(s) is to be of good assay quality (typically >95% purity). The source and lot number, certificate(s) of analysis, and/or other evidence of identity and purity should be obtained. All reference materials should be checked prior to use to determine if there are significant interfering chromatographic peaks at the retention time of the analyte and/or the internal standard using the analytical procedure to be used in the study.

5.3.2.3 Calibration curve

The calibration curve is to be established for each analyte in the sample and consistently applied throughout the method validation. Both linear and well-characterised non-linear calibration relationships are acceptable, however, the simplest workable regression equation should be used. Selection of the regression model should be rationalised. Results from "zero or blank" standards should not be used as part of the calibration curve except for some binding assays. A calibration curve must be freshly prepared and run on each validation day.

- Calibration standards of the test component(s) are to be prepared in the same biological matrix as the samples in the intended study.
- The matrix used for preparation of C standards and QC samples must be from the same species.
- If possible, the matrix used for preparation of C standards should be from the same batch over the whole pre-study validation.
- The origin and lot number of the matrices should be identified and reported.
- The calibration curve should consist of at least six concentration levels.

For chromatographic assays the calibration standards are prepared and analyzed in duplicate over the concentration range studied. The lowest calibration standard concentration should be at the anticipated LLOQ and the highest calibration standard concentration at the ULOQ. The remaining concentration levels are to be appropriately distributed throughout the calibration range in order to adequately define the curve.

For binding assays the calibration standards are prepared singly and analyzed in duplicate (or triplicate according to the specific procedure) over the concentration range studied. The lowest calibration standard concentration should be at or below the concentration corresponding to the expected LLOQ. The highest calibration standard concentration should be at or above the concentration corresponding to the expected ULOQ.

References p. 128

- Back-calculated values for the calibration standards within the working range should meet the following criteria:
  - 15% deviation from expected concentration.
  - At least two-thirds (with a minimum of 6 different levels) of the non-zero standards meeting the above criteria, including LLOQ and ULOQ levels (for chromatographic assays). Excluding the standards should not change the regression model used.

5.3.2.4 Quality control samples

Results from the analysis of QC samples are to be used to validate the analytical method with regard to accuracy, precision, sensitivity, selectivity and stability. Solutions prepared from a weighing different from that used for calibration standard(s) preparation are to be used to prepare the QC samples. The QC samples should cover the anticipated dynamic concentration range of the method.

- QC samples must be prepared at a minimum of four concentrations with one at the anticipated LLOQ, one within 3-times the LLOQ, one approximately midway between the high and low QC concentrations, and one close to the highest C concentration (or at the anticipated ULOQ for binding assays).
- QC samples should be prepared from different batches of matrix on each validation day.
- Intra-day data: A minimum of five replicates prepared from the same matrix for each QC concentration must be analyzed on each validation day. For binding assays, each replicate may consist of 2 or more concentration determinations.
- Inter-day data: Samples must be analyzed on a minimum of three validation assays (six for binding assays), resulting in $n \geq 15$ ($n \geq 30$ for binding assays) at each QC concentration used.
- Acceptance criteria for accuracy and precision

Accuracy	Accuracy (%) = [measured value/expected value] × 100	
Acceptance criteria based on the mean value at each concentration level	Chromatographic assays 85–115% (80–120% at the LLOQ)	Binding assays 85–115% (80–120% at the LLOQ & ULOQ)
Precision	Precision (%) = CV (%) = (Standard Deviation/Mean) × 100	
Acceptance criteria at each concentration level	Chromatographic assays ≤ 15% (≤ 20% at the LLOQ)	Binding assays ≤ 15% (≤ 20% at the LLOQ & ULOQ)

Validation of the method should include demonstration of method performance meeting or exceeding the above accuracy and precision acceptance criteria within (intra-day) and between (inter-day) validation days for each QC sample concentration.

All results other than those rejected for analytical/technical reasons should be used in the calculation of the accuracy and precision of the method.

If the above criteria are not met (e.g. for binding assays showing a higher variability related to compound, matrix or antigen-antibody reaction), new acceptance criteria must be redefined and documented accordingly.

5.3.2.5 Sensitivity

The establishment of the LLOQ must be based on the statistical analysis of QC samples meeting accuracy and precision acceptance criteria detailed in above section.

5.3.2.6 Absolute extraction recovery and matrix effect

The absolute extraction recovery and matrix effect for all analytes should be evaluated, whenever possible, at a minimum of three QC concentrations (low, medium and high) spanning the calibration curve for the analyte(s) and at the concentration used in the method for the ISTD.

- Absolute extraction recovery: comparison of the analytical response of spiked sample to the response of 'blank' sample extract spiked with the analyte.
- Matrix effect: comparison of the analytical response of ëblankí sample extract spiked with the analyte to the response of 'neat/unextracted' solution analyzed directly.

5.3.2.7 Selectivity

The selectivity of the analytical method must be investigated to address the following: For chromatographic assays:

- A minimum of 6 different batches of the same biological matrix should be tested for potential interferences.
- A minimum of 5 replicates of ëblankí samples from the same batch should be prepared and analyzed.

  For each batch of 'blank' samples the mean response of interfering peaks at the retention time of the analyte should be less than 20% of the mean response ($n \geq 5$) of the LLOQ standard.

  The mean response of interfering peaks at the retention time of the ISTD should be $\leq 5\%$ of the mean response ($n \geq 5$) of the ISTD concentration to be used in studies.

  If one batch of the 'blank' samples exhibits interferences exceeding the above acceptance criteria at the retention time of the drug, metabolites or ISTD, additional matrix 'blank' samples should be tested.

  If one 'blank' sample from this subsequent group still shows interference, either the method should be changed to eliminate the interference, or the LLOQ (or the ISTD concentration, depending on the interferences) should be re-evaluated.
- When using ISTD, 'zero' samples should be prepared and analyzed for potential interferences at the retention time of the analyte(s).

- If the method is intended to quantify more than one analyte, each analyte should be chromatographed separately to ensure that impurities or contributions (crosstalk effect) from one analyte do not interfere in the determination of another analyte.
- Any potentially interfering substances (i.e.known metabolites, concomitant medication if already defined, anticoagulants, . . .) are to be investigated/included, as required.

For binding assays:

- A minimum of 6 different batches of the same biological matrix should be tested for potential interferences.
- Potentially interfering/cross-reacting substances (e.g. known metabolites, similar proteins, analogues, anticoagulants, etc.) are to be investigated during method development whenever possible.

5.3.2.8 Stability

Stability of the test component(s) in the stock solution (whenever possible) and in the biological matrix must be evaluated. In addition, the stability of the test component(s) under assay conditions must be evaluated. For investigations concerning non-stock solution stability, samples spiked at two different concentrations (low, high) must be analyzed in triplicate and quantified versus a freshly prepared standard curve. In the event that the average accuracy of the test component(s) is less than 85% at a given concentration, appropriate measures should be undertaken to address component(s) instability.

(1) Stock Solution stability
Stability of the stock/spiking solutions of analyte and ISTD should be evaluated at room temperature for at least 6 hours. If the stock/spiking solutions are refrigerated or frozen for the relevant period, the stability should be documented. After completion of the desired storage time, the stability should be tested by comparing the instrument response with that of freshly prepared solutions.

(2) Short-Term stability
Stability must be evaluated at room temperature in the sample matrix for 4 to 24 hours. The length of time evaluated will be at least as long as the amount of time required for samples to be extracted during the normal assay process.

(3) Long-Term stability
Long term storage stability will be evaluated at approximately 1 week and 1 month when frozen (at the intended storage temperature, e.g. at or below $-18°C$). Quality control samples will be prepared and frozen to evaluate long-term stability up to the needed time interval (e.g. 12 months) but will be analyzed only if deemed necessary. Long-term stability should be demonstrated over a period of time which exceeds the time between the collection and analysis of any given study sample. The results from long-term stability experiments can be documented in an amendment to the analytical method report. Long-term stability investigations may also be conducted on incurred samples from actual studies to supplement the stability data obtained from spiked

samples. True samples spanning the entire therapeutic concentration range (low, high) may be investigated if available. If an analyte is unstable at or below − 18°C, stability should be reassessed at or below − 70°C.

Note: In case of a long term stability assay performed on QC samples, the mean value measured at one concentration after the storage period is compared to the concentration value measured on the preparation day (before storage) and not to the nominal spiked concentration value.

(4) Freeze and Thaw stability
Spiked samples at low and high concentrations must be subjected to a minimum of three 'freeze-thaw' cycles and must be analyzed in triplicate. Samples should be stored at or below − 18°C (or the intended storage temperature) for 24 hours and thawed unassisted at room temperature. When completely thawed, the samples should be transferred back to the original freezer and kept frozen for 12 to 24 hours. This cycle of thawing and freezing should be repeated two more times, then analyzed on the third cycle.

(5) Post-Preparative Stability
Stability must be investigated under routine assay conditions (i.e. during storage in an autosampler, workstation and/or robotic system) on a case by-case or need-to-know basis. Stability of processed samples in the autosampler should be determined at the autosampler temperature that will be used during study sample analysis and over the run time for the batch size to be used in studies.

5.3.2.9 Dilution

Additional QC and/or real samples are analyzed in replicate at various dilutions performed in the biological matrix (e.g. 1:2, 1:10, 1:50) to validate the possible dilution during study sample analysis.

5.3.3 Validation procedure for an existing method

5.3.3.1 Method transfer between laboratories and/or instruments

In cases where a validated method is transferred to another laboratory (including outsourcing laboratories) or to another instrument, the receiving laboratory must qualify the method to insure adequate method performance prior to the analysis of study samples. The intra-day validation (and inter-day, if necessary) described in Section 5.3.2 should be performed, but using only one matrix. The results from the analysis of QC samples must meet the acceptance criteria outlined in Section 5.3.2.

5.3.3.2 Modifications to an existing assay

When changes are made to a previously validated method, the analyst should exercise good scientific judgment as to how much additional validation is needed.

For minor modifications, such as a change in the ratio of solvents for elution, a change in buffer system, the number of extractions of the biological matrix, or a small change in column temperature to obtain better separation, only partial validation may be

recommended (intra-day performance). The effect of minor changes to the method may be monitored by the generation of ongoing within-study QC results from subsequent sample runs.

If the method requires significant modification, the validation will be performed to the extent determined appropriate by laboratory head or his/her designee. Examples of significant changes to a method include new extraction procedures, different incubation times and different stationary or mobile phases.

5.3.3.3 Application to different species or matrices

In cases where a validated method is to be applied to another species or sample matrix, a validation should be performed in the new matrix prior to the analysis of study samples. QC samples at a minimum of four concentrations must be analyzed in five replicates on at least one day. The matrix used for the preparation of C standards and QC samples should be the same. ëBlankí samples from different batches of the new species/ matrix should be tested for potential interferences, if possible.

Note: Care has to be taken to store spiked and/or true samples for testing long-term stability in the concerned species or sample matrix.

If the accuracy and precision results do not meet the criteria outlined in Section 5.3.2, a full method validation including selectivity, recovery and stability experiments, as appropriate, should be performed. If the analyst can justify a less rigorous validation than described above, a brief justification and description must be documented in the raw data of the study. Also, if a limited supply of the appropriate matrix prevents the performance of the testing specified above, it should be clearly documented.

5.3.4 Documentation and archiving

Formally issued laboratory notebooks, daily worksheets and all related project files are the media for recording and storage/archiving of pertinent raw data.

5.4 WITHIN STUDY VALIDATION

5.4.1 Scope

The within-study validation describes the bioanalytical procedures performed for the within-study method validation: standardization, quality control samples, acceptance criteria and criteria for selection of samples for repeat analysis.

5.4.2 Procedure

Before starting any pivotal study, the pre-study validation should have successfully passed.

Separate weighings should be used for the preparation of calibration sample stock solution(s) and QC sample stock solution(s).

When using a commercially available kit, the manufacturerís recommendations and procedures should be followed.

5.4.2.1 Daily calibration curve

Both linear and nonlinear calibration models are used as defined in the pre-study validation.

5.4.2.1.1 Chromatographic assays

Results from 'zero or blank' are not used in the construction of the calibration curve. Calibration curves are processed on each analysis run.

The daily calibration curve must consist of a minimum of six levels of standard in duplicate. A logarithmic distribution of the calibration levels makes a better curve fitting, especially when using weighting factor. The same curve fitting, weighting determined during validation should be used for the standard curve within the study.

5.4.2.1.2 Binding assays

The daily calibration curve should consist of standards prepared singly and analysed at least in duplicate at a minimum of six concentrations. The replicate responses or concentrations are averaged for the construction of the calibration curve. A blank matrix sample should also be prepared and analysed along with the calibration standards.

5.4.2.1.3 Acceptance criteria

For all standard curves (Chromatographic and binding assays), apply the following acceptance criteria:

Acceptance is based upon "back-calculated" concentration values for each standard within the working range. Individual values (or averaged values in case of software limitation) for which the bias is $> \pm 15\%$ from the theoretical value ($> \pm 20\%$ at the LLOQ for chromatographic methods) can be excluded from the calibration curve, and the regression analysis reprocessed, provided that the established model does not change. For the calibration curve to be considered acceptable, maximally 1/4 of the individual standards can fail the above acceptance criteria, but at least six standard levels need to be accepted. However, at least one replicate at the lowest and highest calibration level must be within the acceptance range. In case of binding assays, less than 6 calibration levels within the working range can be accepted.

5.4.2.1.4 LLOQ and ULOQ

The Lower Limit of Quantification is defined for chromatographic assays on a routine basis as the lowest "acceptable" concentration used in the daily calibration curve; for binding assays, the LLOQ is defined as the lowest "acceptable" QC sample concentration. In general there is only one LLOQ for a study (for a given volume of matrix).

The Upper limit of quantification is defined for chromatographic assays on a routine basis as the highest "acceptable" concentration used in the daily calibration curve; for binding assays, the ULOQ is defined as the highest "acceptable" QC sample concentration.

References p. 128

5.4.2.2 Quality control samples

Quality control samples: prepared at least at three different concentrations (low, mid and high) and analysed with each analytical run. For the lowest QC, concentration should not be higher than 3-times the LLOQ for chromatographic assays; for binding assays, the lowest QC sample defines the LLOQ. The highest QC sample concentration should be close to the ULOQ for chromatographic assays (the recommendation will be between 75% and 90% of the ULOQ). For binding assays, the highest QC sample defines the ULOQ. The mid QC should approximately range in the middle of the calibration curve. The logarithmic middle seems more logical due to the dynamic range.

The QC acceptance criteria for individual analytical runs is as follows:

Acceptance is based upon concentration values for each QC sample. At least 2/3 of the QC concentration values must be within 15% of their respective theoretical values, and at least 50% of the values at each QC concentration must be within this range.

If more than 50% of the values at the same QC concentration are outside the acceptance range for the accuracy [85–115%], the run should be rejected and all samples repeated. Only the repeat values will be reported.

Additionally, for binding assays one concentration value is derived from the mean of the duplicate analysis of one sample, for which the CV% should not exceed 20%.

Notes:

- The acceptance of a run is based on both acceptance for C standards and QC samples.
- When an assay has been validated with different acceptance criteria, the within-study validation follows the rules established during the pre-study validation.
- In case multiple analytes are analysed within a same run, only data from the analyte failing the acceptance criteria should be rejected.
- The QC samples should be arranged as considered appropriate within the run. Placement of standards and QC samples within a run should be designed to detect assay drift during the overall run time.
- The minimum number of QC samples should be at least 5% of the number of unknown samples or six total QCs, whichever is greater.

5.4.2.3 Criteria for samples repeat analysis

(1) Signal interference or peak distortion compromising the quantitation of the analyte and/or the internal standard (chromatographic method)

In this case, the sample will be repeated (given sufficient sample volume). Only the concentration of the repeat analysis will be reported. If no sample volume is available for repeat analysis, no result will be reported.

(2) Technical problem

same as (1)

(3) Values above the ULOQ

Repeat the analysis following appropriate dilution (in the appropriate matrix). The dilution should have been validated during the pre-study validation. Only the repeated result will be reported. If not enough sample is available, report concentration as: >ULOQ.

(4) Unexpected value within the concentration-time profile

The analysis should be repeated based on scientific judgment if the obtained result is either twice or one half that expected based upon surrounding concentration values in the concentration *versus* time profile.

- If sufficient sample volume remains, analyse the suspect sample to generate three additional independent concentration values (i.e. in triplicate for chromatographic analysis, three sets of replicates where appropriate for binding assays). The median of the four values (original and the three repeat values) will be reported.
- If only two repeat analyses are possible, the median of the three values (original and the two repeat values) will be reported.
- If only one repeat analysis is possible:
  Repeat value deviates by less than 15% of the original, the original value is reported.
  Repeat value deviates by more than 15% and less than 30% of the original, the mean of both results is reported.
  Repeat value deviates by more than 30% of the original, no result is reported.
- If not enough of the original sample is available, report the value, but tagged with "not enough sample to confirm the value".
- The pre-dose samples (i.e. taken before the first administration) are considered to be part of the concentration vs. time profile.

(5) Values of control samples above the LLOQ

- If a control sample, which was analysed at the expected t_{max} of the concentration vs. time profile, is positive, analysis is repeated (if sufficient sample volume available) together with all samples from the concerned control animal.
- For sparse sampling studies, all the control samples will be analysed. If one control sample is found positive, a single repeat should be generated with a matrix blank from another source (as requested).
  (a) Repeat value and blank are negative (i.e. below the LLOQ): the value will be reported as zero.
  (b) Repeat value is positive and blank negative: the original value will be reported.
  (c) Repeat value and blank are positive: the repeat analysis is not valid and the analyst should define and solve the probable analytical problem, before re-analysis, if possible.

(6) CV% between replicates is not acceptable (binding assays)

If the CV% value (for concentration >LLOQ) between duplicate calculated concentrations is higher than 20% a single repeat should be generated. If a higher variability has been shown during the prestudy validation, a CV% higher than 20% can be accepted.

References p. 128

(7) Cs and/or QCs out of acceptance criteria
If the acceptance criteria for Cs and/or QCs are not fulfilled, the run must be rejected and the analysis of all unknown samples must be repeated. The concentrations found after reanalysis are reported and listed in the sample repeat table.

5.4.3 Documentation

(1) Performance of the method
The intra-day performance for C standards and QC samples should be documented in the raw data.

A summary table of all Cs with the inter-day performance should be reported for all validated runs. In this table, the values of Cs considered as outlier, excluded for the regression analysis of the calibration curve, should also be reported and tagged. However, they are not taken into consideration for the calculations.

All QC results measured in accepted analysis runs are reported in a summary table including inter-assay (overall) accuracy and precision. In this table, the values of QCs considered as outlier are reported, tagged or highlighted, and used for the overall calculations.

Invalidated run should also be reported in the recapitulative table with the date of the run and the reason of the lack of validation.

(2) Repeat analysis
A recapitulative table including all the repeated samples and the reason of reassay should be documented and archived with the raw data for each study.

(3) Chromatograms for clinical studies
- For pivotal bioequivalence studies, chromatograms from 20% of serially selected subjects with standards and QC samples from those analytical runs should be appended to the report.
- In other clinical studies, serial chromatograms from 5% of randomly selected subjects in each study should be reported with standards and QC samples from those analytical runs.

(4) Reintegrated data
In case of manual peak re-integration, the documentation should include the initial and repeat integration results, the reason for the reintegration and the method used for reintegration.

(5) Biological matrix
The biological matrix used for the preparation of Cs and QCs should be identified by its origin and reported.

5.5 DEFINITIONS

Accuracy	Closeness of the concentration value obtained by the method to the known true concentration value of the analyte.
Analysis "Day or Run or Sequence"	A set of C standards, blanks with and without internal standard, if appropriate, QC samples, reference for chromatography if available and unknown samples that are batch processed together.
Analyte	The substance being measured in the analytical procedure, e.g. the test item and metabolites (except internal standards).
'Blank' sample	Sample to which neither analyte(s) nor internal standard has been added.
Calibration curve	A set of prepared calibration standards used to define the relationship between instrument response and known concentrations of the analyte.
Calibration Standard	A biological matrix to which a known amount of analyte has been added. Calibration standards are used to construct calibration curves from which the concentrations of analytes in QC samples and in unknown samples are determined.
Concurrent Functional Testing	consists in maintaining the main functions of the system in a proper state during its operational use. This activity is performed during a vendor qualification and clearly documented.
Concurrent Performance Testing	consists in tracking the relevant performance data during routine analysis and must be performed and documented to ensure *continuous performance* of the system.
Control animal	Animal dosed with the control article or the vehicule, but not with the test article.
Control sample	Sample collected from control animal.
Crosstalk effect	In mass spectrometry, the determination of two analytes at the same retention time which could interfere.

References p. 128

Incurred sample	Sample taken from clinical or pre-clinical studies.
Internal standard	Test compound (e.g. structurally similar analog, stable labeled isotope) added to both standards and samples at known and constant concentration to facilitate quantification of the target analyte(s).
Installation Qualification	Establishing confidence that process equipment and ancillary systems are compliant with appropriate codes and approved design intentions, and that manufacturer's recommendations are suitably considered.
Lower limit of quantification	The lowest concentration of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy.
Matrix effect	The direct or indirect alteration or interference in response due to the presence of unintended analytes (for analysis) or other interfering substances in the sample.
Operational Qualification	Establishing confidence that process equipment and sub-systems are capable of consistently operating within established limits and tolerances.
Performance Qualification	Establishing confidence that the process is effective and reproducible.
Precision	Closeness of individual measures of an analyte when the method is applied repeatedly to multiple aliquots of the same biological sample.
Quality Control sample	A spiked sample used to monitor the performance of a bioanalytical method and to assess the integrity and validity of the results of the unknown samples analyzed in an individual batch.
Recovery	Detector response obtained from an amount of the analyte added to and recovered from the biological matrix, compared to the detector response obtained from a blank extract.
Selectivity	Ability of an analytical method to differentiate and quantitate the analyte in the presence of other constituents in the sample. It refers directly to the ability of the method to produce a concentration dependent response to a single analyte.

Solution	An homogeneous mixture of a liquid (solvent) with a solute, e.g. analytes, internal standards, antibodies, organic and anorganic salts.
Spiked sample	Blank sample spiked with one or more analytes.
Stability	The physico-chemical stability of an analyte in a given matrix under specific storage conditions for given time intervals.
Upper limit of quantification	The highest amount of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy.
Working range	Range of concentration defined between the LLOQ and the ULOQ.
'Zero' sample	Sample spiked with internal standard only.

5.6 ABBREVIATIONS

Accuracy (%)	= 100 × (mean observed or back calculated value)/ expected value
Bias (%)	= 100 × [(mean observed or back calculated value) ñ expected value]/expected value
C	Calibration
cFT	Concurrent Functional Testing
cPT	Concurrent Performance Testing
CV	Precision: Coefficient of variation = 100 × SD/mean
GLP	Good Laboratory Practice
HPLC/MS/MS	High performance liquid chromatography/Tandem mass spectrometry
ISTD	Internal standard
IQ	Installation Qualification
LC	Liquid chromatography
LLOQ	Lower limit of quantification
MS	Mass spectrometry
OQ	Operational Qualification
PQ	Performance Qualification
QC	Quality control
SD	Standard deviation
ULOQ	Upper limit of quantification

References p. 128

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CHAPTER 6

Pharmacokinetic methods for analysis, interpretation, and management of TDM data, and for individualizing drug dosage regimens optimally

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6.1 INTRODUCTION AND OVERVIEW

When a drug has a narrow margin of safety, we must keep its dosage not too low, where it is likely to be ineffective, and not too high, where it is likely to be toxic. We must carefully plan and individualize the dosage for each patient, to achieve some desired target goal such as a serum concentration or effect (the prothrombin time or its International Normalized Ratio, INR, for example), or its profile over time. As therapy proceeds, we should always observe the patient, and should often monitor serum concentrations or the effect at appropriate intervals. These intervals should be frequent enough so that we can evaluate the patient when there are relatively small changes in the total amount of drug in the body between observations, so that if toxicity develops, we can detect it in an early stage of its development, and can make the appropriate adjustment in dosage early, rather than later, after toxicity has become more severe and dangerous.

It is commonly said that one should individualize dosage to body weight and renal function, for example. But a specific therapeutic target goal is usually not explicitly

stated. We usually do this, however, to control either the total amount of drug in the patient's body, or the serum concentration, for example, at a desired specific target value, usually within some general target "therapeutic range" of serum concentrations where most patients (but not all) do well, and where the incidence of toxicity is acceptably low. However, this is approach is appropriate only for the initial regimen, and it also ignores the opportunity to be gentle, moderate, or aggressive in the approach to the patient, according to each individual patient's need for the drug, with appropriate adjustments of dosage as necessary.

The expected risk of toxicity should be no greater that that which is warranted by the patient's need for the drug. In many cases, if the patient's need is not great, or is not acute, the target goal should first be one that is associated with a only low incidence or risk of toxicity, leading to a gentle dosage regimen. Based on the patient's response, the target goal can then be revised upward and a higher dosage given to achieve it. This, for example, is behind the "start low, go up slow" dosage policy so well advocated by Cohen [1].

6.1.1 Problems with "Therapeutic Ranges"

Therapeutic drug monitoring (TDM) cannot be done by the laboratory unassisted. Some laboratories have attempted to do this, without seeing the patient, reporting the measured serum concentration along with a statement of the usual therapeutic ranges of the concentrations over which most patients, but certainly not all, usually have done well. This is not optimal TDM. One simply cannot do optimal TDM without seeing the patient clinically.

Fig. 6.1 shows the usual means by which therapeutic ranges appear to have been obtained. It is usually stated that first, there is a "significant" incidence of therapeutic effects with increasing serum drug concentrations. This defines the beginning of the therapeutic range. Later on the incidence of toxic effects also becomes "significant", and the "toxic range" has been entered. The eye is drawn to the bends in each line in Fig. 6.1, and the classification of the apparent "therapeutic range" has been developed, published, and usually accepted without apparent further thought. This procedure does not consider the need to develop a gentle dosage regimen for a patient who needs only a gentle touch, or a more aggressive one for a patient who really needs the dosage "pushed". Another problem has been that special populations of patients have not had their special needs recognized. For example, it is well known that patients with atrial fibrillation need higher serum concentrations of digoxin, for example, usually averaging 2.0 ng/ml, (often said to be the top of the therapeutic range of digoxin serum concentrations for patients having regular sinus rhythm), for full control of their ventricular rate, and yet this has not been followed up by setting a special therapeutic range for patients with atrial fibrillation. It is a good example of how we have taught clinicians, in the interests of so-called "science", to turn away from their patients and their individual clinical situations, and to look at some measured laboratory value in a book somewhere which actually may or may not be relevant or optimal for that particular patient.

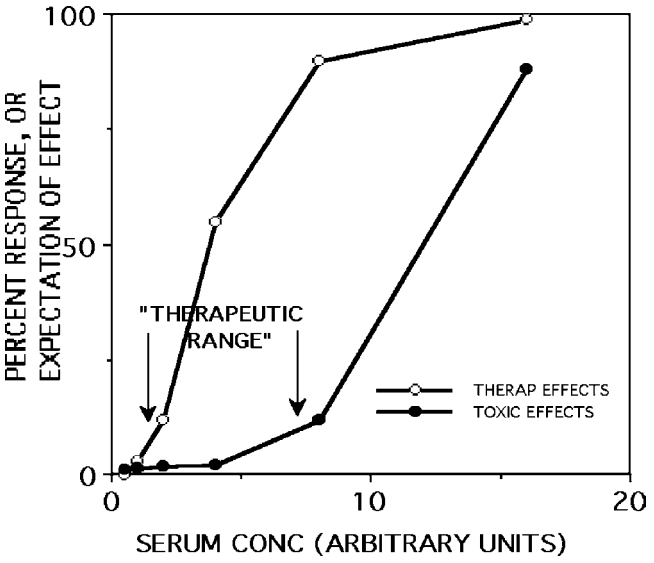

Fig. 6.1. General relationships usually found between serum drug concentrations and the incidence of therapeutic and toxic effects. The eye is drawn to the bends in the curves, and the therapeutic range is classified in relation to these bends. This qualitative procedure of classification discards the important quantitative relationship of the incidence of toxic effects versus serum concentration.

Those risks associated with being below the therapeutic window are usually associated with lack of effect. Those associated with being above the window are usually associated with toxicity. Each of these outcomes – subtherapeutic, therapeutic, or toxic – has not only its associated probability but also its own positive or negative quantitative utility function of goodness or badness. Optimizing such a complex set of probabilities and utilities becomes a most complex process, and is poorly amenable to rigorous clinical decision analysis.

The risks and benefits of using a therapeutic range or window are quite complex from the point of view of decision analysis. One could, for example, develop a dosage regimen to maximize the probability of having the patient's serum concentration be within some desirable therapeutic window. This sounds good at first, but the decision-making process rapidly becomes quite complex. One must also weigh the benefit and the probability of a desirable response against the risks, and their probabilities, of being outside that window, either below or above it.

6.1.2 Setting specific target goals based on need

A more intuitive and individualized approach is to have no "window of neutrality" about a target, as in a therapeutic range. Based on the general relationship between serum concentration and incidence of toxicity as shown in Fig. 6.1, for example, one can select a specific target serum concentration goal to be achieved for a specific patient at a

specific time after the dose. One does not want the patient to run any greater risk of toxicity than is justified by the patient's clinical need for the drug. Within that constraint, however, one wants to give the patient as much drug as possible, to get the maximum benefit.

In this approach, the risks of being just slightly above the desired target goal are only minimally different from those associated with being just slightly below it, in the sense of an infinitesimal difference as in calculus. Because of this, it appears easier, and also more intuitive for a clinician, to choose a specific target goal, and then to attempt to achieve or hit that goal with the greatest precision (least error) possible.

Because of all this, it is not enough any more for a clinical laboratory simply to report a measured serum concentration and the usual therapeutic range. A responsible clinician needs to be involved as well, to see the patient and to manage the clinical problem which the patient presents, aided by the data from the lab, and also aided by useful models and software for planning, monitoring, and adjusting drug dosage regimens for these patients. All this can easily be done using software developed for PC's, by clinical pharmacologists or clinical pharmacists using laptop machines, entering the data from the chart at the nurses station, doing the analyses, printing out the results for the chart, writing the appropriate orders, and moving on to see the next patient.

Without selecting a specific target goal, there can be no truly individualized drug therapy. Individualized drug therapy begins by setting a specific individualized target goal for each patient. The task of the clinician is to select, and then to hit, the desired target goal as precisely as possible. As soon as the initial regimen is given, the clinician's task is then to observe the patient's clinical response at appropriately frequent intervals, and to reevaluate whether the target goal was hit precisely enough or not, whether the target goal was correctly chosen or not, or if it should be changed and a new dosage regimen developed to hit the new target goal. This is the basis of the "target-oriented, model-based, approach to individualized drug dosage for each individual patient [2].

6.2 THE NEED FOR MODELS

Pharmacokinetic models are extremely useful. While no model is an exact description of reality, which is almost always more complex, models describe in useful quantitative terms the behavior of drugs as they are given to patients – the time course of their absorption, serum concentrations, pathways, exchange rates to and from other compartments, their various therapeutic and toxic effects, and their metabolism and excretion. Often these processes and pathways may have important relationships to other clinical descriptors or covariates such as body weight, age, renal function, smoking status, gender, and genetic CYP450 makeup, for example.

Pharmacokinetic and pharmacodynamic (PK/PD) models provide the tool to apply past experience to the care of new patients. Past experience with drug behavior is now usually stored in the form of a population PK/PD model. This model is then used to design the initial dosage regimen for the next patient who appears to belong to that particular population.

The dosage regimen to hit the therapeutic target goal is computed and given. The patient is monitored both clinically and by measuring the serum drug concentrations. The serum concentrations should be used not only to note if they are within some general "therapeutic range", but most importantly (and most usefully) to make a specific model of the behavior of the drug in each individual patient, based first on information from the population PK/PD model, and then, using Bayes' theorem, developing an individualized model that best describes the behavior of the drug in that particular patient. Bayesian approaches balance the patient's individual data against the more general information from the population model, and make an individualized model that usually predicts future concentrations better than techniques which do not use this approach.

Using the patient's individualized model, one can then calculate what the patient's probable serum concentrations were at all other times when they where not measured, even when he or she was not at all in a steady state, even during highly unstable clinical situations in very acutely ill patients with great changes in their clinical status and renal function over time. One can also reconstruct and see graphically the computed concentrations of drugs in a peripheral nonserum (tissue) compartment or in various effect compartments. The patient's individualized model permits making dosage adjustments without waiting for a steady state before sampling serum concentrations, and can take into consideration practical clinical situations, such as handling data of different dosage regimens (with unequal doses and unequal dosing intervals), and totally arbitrary time intervals between drug doses and blood sampling.

These important physiological, pharmacological, pharmacokinetic and pharmacodynamic relationships cannot be seen or understood without PK/PD models. By comparing the clinical behavior of the patient with the behavior of the patient's model, one can evaluate the patient's clinical sensitivity to the drug, and can adjust the target goal appropriately. For digoxin, for example, the inotropic effect of the drug correlates best with the computed concentrations of the drug in the peripheral compartment (ug/kg of body weight, for example) rather than with the serum concentrations. The excellent model made by Reuning and colleagues for digoxin [3] has been highly useful clinically [4].

6.3 MAXIMUM APOSTERIORI PROBABILITY (MAP) BAYESIAN INDIVIDUALIZATION OF DRUG DOSAGE REGIMENS

Bayes' theorem describes in quantitative terms how we learn from experience. It describes the important sequential relationship between:

(1) The estimated probabilities of certain events (for example, in PK/PD terms, a patient's apparent volume of distribution, and the clearance or rate constant for elimination of a drug, or an effect parameter), that are probably present in a patient before we have had any chance to know anything about his/her response to the drug (or the serum concentrations), because we have not given him/her the drug yet, or have not had any measured serum concentrations or other feedback responses become available to us yet. These initial estimates constitute the Bayesian "prior",

as they state the situation before (prior to) knowing the next set of information. These are then combined with:

(2) The measured serum concentrations and/or other responses that are subsequently found in that patient, based on whatever dosage the patient has received, and then

(3) The revised (Bayesian posterior) probabilities of these PK/PD parameter values after (posterior to) taking into account the new information.

In the beginning, before the drug is given or before any feedback information is available, all we have is our past experience about the behavior of the drug in a population of similar patients. This is why one of our most important tasks as clinicians is to store our experiences with patients in a form that can be used in the future to apply that experience optimally, usually using Bayes' theorem, to the care of the next similar patient. This is why it is important to make population PK/PD models of the behavior of the drug in the actual patients we treat, not just in research clinical trials, to obtain and to store that important clinical past experience optimally for future use and application.

The Maximum Aposteriori Probability (MAP) Bayesian approach to individualization of drug dosage regimens was introduced to the pharmacokinetic community by Sheiner et al. [5]. In this approach, "parametric" population models are used as the initial Bayesian priors. In these parametric models, the distributions of the PK/PD parameters in the structural model (the apparent volume of distribution, clearances, rate constants) are described by statistical parameters such as their means, standard deviations (SD's), and the correlations between them. This is what the word "parametric" means in this case – that the PK/PD model parameter distributions in the population are assumed to belong to special parametric families of distributions, usually either normal or lognormal, which are completely described by their means, SD's, and correlations.

The MAP Bayesian approach is a modification of the weighted least squares regression procedure, in which the MAP Bayesian objective function combines two types of data – the measured serum levels found in that patient and the parameter values in the population model which forms the Bayesian prior. Their contributions to the MAP Bayesian posterior individualized patient model are shown in the objective function below:

$$\sum \frac{(\mathrm{C_{obs}} - C_{\mathrm{mod}})^2}{SD^2_{(Cobs)}} + \sum \frac{(\mathrm{P_{pop}} - P_{\mathrm{mod}})^2}{SD^2_{(Ppop)}}, \tag{1}$$

where C_{obs} is the collection of observed serum concentrations, $SD^2{}_{(Cobs)}$ is the collection of their respective variances, and C_{mod} is the model estimate of each serum concentration at the time it was obtained. Similarly, P_{pop} is the collection of the various mean population model parameter values, $SD^2{}_{(Ppop)}$ is the collection of their respective variances, and P_{mod} is the collection of the Bayesian posterior model parameter values.

Each serum data point (each C_{obs}) can be given a weight according to its Fisher information [6], the reciprocal of its variance. Similarly, each population parameter

value (each P_{pop}) is given a weight according to the reciprocal of its variance [6]. Population parameters having greater diversity, and therefore greater variance, contribute less to the individualized model than do population parameters having smaller variances. Similarly, a precise C_{obs} will draw the fitting procedure more closely to the respective observed concentration than a less precise C_{obs}. The more serum data are obtained, the more that information dominates the determination of the MAP Bayesian posterior parameter values (P_{mod}) in the patient's individualized pharmacokinetic model.

Like the conventional weighted least squares regression procedure, the MAP Bayesian method can provide correct weighting of serum concentration data according to its credibility based on Fisher information [6]. It thus has the potential for obtaining good estimates of the individual pharmacokinetic parameter values. Second, like the conventional weighted least squares regression procedure, the MAP Bayesian procedure can fit the model to data of doses and serum concentrations acquired over many dose intervals, usually the patient's entire dosage history. There is no longer any reason to do the traditional "single dose" pharmacokinetic study. Further, there is no need for the patient to be in a steady state or for the serum data to be only post-distributional. Studies and population pharmacokinetic/pharmacodynamic modeling can be done on the actual patients being treated, as they are receiving their therapy. But the MAP Bayesian approach has an additional advantage. In contrast to older least squares methods of fitting of PK/PD data, this procedure can avoid the old requirement of needing at least one serum concentration for each parameter to be fitted, or at least two serum concentrations in the most simple traditional linear 1-compartment PK model. The MAP Bayesian method can fit using only a single serum concentration data point if needed. This is because the procedure already has one data point for each parameter – the collection of the population parameter values themselves. Because of this, the MAP Bayesian procedure can start to fit with only a single serum concentration. This feature of the MAP Bayesian method allows one to handle the often very poor and sparse data which is so often present in many clinical strategies of therapeutic drug monitoring and dosage adjustment.

Having made the patient's individualized model, one then uses it to reconstruct the past behavior of the drug in the patient to date. One can examine a plot of the behavior of this model, usually over the entire duration of the patient's past therapy. One can evaluate the clinical sensitivity of the patient to the drug by looking at the patient clinically and comparing his/her clinical behavior with the individualized pharmacokinetic model. In that way, one can evaluate whether the initial target goal was well chosen or not. One can choose a different target goal if needed, and once again one can compute the dosage regimen to hit it. In this way, dosage can continue to be adjusted to the patient's body weight, renal function, and available serum concentrations, for example, to hit the desired target goal, usually with increasing precision during the course of the patient's therapy.

Two other fitting procedures, now coming on the scene, hold promise of doing better than the MAP Bayesian method. One is the "Multiple Model" (MM) method of fitting data and designing drug dosage regimens [7]. It is based on "nonparametric" population models [8,9] and their individualized Bayesian posterior pharmacokinetic models. The

other is the "Interacting Multiple Model" (IMM) sequential Bayesian method [10]. These methods of Bayesian analysis will be discussed more fully later on.

6.4 ANALYZING ASSAY AND ENVIRONMENTAL SOURCES OF ERROR

6.4.1 Overview

Very often, when an assay is calibrated and its error is determined, the results are used simply to determine whether or not the assay is acceptably precise. Measures of inter-day and intra-day variability are made. After the assay is determined to be acceptably precise, this information is usually no longer used. However, when analyzing TDM data or pharmacokinetic data, it is useful to assign a measure of credibility to each data point to be fitted or analyzed. Because of this, statements of inter- and intra- day variability are not very useful. Instead, one can develop an estimate of the overall error with which any single measurement having a certain value is likely to be made, as samples progress through the analytic assay system in a laboratory.

6.4.2 Determining the assay error polynomial

In the iterative two-stage Bayesian (IT2B) and the nonparametric adaptive grid (NPAG) population modeling programs in the USC*PACK collection [11], for example, and also in the clinical programs for Bayesian adaptive control of dosage regimens, one is encouraged, first of all, to determine the error pattern of the assay quite specifically, over its entire working range, by determining several representative assay measurements in at least quadruplicate, and to find the standard deviation (SD) of each of these points. For example, one can measure, in at least quadruplicate, a blank sample, a low one, an intermediate one, a high one, and a very high one. One can then fit the relationship between the serum concentration (or any other response or effect) and the SD with which it has been measured, with a polynomial of up to third order, so that one can then compute the Fisher information, for example, as a useful measure of the credibility of each serum concentration data point [4,6]. One can then express the relationship as

$$SD = A_0 + A_1C + A_2C^2 + A_3C^3 \quad (2)$$

where SD is the assay SD, A_0 through A_3 are the coefficients of the polynomial, C is the measured concentration, C^2 is the concentration squared, and C^3 is the concentration cubed. A representative plot of such a relationship, using a second order polynomial to describe the error pattern of an EMIT assay of gentamicin, is shown in Fig. 6.2.

6.4.3 No lower limit of quantification for pharmacokinetic work and TDM

If the serum specimen is one which is obtained for purposes of toxicology, where there is no other information except the assay result itself to tell you if the drug is present or

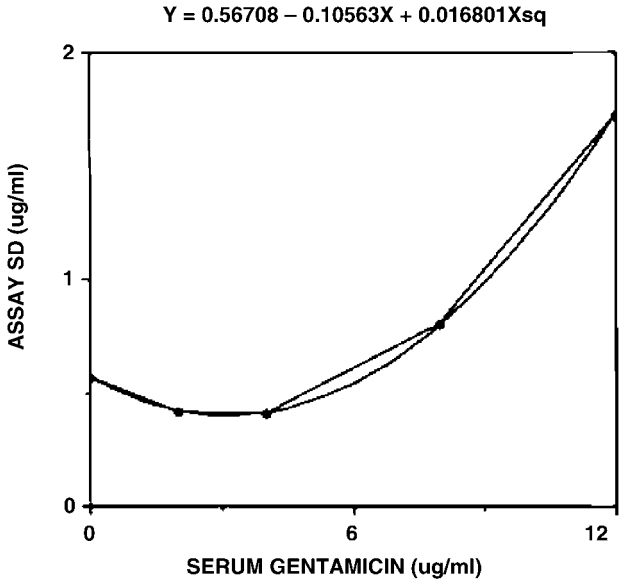

Fig. 6.2. Graph of the relationship between serum Gentamicin concentrations, measured by Emit assay in at least quadruplicate (the dots) and the standard deviations (SD's) of the measurements. The relationship is captured by the polynomial equation shown at the top. Y = assay SD, X = measured serum concentration, Xsq = square of serum concentration.

not, then there is clearly a point below which one cannot be sure there is any drug present in the sample or not. Then the usual lower limit of detection or quantification is usually taken to be 2 or 3 SD's above the blank value. This is a common practice.

However, when one is doing pharmacokinetic studies and analyses, and TDM, this is clearly NOT the case. Other information is also available. One knows when the sample was obtained and when the last dose was given. Since most drugs disappear with a T1/2, a clearance, or a rate constant, the final molecule of drug is never lost. It is clear, from the other information that accompanies each such sample, that the drug is there.

In TDM and pharmacokinetic work, the question is not whether the drug is present or not. That is the wrong question. The drug is there. The question to be answered is rather, HOW MUCH drug is present? Because of this, there is actually no lower limit of detection for pharmacokinetic studies. One can use the assay error polynomial to give correct weighting of a measured drug concentration all the way down to, and including, the blank, as illustrated in Fig. 6.2. The weighting can be done according to the Fisher information of each serum concentration data point [6]. Determining the assay error polynomial is a cost-effective way of using a representative set of samples, covering the working range of the assay, to obtain a good estimate of the SD with which any single sample is then probably measured. This is discussed further in [4]. For toxicology, there is clearly a lower limit of quantification. It is a still negotiable number of SD's above the blank. But for PK work and for TDM, there is clearly no such lower limit. The drug is

present. The correct measure of credibility can be given to the measurement based on the assay error polynomial, all the way down to and including a blank.

6.4.4 The SD is what is important, not the CV

The assay error is often expressed as a percent coefficient of variation (CV), the SD divided by the measurement. Then it is said that as the measured concentration approaches zero, the CV becomes infinite, and the signal gets lost in the noise. This is true only if one looks at the data from the point of view of the CV. However, the assay SD, the variance, and the Fisher information, the reciprocal of the variance, are always finite, even as the value of the measurement goes all the way to zero. The correct measure of the credibility of any measurement can always be made. Because of this, there is no lower limit of quantification of a serum drug concentration when it is obtained appropriately for TDM, PK/PD analysis, or population modeling purposes [4].

Furthermore, while it may "seem logical" that an assay with a constant percent CV is "equally precise" over its entire range, this is clearly not true with respect to the real measure of credibility, the Fisher information. Consider an assay, for example, with a CV of 10%. If a measured value is 10 units, then its SD is 1.0, its variance is also 1.0, and its weight, the reciprocal of its variance, is also 1.0.

Now consider a similar measurement with a value of 20 units. Its SD is now 2.0, its variance is 4.0, and its weight, the Fisher information, is now $\frac{1}{4}$. So it is not at all true that a constant percent error reflects a constant credibility of an assay over its range, when one considers the Fisher information [6] as an index of assay credibility.

6.4.5 Determining the remaining environmental error

In addition, a parameter which we have called gamma, a further measure of all the other environmental sources of intra-individual variability, can also be computed. It is used in the USC*PACK programs [11] as a multiplier of each of the coefficients of the assay error polynomial as described above. The nominal value of gamma is 1.0, suggesting that there may be no other source of variability that the assay error pattern itself. The true value of gamma is usually (though not always) greater than 1.0. It includes not only the various environmental errors such as those in preparing and administering the doses, recording the times at which the doses were given, and recording the times at which the serum samples were obtained, but also the errors in which the structural model used fails to describe the true events completely (model misspecification), and also any possible changes in the model parameter values over time, due to the changing status of the patient during the period of data analysis. Gamma is thus an overall reflection of all the other sources of intraindividual variability besides the assay error. In this way, one can compare the SD due to the total system noise with that of the assay SD by itself.

Determining gamma will help us to detect the presence of environmental variability and/or the proposed model or data inadequacy. If gamma is relatively small, it suggests

that in that particular data set the sum of the environmental sources of noise may be small in comparison with the assay error, and the model is well-chosen for this data. If it is large, it suggests that, in this fit, the overall environmental noise may be significant.

The USC*PACK IT2B population modeling program can also be used to compute estimates of the various combined assay error and environmental polynomial error coefficients, if one has no knowledge of what the assay error pattern is, or if the measurement is one which is impossible to replicate to determine its error. In this case, gamma is not determined separately, but is included in the various other polynomial coefficients [11].

6.5 EXAMPLES OF MAP BAYESIAN TARGET-ORIENTED, MODEL-BASED, APPROACHES TO PATIENT CARE

6.5.1 Gentamicin therapy

With a 1-compartment pharmacokinetic model in which the elimination rate constant (Kel) was made up of a nonrenal component (Knr) and a renal component having a slope (Kslope) relationship to creatinine clearance (CCr) so that Kel = Knr + Kslope × CCr [12], the MAP Bayesian procedure resulted in significantly better prediction of future serum concentrations (Fig. 6.3) than predictions made using linear regression

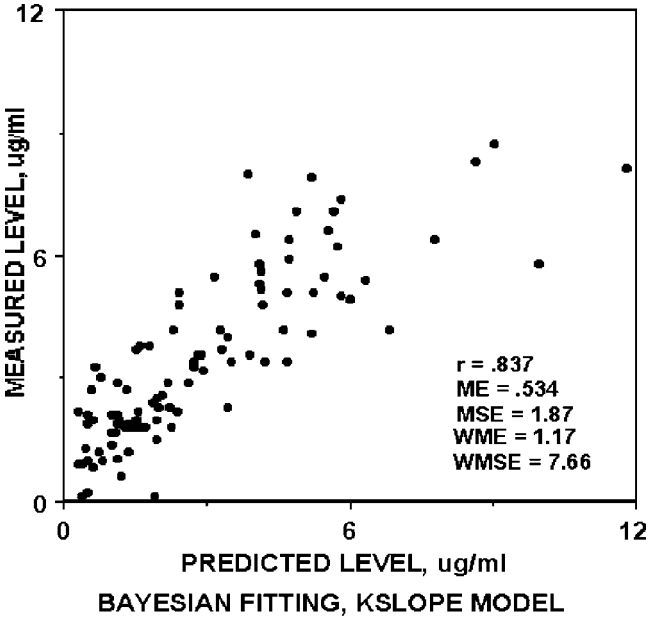

Fig. 6.3. Predicted versus measured serum Gentamicin concentrations found with M.A.P. Bayesian fitting and the Kslope model. r = correlation coefficient, ME = mean error, MSE = mean squared error. WME = mean weighted error. WMSE = weighted mean squared error. See text for discussion.

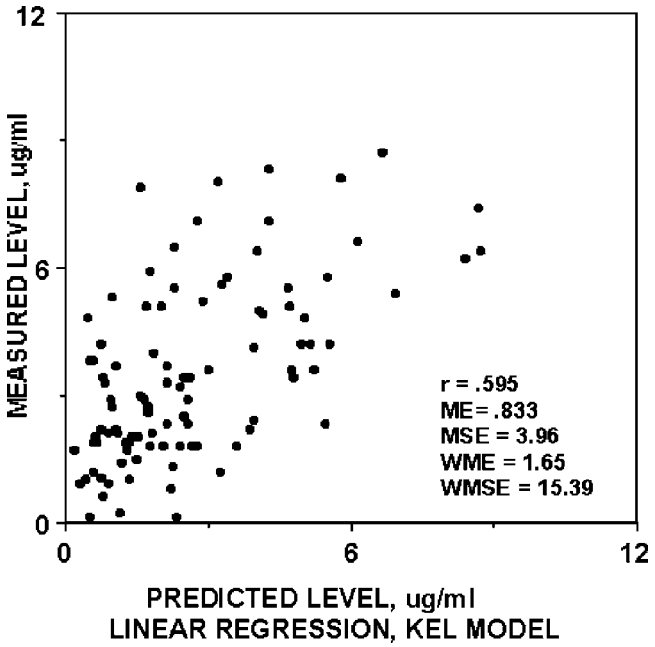

Fig. 6.4. Predicted versus measured serum concentrations found with linear regression on the logarithms of the serum concentrations. Other symbols as in Fig. 6.3.

(Fig. 6.4). In contrast to most patients in the literature, who may have either normal or reduced renal function but whose renal function is stable, many patients in the above study were highly unstable and had changing renal function, to a quite significant degree, during their therapy [13].

Because the software used in that study [11,13] was specifically designed to operate in the presence of significant changes in renal function from dose to dose, it has also been useful in the analysis and management of aminoglycoside therapy for patients who must undergo periodic hemodialysis. The key is to get serum samples of both the drug and of serum creatinine concentrations both before and after dialysis. In our experience, for current dialysis equipment, and for the aminoglycoside antibiotics, the apparent increase in creatinine clearance during dialysis with most current equipment is about 50 ml/min above the patient's baseline value.

The baseline value of CCr can be estimated by examining the most recent rising pair of serum creatinine values, after one dialysis for example, and just before the next one. This is why the pair of serum creatinine samples pre- and post-dialysis is useful. In addition, when a patient goes on dialysis, one can record this as giving a dose of the drug, where the amount of the dose is 0.0 mg. With this dose of zero, the infusion time can be stated as very short, 0.1 hr, for example, and the creatinine clearance can be directly entered as being 50 ml/min above that of the patient's baseline. Finally, when the patient goes off the dialysis, another dose of zero is entered at that time, and the patient's creatinine clearance is set back to the baseline value [11].

6.5.2 Timing the aminoglycoside dose and the dialysis

Another corollary for dialysis patients is that while most of them are given their dose of drug soon after the dialysis, this can cause a problem. The serum aminoglycoside concentrations in such patients have extremely long half-times, and these patients are often the ones who have the greatest incidence of renal toxicity and ototoxicity, because their serum concentrations stay so high for so long after each dose, even though the doses themselves are adjusted to keep the total area under the serum concentration curve at an appropriate value constrained by the desirable target peak and trough goals. There will be high concentrations for a long time, and then low ones, which may not be effective.

Instead of this, it may be more prudent and useful to give the dose before dialysis, about 2 or 3 hours before dialysis. In this case, one gets the desired peak value for good bacterial kill with this "concentration-dependent" drug. Then the dialysis helps to mimic the renal function of a patient with more normal (or less abnormal) renal function, reducing the serum concentration more rapidly, and helps to achieve a serum concentration profile somewhat more like that of a patent with less impaired renal function. These clinical protocols can easily be planned with appropriate software [11].

6.6 OTHER STUDIES OF OUTCOME AND COST OF TDM

6.6.1 Gentamicin therapy

Probably the best examination to date of the utility of the MAP Bayesian approach to individualize drug dosage regimens for patients has been the work of van Lent-Evers et al. [2]. They compared the model-based, target goal approach to aminoglycoside therapy with a more conventional therapeutic drug monitoring strategy. The mean peak and trough concentrations in the study group were 10.6 ± 2.9 μg/ml and 0.7 ± 0.6 μg/ml respectively versus 7.6 ± 2.2 and 1.4 ± 1.3 μg/ml respectively, both significant differences. The peaks were significantly higher and the troughs significantly lower in the study group. Overall mortality was 9 of 105 (9%) in the study group versus 18 of 127 (14%) in the control group, not a significant difference ($p = 0.26$). However, in those patients who had obvious infections present on admission, mortality was only 1 of 48 in the study group versus 9 of 62 in the control group, a significant difference ($p = 0.023$). In addition, nephrotoxicity was only 2.9% in the study group versus 13.4% in the control group.

While the clinical outcome was significantly improved (more effective, less toxic) with the use of this model-based, target-oriented approach to monitoring and dosage individualization, it was interesting to see that hospital stay was also significantly reduced, from 26.3 ± 2.9 days overall in the control group to 20.0 ± 1.4 days in the study group ($p = 0.045$). For patients with infections present on admission, the stay was similarly reduced, from 18.0 ± 1.4 days in the control group to 12.6 ± 0.8 days in the study group. Thus in both patient groups, those with and also without infections on

admission, hospital stay was reduced by about six days with the use of this approach to serum concentration monitoring and model-based dosage individualization.

Further, despite the added effort and cost to implement this therapeutic approach, the overall cost per patient was reduced from 16,882 ± 17,721 Dutch florins in the control group to 13,125 ± 9,267, a significant difference ($p < 0.05$). In the patients with infections on admission, the cost was reduced from 11,743 ± 7,437 Dutch florins to 8,883 ± 3,778 florins, an even more significant difference ($p < 0.001$). Thus in a sizeable group of patents, the model-based, target-oriented method of TDM and of individualizing aminoglycoside dosage regimens not only resulted in better outcomes, but also in shorter hospital stays than conventional TDM, and at a net cost savings of about US$1000.00 per patient [2].

6.6.2 Amikacin therapy

MAP Bayesian target-oriented, model-based adaptive control has been used to manage amikacin therapy in geriatric patients, often for extended periods, by Maire et al. [14]. In their patients, whose renal function was often quite reduced but who were generally more clinically stable, visibly better prediction (and therefore control) of serum concentrations was seen with MAP Bayesian analysis than with the unfitted population model, showing the utility of TDM and of model-based, target-oriented dosage adjustment.

The results of Maire et al. [14] in these clinically more stable patients are shown in Fig. 6.5, left. They are better than those found in the gentamicin patients with unstable renal function [13] shown in Fig. 6.3 above. Further, Fig. 6.5, right, shows the much

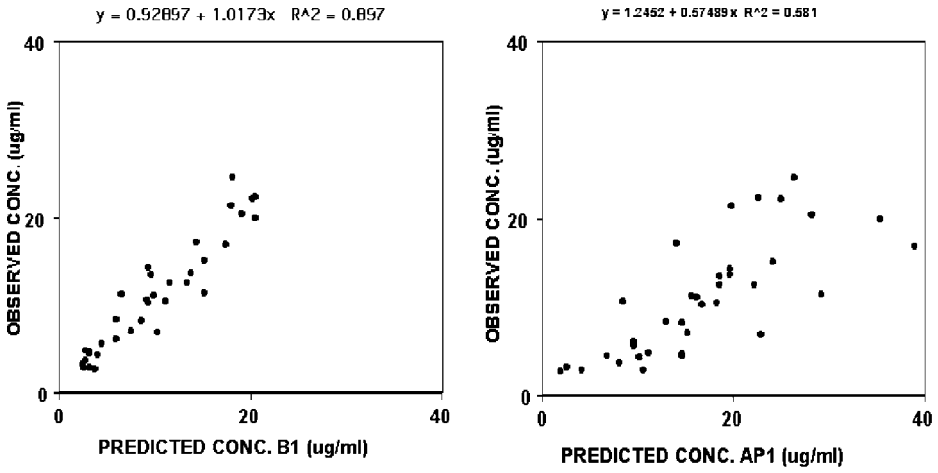

Fig. 6.5. Left: Predicted versus observed serum Amikacin concentrations found with M.A.P. Bayesian fitting, 1 compartment Kslope model (B1). Right: Predicted versus observed serum Amikacin concentrations found with A Priori population 1 compartment Kslope model (AP1).

poorer predictions based simply on the population model for Amikacin, without any fitting to the serum data.

6.6.3 Vancomycin therapy

Vancomycin therapy was evaluated by Hurst et al [15] using a two-compartment (central plus peripheral compartment) model. Using traditional linear regression, extremely poor prediction was found, as shown in Fig. 6.6, left. In contrast, the 2 compartment model, coupled with MAP Bayesian fitting, led to significantly better prediction of future serum concentrations than did the linear regression method, as shown in Fig. 6, right.

6.6.4 Digoxin therapy

The digoxin population model used in the USC*PACK MAP Bayesian software [3,4,11] is based on that described by Reuning, Sams, and Notari [3]. Their two-compartment model uses both a central (serum) and a peripheral (nonserum) compartment. Computed concentrations of drug in the peripheral compartment correlate much better with inotropic effect than do serum concentrations [3,4,11]. The USC*PACK digoxin software [11] not only uses a modified version of their model, but also develops dosage regimens to achieve desired target goals in either the central (serum concentration) compartment or in the peripheral (tissue or effect) compartment.

The following example, shown in Fig. 6.7, is illustrative. A 58 year old man developed rapid atrial fibrillation at another center, after missing his usual daily dose of

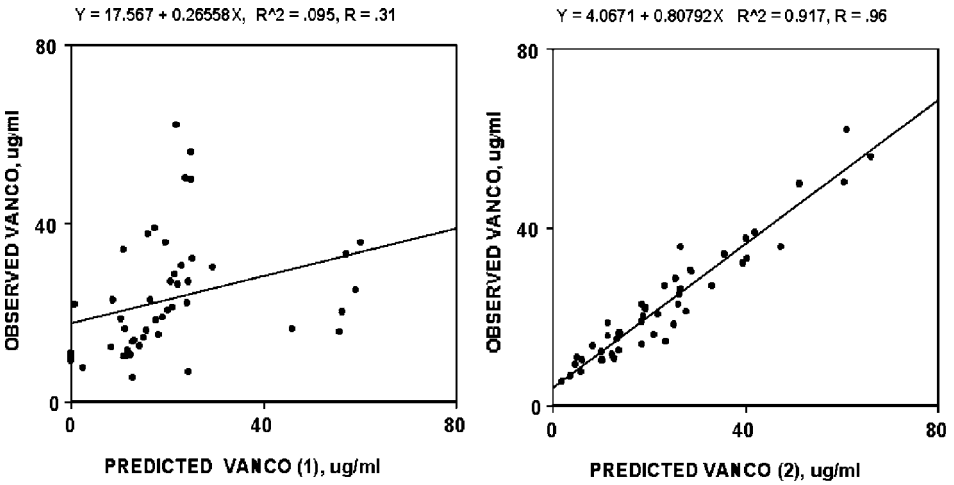

Fig. 6.6. Left: Predicted versus observed serum Vancomycin concentrations found with Linear regression. Right: Predicted versus observed serum Vancomycin concentrations found with a 2-compartment Kslope model and MAP Bayesian fitting).

0.25 mg. He was clinically titrated with several intravenous doses of digoxin, and converted to sinus rhythm. The problem then was to select a successful dosage regimen for the patient. He was placed back on his original oral maintenance dosage. After a day, atrial fibrillation recurred. He again was titrated with intravenous digoxin and again converted to sinus rhythm. Again, the problem was to select a successful dosage regimen for the patient. Once again, he was placed on his original oral maintenance dosage, and once again, after about two days, atrial fibrillation recurred, as the patient excreted the extra digoxin that had been given with the titration step. For a third time he was titrated with several intravenous doses of digoxin, and for a third time he converted to sinus rhythm. A week of hospital time had been consumed during this period of his care. The same question remained – now that sinus rhythm had been restored, and for a third time, what digoxin dosage regimen should this patient now receive?

At this point the USC*PACK MAP Bayesian digoxin software [11] was used, in a telephone consultation, to analyze the patient's situation. The raw data of three serum concentrations, all taken during the post-distributional phase after a dose, showed almost no correlation with the patient's clinical behavior. As shown in Fig. 6.7, he was back in atrial fibrillation when the first serum concentration of 1.0 ng/ml was obtained (the first black rectangle) and had converted again to sinus rhythm when the second and third serum concentrations of 1.0 and 1.2 ng/ml were obtained (the 2nd and 3rd black rectangles) with a lapse back into atrial fibrillation in between them.

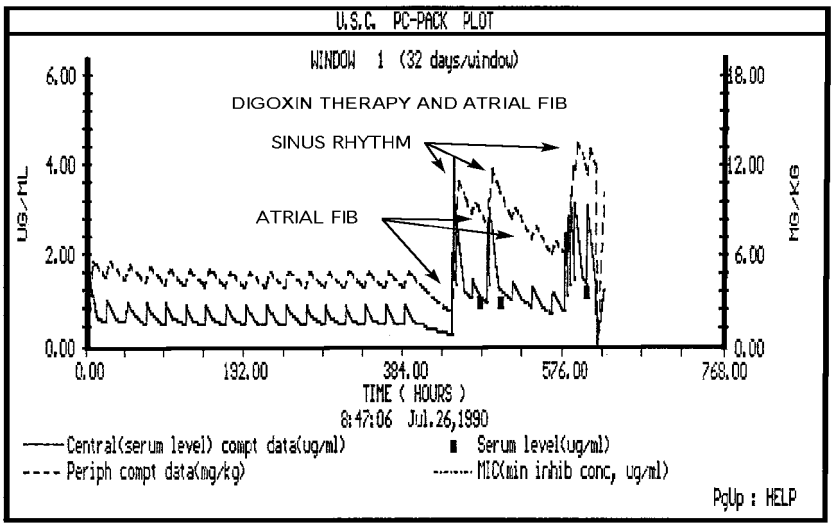

Fig. 6.7. Screen plot of patient with atrial fibrillation who was successfully converted to sinus rhythm with IV digoxin three separate times, but who relapsed into atrial fibrillation twice when put back on his previous maintenance dose. Sinus rhythm was consistently present when peripheral body glycoside concentrations were 10–13 μg/kg (right hand scale, and not mg/kg as labeled). Selection of a therapeutic goal of 11.5 μg/kg in the peripheral compartment led to a dosage regimen of 0.5 and 0.625 mg/day. On that regimen, the patient could be discharged home in sinus rhythm and was still in sinus rhythm when seen in clinic 2 weeks later.

A most important question arises: how can it be that the patient was in atrial fibrillation at one time with a serum concentration of 1.0 ng/ml, and in sinus rhythm at another time, with exactly the same serum concentration? Experiences of this type have made many cardiologists feel that monitoring of serum digoxin concentrations is not useful. Two questions actually need to be asked here. (1) Was the patient in a steady state at the time each of the serum samples were drawn? The answer clearly is no – the patient was not at all in a steady state when either of the two samples was drawn. (2) Were the serum samples obtained at the same time after the dose? Again, the answer is no. Because of this, one cannot use the conventional interpretation of serum concentrations which is based on such steady-state premises. Without the use of a model, the raw data of the above serum concentrations was of little or no use.

However, when the 2-compartment digoxin population model was fitted to the data of his various doses and these serum concentrations, the resulting fitted model was very informative, and quite good correlation was seen between the computed peripheral tissue concentrations and the patient's clinical status. As shown in Fig. 6.7, sinus rhythm was present in this patient whenever his peripheral compartment tissue concentrations were between 10.0 to 13.0 μg/kg. Based on this correlation, a target goal of 11.5 μg/kg (the middle of the patient's response range) was chosen for the desired peripheral target peak compartment body concentration. The resulting regimen to achieve the target goal was 0.25 mg for the first day, and then averaged 0.57 mg/day.

Following this consultation, he was given 0.25 mg the first day, and then placed on a maintenance regimen of 0.5 and 0.625 mg on alternating days, an average of 0.56 mg/day. On this regimen he remained in sinus rhythm. He was able to leave the hospital in sinus rhythm, and was still in sinus rhythm without evidence of toxicity when seen in the clinic two weeks later.

When one considers that an entire week had elapsed before the above consultation, with three successful conversions to sinus rhythm but two failures to arrive at a useful regimen to maintain that conversion, all involving the patients, the physicians', and the ward personnel's time, effort, and money, it is clear that the above pharmacokinetic consultation and the target-oriented, model-based dosage recommendation was quite cost-effective.

6.6.5 Lidocaine therapy

A target-oriented, model-based approach was used to manage lidocaine therapy [16]. Patients with myocardial infarcts who had arrhythmias requiring Lidocaine were retrospectively evaluated. Seventy eight patients had received conventional non-pharmacokinetically oriented therapy, and an equal number of patients had received pharmacokinetically designed, target-oriented, model-based infusion regimens. Of the conventional therapy patients, eight developed ventricular fibrillation, one became toxic, and 33 required additional lidocaine to control their arrhythmias. In contrast, only two patients receiving the target-oriented, model-based regimens developed ventricular fibrillation, a suggestive though not significant difference ($p = 0.12$). One patient became toxic, and the two who developed ventricular fibrillation were the only patients who

required more lidocaine to control their arrhythmias ($p < 0.001$). The pharmacokinetically designed regimens not only achieved more effective serum concentrations [16] but also suggestively reduced the incidence of ventricular fibrillation, and significantly reduced the incidence of breakthrough arrhythmias [16,17].

6.6.6 Busulfan therapy

In a comparison with an equal number of historical control subjects by Bleyzac et al. [18], children who underwent bone marrow transplantation for various hematologic problems and malignancies had their Busulfan therapy given as a standard regimen during the process of transplantation versus having it given using MAP Bayesian adaptive control. Twenty nine patients composed each group. The patients receiving Busulfan guided by MAP Bayesian adaptive control had the incidence of veno-occlusive disease reduced from 24.1 to 3.4% ($p < 0.05$). In addition, graft failure was reduced from 12.0% to 0%. Survival was increased from 65.5 to 82.8%. Because of this, MAP Bayesian adaptive control of the Busulfan dosage regimens in this study made visible and significant improvements in the care of these patients [18].

6.7 WHY WE REALLY MONITOR SERUM CONCENTRATIONS: FOR CLINICIAN-MANAGED, MODEL-BASED, TARGET-ORIENTED INDIVIDUALIZED DRUG THERAPY

Traditional approaches to therapeutic drug monitoring were originally designed for use only in steady state situations, and usually employed only 1-compartment models. They developed dosage regimens only for such steady state situations, and were oriented to keeping serum concentrations within a general therapeutic range rather than to achieving a specific target goal for a specific individual patient. Such approaches made it impossible to deal with patients in some of their most important clinical moments, as, for example, during changing renal function or dialysis, or when certain "golden clinical moments" must be captured, and a dosage regimen developed to achieve and maintain a desired target goal immediately, without waiting for a steady state, as in the case of the above patient receiving digoxin.

The above patient on digoxin (Fig. 6.7) shows how truly individualized drug therapy begins with clinical selection of a specific therapeutic goal for each patient, based on that individual patient's need for the drug. One then should achieve that goal with the greatest possible precision, without any zone of indifference about it. The approach to that patient was highly cost-effective, when compared to the fact that an entire week of hospital time was spent in the previous attempts at dosage adjustment without the aid of a model-based, target-oriented method to aid in the evaluation and interpretation of the TDM data they had obtained.

That patient's case also emphasizes the fact that it is frequently not useful to measure serum drug concentrations simply to see whether or not they are in some general "therapeutic range", nor even to correlate them with the patient's clinical behavior,

although that is often possible, but significantly not so in that patient. That patient clearly shows that the real reason for monitoring serum concentrations is rather to find out how the drug (and its model) actually behaves in each individual patient, especially in non-steady-state situations, and to correlate the behavior of each patient's individual model with his/her own individual clinical behavior. Only then can one optimally evaluate each patient's clinical sensitivity to, and specific need for, a drug. MAP Bayesian adaptive control, managed by looking at the patient clinically, using model-based, target-oriented individualized drug therapy, has brought a precision and capability to drug dosage adjustment and to TDM which was not possible with older obsolete approaches based on linear regression or simply on raw data of the serum concentrations alone.

6.8 OPTIMAL TDM MONITORING STRATEGIES

Often serum samples have been obtained at the trough, just before the next dose, after distribution of the drug is complete in the body after a dose, when the errors in recording the time at which the previous dose was given and the time at which the sample was drawn make the least difference in the value of the measured serum concentration. It is not generally realized that because of this, one has deliberately selected the least informative time, containing the least information concerning the actual behavior of the drug. Specifically, there is minimal information about the processes of absorption, distribution, elimination, their relationship to the dose and to the time course and profile of the serum concentrations, and to the drug effects.

It is often much more useful and informative to obtain serum samples when they contain the most information about the various processes described above. One can use a model, and can make small variations in the model parameter values, and note their effect upon the profile of the serum concentrations. At what time do changes in the model parameter values cause the greatest changes in the serum concentration profile? These are the times when the serum concentrations are maximally sensitive to changes in the various model parameter values. These are the times when getting the serum samples lets one best "see through" the many clinical uncertainties, and best understand the behavior of the drug, by permitting the most precise parameter estimates to be made for the chosen model. These times can be calculated using the well-known D-optimal sampling strategies, based on the work of D'Argenio, for example [19]. Some typical examples of these D-optimal times are illustrated in Figs 6.6 through 6.10. These are shown for an assay that has a constant error over its entire working range. Since that is often not realistic, these D-optimal times can be adjusted for any assay error polynomial of the type described earlier, and the correct, somewhat different times then used to optimize the process of TDM. An excellent routine, part of the ADAPT II package [20], has been developed for this purpose.

D-optimal strategies can easily be employed for aminoglycoside therapy in routine clinical care. For example, instead of waiting 30 minutes after the end on the infusion to get a so-called "peak" sample, and then a trough sample, one can start by getting a true peak sample, out of the opposite arm at the end of the intravenous infusion, and

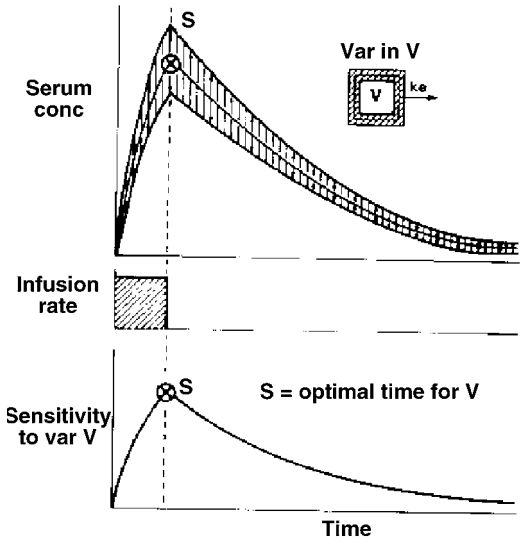

Fig. 6.8. In the context of intermittent intravenous (IV) drug administration, with other things being equal, the greatest change in serum concentration resulting from a change in the apparent volume of distribution (V) is at the true peak, at the end of the IV infusion. Measurements at other times are associated with smaller changes, and are therefore less informative. In general, high concentrations are informative for V, and lower ones are less so.

then to skip one or more dose intervals before getting the next sample about 2 or 3 hours before the trough. For example, it is useful to wait, whatever the dose interval is, until about 21 hours into the regimen, if the patient's creatinine clearance at least 40 ml/min/1.73 M^2. Because of this, it is easy to center the patient's aminoglycoside doses about three hours after routine morning blood drawing time. In that way it is easy to make the routine blood sample at that time be quite close to a D-optimal sample.

For monitoring of digoxin, a peak sample taken about $1\frac{3}{4}$ hours after an oral dose, and a trough sample are a useful pair. For information on the exchange rate constants between the serum and the peripheral digoxin compartment, samples at $\frac{1}{2}$ hour and at 7 hours after the dose are also approximately D-optimal times. In general, considerations of D-optimal sampling strategies also suggest that if possible, it is useful (though not always necessary) to obtain at least one sample for each parameter to be estimated in the patient's model when doing therapeutic drug monitoring.

6.8.1 More general comments

We need to monitor drug therapy better in general. It is distressing to see patients with multidrug resistant TB, for example, dosed without such monitoring. Since many patients with multidrug resistant TB simply absorb the drugs poorly, it wastes the patient's lives by treating them with an unmonitored regimen, simply waiting to see if

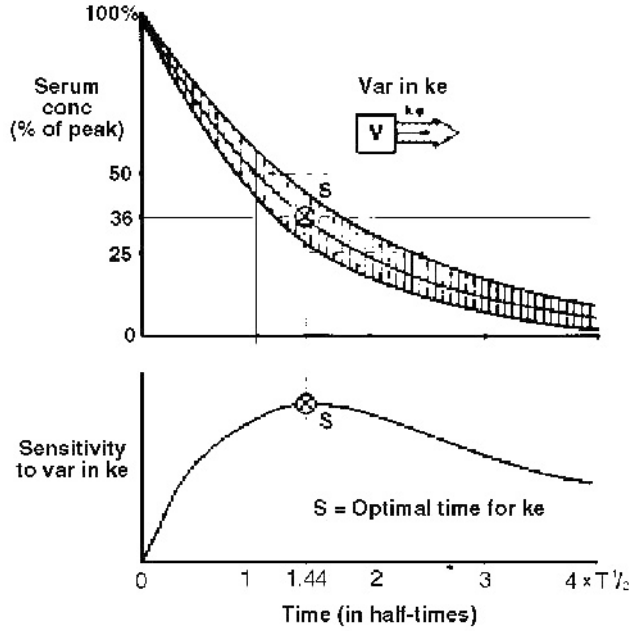

Fig. 6.9. In the context of intermittent intravenous (IV) drug administration, with other things being equal, the greatest change in serum concentration resulting from a change in the rate constant of elimination is when the concentration has fallen to 36% of the true peak. Measurements at other times are associated with smaller changes, and are therefore less informative. A fairly broad band of relatively high sensitivity is seen, but trough measurements are significantly less and less informative the more they are below 36% of the peak.

their sputum smears and cultures eventually become negative. It is much more useful, and may well be more cost-effective in these patients, to know early in the course of therapy whether or not the serum concentrations achieved on a given regimen are likely to be effective, and to make sure that they are.

Further, we spend a great deal of money on expensive treatments for patients with cancer and AIDS, and we follow the viral load, and the measures of hematological toxicity. But we are not yet optimizing this process, and we should. We treat cancer patients with methotrexate to a desired area under the serum curve (AUC), but we usually do not ask if that AUC is really optimal for each individual patient, or if it is really reliable in the first place. Many population models of methotrexate, for example, may well have unreliable parameter values and AUC's, as sampling often is not begun until after at least 6 or 8 hours after the dose, as the monitoring was done simply to see if the drug was essentially gone or if rescue with leucovorin was indicated. Little information on the actual processes of absorption, distribution, and elimination of the drug is available from such studies, when sampling is so delayed and suboptimal.

Monitoring serum concentrations and determining their relationship to the hematocrit, leukocyte count, and platelet count, for example, would permit therapy to be optimized within the constraints of tolerable measures of toxicity for each individual

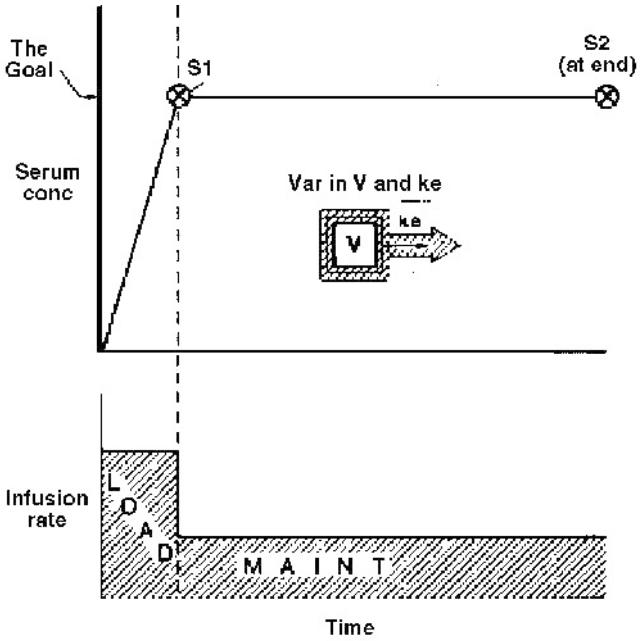

Fig. 6.10. In the context of loading followed by maintenance intravenous (IV) drug administration, with other things being equal, the greatest changes in serum concentrations are first, at the end of the loading infusion, just as at the end of the infusion in Fig. 6.8, and then at the steady state after it is achieved after about 5 drug half-times have elapsed on the maintenance infusion. Measurements at other times are associated with smaller changes, and are therefore less informative.

patient. This would be most useful for optimizing therapy for patients with AIDS, with cancer, with transplants, and with various infectious diseases. There is a great deal of work to be done in this area!

6.9 SPECIAL CASES: ENTERING INITIAL CONDITIONS – CHANGING POPULATION MODELS DURING THE FITTING PROCEDURE

Most pharmacokinetic analyses have dealt with patients (and their pharmacokinetic models), who have had stable values for their various parameters such as volume of distribution, rate constants, clearances, etc. However, this is not always so, even though one can express a rate constant as an intercept plus a slope times a descriptor of elimination such as creatinine clearance [21] or cardiac index, so that renal function or cardiac index can change from dose to dose during therapy, and the patient's drug model can keep up with these changes as they take place.

Probably the most serious problem in analyzing pharmacokinetic data in patients is caused by sudden significant changes in a patient's volume of distribution (Vd) of the central (serum concentration) compartment, without any change in any currently known

clinical descriptor such as body weight. It is generally known, for example, that patients in an ICU setting have larger values for the Vd of gentamicin and other aminoglycosides than do general medical patients. Indeed, young very healthy people who suddenly require an aminoglycoside for a perforated or gangrenous appendix often have even smaller values for Vd [11].

6.9.1 An aminoglycoside patient with a sudden change in clinical status and volume of distribution

An interesting 54 year old woman in Christchurch, New Zealand, was seen through the courtesy of Dr. Evan Begg in the fall of 1991. She was 69 inches tall, weighed 80 kg, and her serum creatinine on admission was 0.7 mg/dL. She had a pyelonephritis, and was receiving tobramycin 80 mg approximately every 8 hours. She had a measured peak serum concentration of 4.6 and a trough of 0.4 μg/ml respectively, and had been felt by all to be having a satisfactory clinical response. During this time, her Vd was 0.18 l/kg, based on those two serum samples. However, on about the 6th day, she suddenly and most unexpectedly relapsed and went into clear-cut septic shock.

Following her surprising relapse on therapy, she was aggressively treated with much larger doses, 300 mg every 12 hours during this time. Her serum tobramycin concentrations rose to peaks of 10.1 μg/ml. During this period of sudden septic shock, her serum creatinine also rose, from 0.7 to 3.7 mg/dL, and her estimated CCr fell to 18 ml/min/1.73m^2. After about another 10 days she improved. At that time, her serum tobramycin concentrations rose to a peak of 16, and it was necessary to sharply reduce the dose to 140 mg about every 12 to 24 hours. Her serum creatinine fell to 1.1 to 1.3 mg/dL, and her CCr rose to 57 ml/min/1.73m^2.

It was simply not possible to get a good MAP Bayesian fit to all the serum data over the entire time period. Most samples were obtained during her second, sickest phase, and they dominated the fit. The ones at the beginning, prior to the sepsis, and at the end, after her improvement, were not at all well fitted.

Because of this, the data was divided into three parts – an initial one before her relapse into sepsis, a second one when she was septic, and a third one following improvement, but before it was felt safe to discontinue therapy. Each data set was fitted separately, using the USC*PACK programs [11].

During the first data set, the first 6 days, when her clinical behavior was that of a general medical patient, not gravely ill, her Vd was 0.18 L/kg as described above. The problem then was to pass on the ending values of the serum and peripheral compartment concentrations as initial conditions for the fitting process for the second data set. This was done, using that feature of the USC*PACK clinical software [11], which was developed specifically for this purpose.

A major change in her Vd was then seen when fitting the data obtained during the second, septic, phase. The Vd rose from 0.18 L/kg in the previous phase to 0.51 L/kg, and the Kslope, the increment of elimination rate constant per unit of CCr, fell to zero. However, the Kcp, the rate constant from serum to peripheral compartment, rose to 0.255 h^{-1}, suggesting that she was "third-spacing" the tobramycin somewhere. The

ending concentrations in the central (serum) compartment for this data set were 2.09 ug/ml, and for the peripheral compartment were a very high 44.1 μg/kg.

These ending values were again passed on as initial conditions to the third part of her data set, that of recovery. During this time the serum peaks were 16 and 12 μg/ml, and the dose was reduced to 140 mg every 12–24 hours. Her Vd during this third phase, that of recovery, when she was no longer seriously ill, had fallen greatly to 0.15 L/kg, close to her previous initial value as a general medical patient.

The ability to enter stated initial conditions permitted changing population models during the various phases of the patient's overall fitting procedure, and allowed intelligent analysis of this patient's data, especially as quite significant concentrations were present not only in the central (serum) compartment, but also in the peripheral compartment, during the transition from the patient's second to the third, recovery, phase.

At the Cleveland Clinic, Drs. Marcus Haug and Peter Slugg [22] have spoken of "Vd collapse", when the Vd would drop from a larger to a smaller value. They showed that this change was a clinical indicator of incipient recovery of the patient. The present patient not only demonstrated such Vd collapse later on, as she got better, but also its opposite, Vd expansion, as she made the earlier transition from being a general medical patient with a pyelonephritis to a seriously ill ICU patient with life-threatening septic shock.

Not only do different populations of aminoglycoside patients have different values of Vd, but each individual patient goes through these transitions, as demonstrated by this patient. The analysis of this patient's data was greatly facilitated, and indeed was only possible, using the MAP Bayesian approach, by breaking the dosage history up into several parts. Each part was then analyzed, and the ending concentrations from one part were passed on to the next data set as initial conditions or concentrations of drug present prior to the first dose given in the next data set, with the appropriate population model, if needed, as well.

6.10 LINKED PHARMACODYNAMIC MODELS: BACTERIAL GROWTH AND KILL

In this section we will describe the linkage of a nonlinear pharmacodynamic model of effect to the basic linear pharmacokinetic model, and show some applications in clinical software of models describing bacterial growth in the absence of a drug and its kill by an antibiotic.

6.10.1 General considerations

Let us assume that an organism is in its logarithmic phase of growth in the absence of any antibiotic. It will have a rate constant for this growth – a doubling time. The killing effect of the antibiotic can be modeled as a Michaelis-Menten or Hill model. The model generates a rate for this effect. The rate of growth or kill of an organism depends upon

the difference between these two rate constants. The killing effect will be determined by the Emax, representing the maximum possible rate constant for kill, the EC_{50}, the concentration at which the effect is half maximal, and the time course of the concentrations at the site of the effect achieved with the dosage regimen the patient is given. Both the growth rate constant and the Emax can be found from available data in the literature for various organisms. The general growth versus kill equation is

$$\frac{dB}{dt} = (K_g - K_k) \times B \tag{3}$$

and

$$K_k = \frac{E_{\max} \times C_t^n}{EC_{50}^n + C_t^n}, \tag{4}$$

where B is the number of organisms (set to 1.0 relative unit at the start of therapy), K_g is the rate constant for growth, K_k is the rate constant for killing, Emax is the maximum possible effect (rate of killing), EC_{50} is the concentration at which the killing rate is half maximal, n is the Hill or sigmoidicity coefficient, C_t is the concentration at the site of the effect (serum, peripheral compartment, effect compartment, or in the center of a spherical model of diffusion), at any time t, and $\times$ indicates multiplication.

The EC_{50} can be found from the measured (or clinically estimated) minimum inhibitory concentration (MIC) of the organism. This relationship was developed by Zhi et al. [23], and also independently by Schumitzky [24]. The MIC is modeled as a rate of kill that is equal to but opposite in direction to the rate constant for growth. The MIC thus offsets growth, and at the MIC there is neither net growth nor decrease in the number of organisms. At the MIC,

$$dB_{dt} = 0 \text{ and } K_k = K_g, \tag{5}$$

and

$$MIC = \left[\frac{K_g \times EC_{50}^n}{E_{\max} - K_g} \right]^{\frac{1}{n}}. \tag{6}$$

In this way, the EC_{50} can be found from the MIC, and vice versa.

The input to this effect model can be from either the central or the peripheral compartment concentrations of a pharmacokinetic model, or from the center (or any other layer) of a spherical model of diffusion [25]. The sphere may represent an endocardial vegetation, an abscess, or even a small microorganism. In the latter case, one can adjust the sphere diameter and the diffusion coefficient so that the concentrations in the center of the small sphere lag behind the serum concentrations and cross below the MIC about 6 hours after the serum concentrations do, to simulate a post-antibiotic effect of about 6 hours, for example. The effect relationship was modeled by Bouvier D'Ivoire and Maire [26], from data obtained from Craig and Ebert [27], for pseudomonas and an aminoglycoside.

Let us first examine these effect models with relationship to the dosage regimen of amikacin. We had considered a hypothetical 65 year old man, 70 inches tall, weighing 70 kg, having a serum creatinine of 1.0 mg/dL. The patient's dosage regimen consisted of an initial dose of 850 mg of amikacin followed by 750 mg every 12 hours thereafter. On that regimen, predicted serum concentrations were 43 μg/ml for the peak and 3.2 for the trough, possibly a bit low, as the MIC of the organism was stated to be 8.0 μg/ml. Fig. 6.11 is a plot not only of the predicted time course (the first six days) of serum amikacin concentrations for the above patient, but also of its ability to kill microorganisms using the model made by Bouvier D'Ivoire and Maire [26], based on the data of Craig and Ebert [27]. The serum concentration profile is presented as the input to the bactericidal effect model.

The model always assumes an initial inoculum of one relative unit of organisms. The scale of the relative number of organisms is shown on the right side of Fig. 6.11, while the scale of the serum concentrations is on the left. As shown in the figure, the serum concentration profile resulting from that regimen appears to be able to kill such an organism well in this particular patient. As the serum concentrations fall below the MIC with the first dose, however, the organisms begin to grow again, but the second dose kills them again, with slight regrowth once again toward the end of that dose interval. The third dose reduces the number of organisms essentially to zero. Use of this effect model

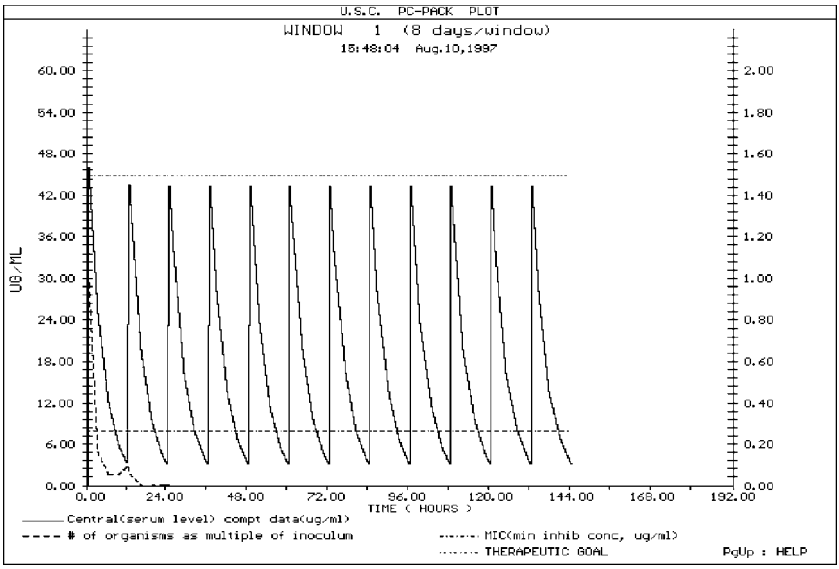

Fig. 6.11. Predicted killing effect of the regimen. Input from the central (serum) compartment profile of serum concentrations. The regimen is likely to kill well for a bloodstream infection (sepsis). Solid line and left hand scale – profile of serum amikacin concentrations. Dashed line and right hand scale – relative numbers of organisms, with 1.0 relative unit present at the start of therapy. Upper horizontal dotted and dashed line – original peak serum goal of therapy. Lower horizontal dashed line: the patient's MIC of 8.0 μg/ml.

suggests that such a serum concentration profile should be effective in killing an organism having an MIC of 8.0 μg/ml, even though the serum concentrations are below the MIC about one third of the time, as the high peaks are effective in the killing.

Such models of bacterial growth and kill permit one to incorporate known in vitro data of the logarithmic growth rate of the organism and the maximum kill rate achieved with the antibiotic, to integrate it with data of the MIC of each individual patient's organism, and to model the growth and decline of the relative numbers of organisms. These Zhi models have correlated well, in the tobramycin patient described earlier, with her unexpected relapse from having an apparently satisfactory response to therapy to becoming a seriously ill patient with septic shock, and with her subsequent recovery later on, as effective serum concentrations were achieved and maintained.

The Zhi model, however, does not describe the decline of bacterial growth rate seen over time, which may reach a maximum number of organisms, as found by Mouton, Vinks and Punt [28]. The organisms are always assumed to be in their logarithmic growth phase, the maximum growth rate possible. In addition, the Zhi model does not describe the increase in bacterial resistance found over time, and the emergence of resistant organisms. However, one can estimate clinically the maximum possible MIC which the emerging resistant organism might reach, and examine the behavior of the model under that condition. In this case, the Zhi model becomes a useful example of a "worst case" scenario model, with the resistant organisms being so from the very start of therapy, and with the logarithmic growth rate always being in effect, never slackening. If a given dosage regimen, generating a certain serum concentration profile, can kill well using the Zhi model, one might expect it probably to do at least as well in clinical circumstances, where the growth rate may (or may not) slacken with time and may (but may not) reach a maximum number of organisms, and the resistant organisms emerge more slowly with time.

Clearly, further work in this area is needed, but models of this type are beginning to provide a useful new way to perceive, analyze, and evaluate the efficacy of antibiotic therapy. Similar approaches may also be useful in analyzing therapy of patients with AIDS, using the PCR assays, and with cancer. They appear to offer better information than the empirical correlations between peak/MIC ratios and the percent of the dose interval that the serum concentrations are at least the MIC. They do a more complete job of describing the behavior of so-called "concentration-dependent" and "concentration independent" or "time-dependent" drugs, which is often an artificial classification. Zhi models describe both such types of behavior, in which the maximum rate of kill often may be reached, with some drugs such as vancomycin, for example, at about 5 times the MIC. The model better captures such behavior. The classifications into "concentration independent" or "time dependent" seem do not do as well in this regard.

6.11 OTHER LINKED PHARMACODYNAMIC MODELS: AMINOGLYCOSIDE NEPHROTOXICITY AND OTOTOXICITY

Other models of effect have also been linked to the basic pharmacokinetic model. Rougier et al. have modeled the saturable uptake of aminoglycosides by renal cortical

cells, with the resulting reduction of creatinine clearance and the rise in serum creatinine concentrations in patients who displayed evidence of nephrotoxicity from amikacin [33]. They showed that all such therapy caused some decrease in renal function. However, when their model was presented with simulated serum concentration profiles based on a 2-compartment population model and on dosage schedules given to real patients either as three times daily or as once daily, renal toxicity was greatest with a thrice daily schedule and less with a once daily schedule, for approximately the first week of therapy. After one week, with more prolonged therapy, the differences in toxicity became less with the two schedules, as toxicity continued to increase in both groups with continued treatment.

Rougier et al. also made a model of the probability of occurrence of nephrotoxicity, also using a saturable model based on areas under the serum concentration curves [34]. They presented this linked model with three different simulated dosage schedules having the same total daily dosage – 1600 mg every 48 h, 800 mg every 24 h, or 267 mg every 8 h. Good fits to observed serum creatinine concentrations in patients were seen. Toxicity was greatest with dosage every 8 h, in between with every 24 h, and least with every 48 h. Greater accumulation was seen with lower assumed values of K_m. These models appear useful to incorporate into clinical software for individualizing therapy and also into population modeling approaches to capture such relationships.

A saturable model of uptake onto possible toxic binding sites in the vestibular apparatus was used by Berges et al. [35] to evaluate and compare the area under the exposure curve (AUC's) found with a Michaelis-Menten model versus that of several linear models, in a group of patients who had documented vestibular toxicity from gentamicin. They compared the AUC's with those found from a simulated reference regimen of 5 mg/kg/day for 10 days, as given to a simulated male patient age 65, 70 inches tall, 70 kg in weight, with a serum creatinine of 1.0 mg/dL/. The AUC's were found for the serum compartment, for the peripheral (tissue) compartment of the basic linear model, and for a Michaelis-Menten saturable effect compartment, using assumed Km values of 5.0 and 0.5 μg/ml respectively. Using the serum AUC, one of the 8 ototoxic patients had a value less than that seen with the reference regimen, while the other 7 had greater exposure values. Using the peripheral compartment AUC, again, the same patient had an AUC less than that of the reference regimen. However, using the Michaelis-Menten saturable model and the Km value of 5.0 μg/ml, all ototoxic patients had AUC values greater than that from the reference regimen. With the Km of 0.5 μg/ml, all ototoxic patients had AUC values greater than twice that seen from the reference regimen. This analysis of gentamicin behavior in patients with documented vestibular toxicity strongly suggests that clinically, ototoxicity is also better described with a saturable model than with a linear one. These models can easily be incorporated into current clinical software to aid in the analysis of nephrotoxicity and ototoxicity in patients receiving aminoglycosides. Similar effect models may well be capable of describing the effects of drugs on the hematopoetic system in patients receiving cancer chemotherapy, to aid in their dosage optimization on a more individualized basis than is currently done, keeping each patient's hemoglobin, leukocyte or granulocyte count, and platelet count at acceptable target values.

6.12 LIMITATIONS OF CURRENT MAP BAYESIAN ADAPTIVE CONTROL

The maximum aposteriori probability (MAP) Bayesian approach to adaptive control and dosage individualization is straightforward and robust. However, it does not represent an optimal approach to dosage individualization. It has two significant limitations.

The first limitation is that the pharmacokinetic model parameter values used to describe the behavior of the drug are assumed to be either normally or log-normally distributed. This is often not so. Many drugs, for example, have clusters of both rapid and slow metabolizers within the population, and therefore may well have multimodal population parameter distributions for the elimination rate constant. Furthermore, the volume of distribution for drugs such as the aminoglycosides is affected by the patient's clinical state as a general medical patient or a patient in an intensive care unit, for example. Because of this, parameter distributions are often asymmetrical, neither normally nor lognormally distributed, and are therefore not optimally described by means, medians, modes, and variances. This point reflects the significant problems associated with making "parametric" population models, and based on single point estimates of such mean or median parameter values, to develop dosage regimens. In such a situation, one is acting based only on the estimated central tendencies of the parameter distributions, rather than on the full, often genetically polymorphic, parameter distributions, with their clusters of genetically determined fast and slow metabolizers of drugs, for example. The problem is largely overcome by making "nonparametric" population models which describe the entire most likely joint parameter distribution within the population, with up to one support point (set of parameter values, and its estimated probability) for each subject studied in the population [8,9].

The second limitation is that there is no tool in the MAP Bayesian strategy to estimate and predict the precision with which a desired dosage regimen developed to hit a desired target goal actually is likely to do so. The method lacks a vital performance criterion.

The separation, or heuristic certainty equivalence [29], principle is well known among the stochastic control community, but less so among the pharmacokinetic community. It states that when the task of controlling the behavior of a system is separated into the steps of:

(1) Obtaining the best single point parameter values in the model describing the behavior of the system, and then,
(2) Using these single point values to design the inputs to control the system,

that, the task of hitting the target goal is usually performed suboptimally. Yet this is exactly what the MAP Bayesian, and all methods which estimate single values for each model parameter, do.

There is no performance criterion to optimize in the MAP Bayesian dosage strategy (such as the estimated precision with which the desired target will be hit, for example) as there is only one set of parameter values, and the target is simply assumed to be hit exactly.

6.13 OVERCOMING THE SEPARATION PRINCIPLE: "MULTIPLE MODEL" DESIGN OF MAXIMALLY PRECISE DRUG DOSAGE REGIMENS

The above limitations are overcome by the combination of nonparametric population models [8,9] and the "multiple model" design of dosage regimens [30]. Nonparametric population models have been discussed elsewhere [8,9]. Their strength is that they are consistent, statistically efficient, and have good properties of statistical convergence [31]. They are not limited by the assumption that the parameter distributions must be Gaussian or lognormal, as in parametric methods. Instead of simply using parameter means, variances, and correlations between them, as point estimates of a distribution, the nonparametric methods estimate the entire parameter distributions themselves. The final distributions obtained are discrete, not continuous. They consist of discrete sets of parameter estimates, along with an estimate of the probability of each set [8,9]. Up to one set (support point of the distribution) is obtained for each subject studied in the population. This closely approaches the ideal population model (which can never be attained), which would consist of the correct structural model of the drug system, along with the exact value of each parameter in each subject if it would somehow be possible to know those values.

When a parametric population model is used as the Bayesian prior to design an initial dosage regimen for the next patient one encounters, one has only a single estimated value for each parameter. Because of this, only one prediction of future concentrations can be made. The action taken is therefore based only on the estimates of the central tendencies of the parameter distributions, and not on the entire distributions, which may well be multimodal, due to genetic polymorphism in the distribution and metabolism of drugs. The dosage regimen is simply assumed to achieve the target goal exactly, as shown in Fig. 6.12. Figure 6.12 shows the results of an infusion regimen of lidocaine, based on the mean population parameter values for that drug, which was designed to achieve and maintain a target serum concentration of 3 μg/ ml. As shown, this regimen, based on the single mean population parameter values, hits the target exactly, but only when the patient has parameter values which are exactly the population mean values. However, as shown in Fig. 6.13, when that regimen, based on the mean parameter values used in Fig. 6.12, was given to the combination of the actual 81 diverse nonparametric population support points from which these mean parameter values were obtained, an extremely wide distribution of predicted serum concentrations was seen, representing the diversity in the parameter values in the patients studied in the population. The predicted serum concentrations actually covered much more than the usual therapeutic range of 2 to 6 μg/ml.

In contrast, if one has a nonparametric population model [8,9], with its multiple sets of model parameter values (81 in this case), one can make multiple predictions, instead of only one, of the future serum concentrations resulting from any candidate dosage regimen which is "given" to all the models in the population discrete joint density. Based on these multiple models in the population (the discrete joint density), one can compute the weighted squared error with which a candidate regimen is predicted to fail to achieve the desired target goal at a target time. Other regimens can then be

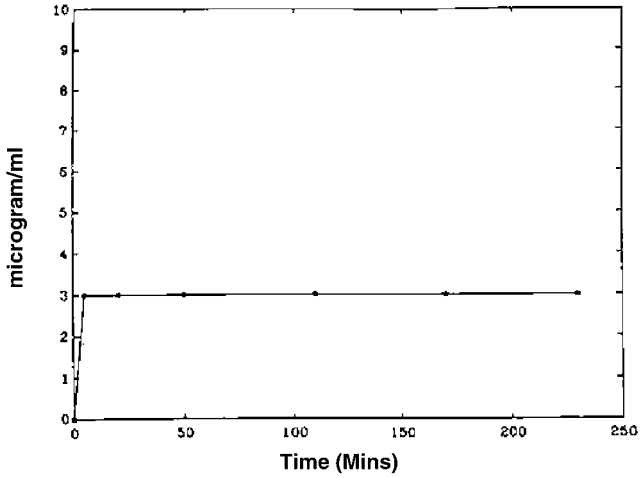

Fig. 6.12. Using lidocaine population mean parameter values, an infusion regimen designed to achieve and maintain a target goal of 3 ug/ml does so exactly when the patient, as here, has exactly the mean population parameter values. Horizontal axis: time in minutes into the regimen. Vertical axis: serum lidocaine concentration.

considered, and the optimal regimen can be found which is specifically designed to achieve the desired target goal with the least weighted squared error [30].

This approach, using the multiple models of the patient provided by the multiple support points in the nonparametric population model, avoids many of the limitations in

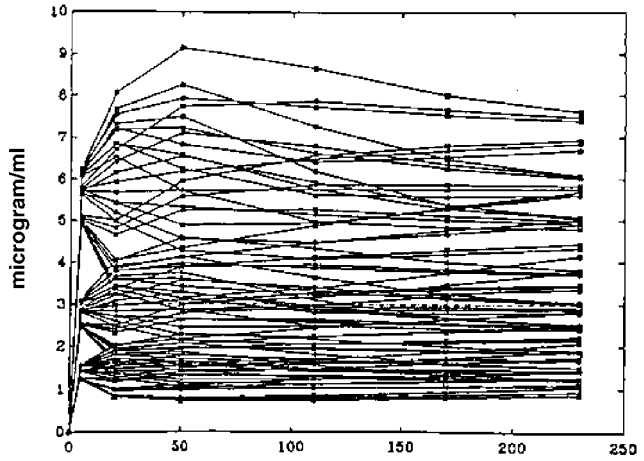

Fig. 6.13. Result when the above lidocaine infusion based on population mean parameter values is given to the 81 diverse support points from which the population mean values were obtained. Great diversity in the predicted responses is seen.

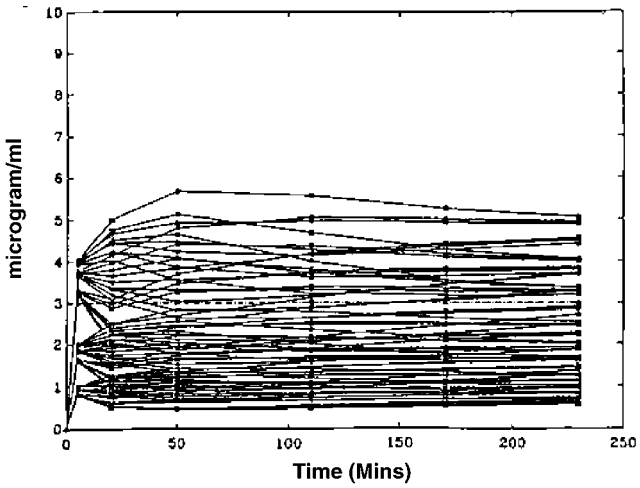

Fig. 6.14. Predicted response of the 81 support points (models) when the regimen obtained by multiple model dosage design is given. The target is achieved with visibly greater, and optimal, precision, compared to that in Fig. 6.13.

the separation principle. This is the real strength of the combination of nonparametric population models coupled with "multiple model" dosage design [30].

As shown in Fig. 6.14, the multiple model (MM) dosage regimen, based on the same nonparametric population model, but now using all the information contained in each of the 81 support points (instead of only the mean values), obtained much more precise achievement of the target goal, because it was specifically designed to do so. The error in the achievement of the therapeutic target goal is much less, and the dispersion of predicted serum concentrations about the target goal is much less. This comparison illustrates the dangers of using mean parameter values to compute dosage regimens for patients, rather than using the much richer information contained in the collection of all the multiple support points in the nonparametric population model.

6.13.1 Obtaining "multiple model" Bayesian posterior joint parameter distributions

With the MAP Bayesian approach to posterior parameter values, the single most likely value for each parameter is obtained when they altogether minimize the objective function shown in equation (1). In contrast, the MM Bayesian approach, using the nonparametric joint densities and their multiple support points, preserves the multiple sets of population parameter values, but specifically recomputes each of their Bayesian posterior probabilities, based upon the serum concentrations obtained. Those combinations of parameter values (support points) that predict the measured concentrations well, become more probable. Those that predict them less well become less so. In this way, the probabilities of all the nonparametric population model support points become

revised, using Bayes' theorem [32]. A smaller number of significant points is usually obtained. When the regimen for the next cycle is developed, these revised models, containing their multiple revised MM Bayesian posterior support points, are used to develop it. The regimen is again specifically designed to achieve the desired target goal with maximum precision (minimum weighted squared error).

6.13.2 Other Bayesian approaches

Three sequential Bayesian approaches have also been used by us to incorporate feedback from measured serum concentration data. The first is the sequential MAP Bayesian approach, in which the MAP posterior parameter values are sequentially updated after each serum concentration data point is obtained. That posterior set of

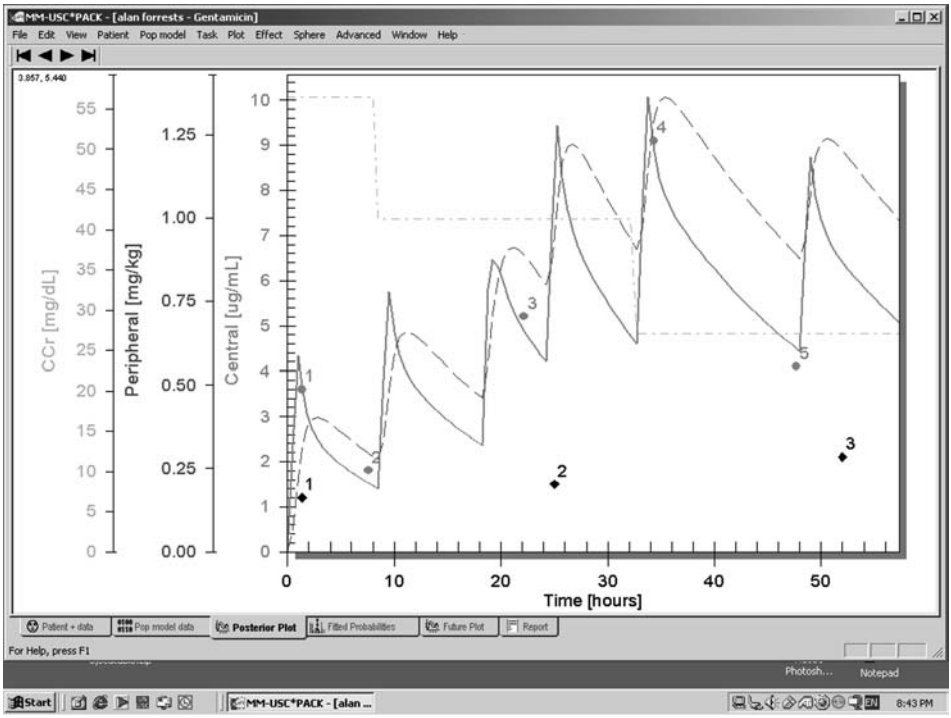

Fig. 6.15. A patient on gentamicin with changing renal function. Serum creatinine concentrations – lower 3 diamond-shaped dots. Creatinine clearance was estimated from the serum creatinine data and the patient's age, gender, height, and weight. It is plotted for each dose of gentamicin which the patient received (left hand scale and stepwise constant line of short and longer dashes). Serum gentamicin concentrations – upper 5 round dots. Solid line: weighted average trajectory of the patient's individual MM Bayesian posterior serum concentration PK profiles fitted to the measured serum concentration data. Line of long dashes: weighted average fitted values of gentamicin concentrations in the peripheral (nonserum) compartment. A good fit to all the data was seen using the MM Bayesian procedure, assuming fixed parameter distributions throughout.

parameter values becomes the Bayesian prior for the analysis of the next measurement, and so on. This sequential procedure shows changes in estimated parameter values following each new measured serum concentration, until all the serum measurements have been analyzed. However, at the end of each full feedback cycle, (after all the samples in each new full cluster of data points have been analyzed), at the time the next dosage regimen is to be developed, this method has learned no more with respect to developing that dosage regimen than if it had fitted all the data together at once, even though it shows changing estimated MAP Bayesian parameter values sequentially. This is because the method is designed to estimate only one single fixed set of parameter values that fit the Bayesian objective function best, over all the data points available up to that time.

The second approach is the sequential MM Bayesian one [32]. Here the MM Bayesian posterior joint density is also sequentially updated after each data point, as described earlier above. Still, at the end of each feedback cycle, this procedure similarly has learned no more with respect to developing the next dosage regimen than if all the data in that cluster were fitted simultaneously. The procedure is still looking for a hypothetical single set of parameter values which best describes all the data. When this fails to be the case, combinations of support points are found which fit best. The

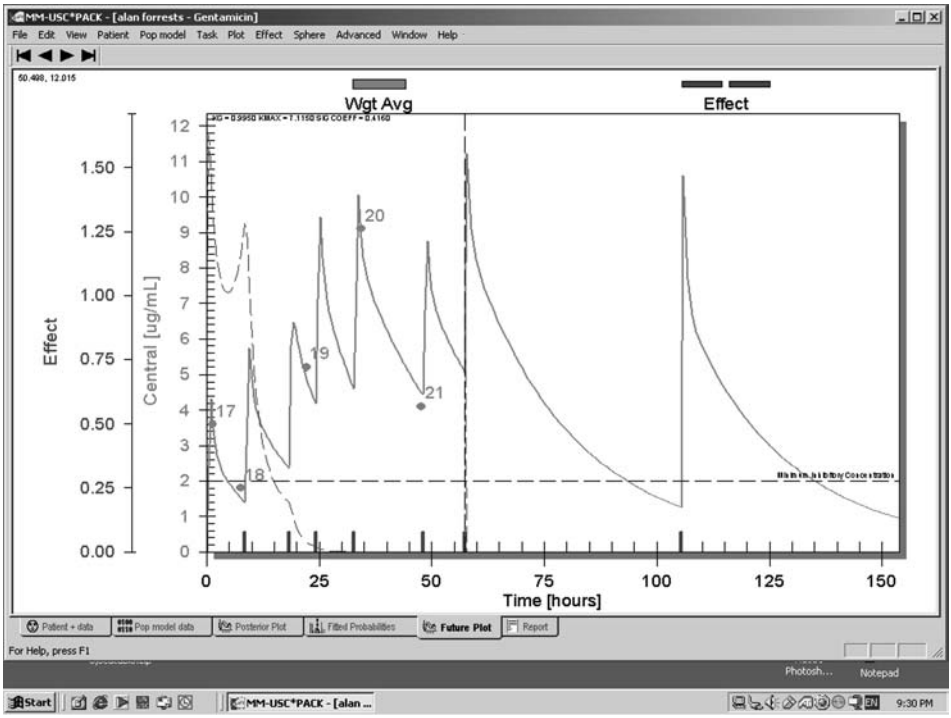

Fig. 6.16. Fitted past and predicted future serum gentamicin concretions (solid line) in the patient described above, on the suggested regimen. Dashed line: fitted and predicted relative numbers of viable organisms using the linked Zhi model.

procedure still estimates a fixed and unchanging single combination of models (support points), which best fit the data up to that time.

A third approach is the interacting multiple model (IMM) sequential Bayesian approach [10]. This method permits the true patient being sought for actually to change, with a certain stated probability, from one model or support point to another during the sequential Bayesian analysis, if such a change is calculated to be more likely, given the measured data. Because of this, the IMM method, originally developed by the aerospace industry and the military to track missiles and aircraft taking evasive action, permits detection of changing drug behavior (pharmacokinetic parameter distributions) during the sequential analysis procedure. It provides an improved method to track truly changing parameter distributions and the behavior of a patient during the evolution of his/her clinical therapy. For example, it permits an improved ability to detect and to quantify changes in the volume of distribution and other parameters of aminoglycoside drugs during changes in a patient's clinical status which are not captured by the use of conventional clinical descriptors.

Using carefully simulated models in which the true parameter values changed during the data collection, the integrated total error in tracking a such simulated patient was

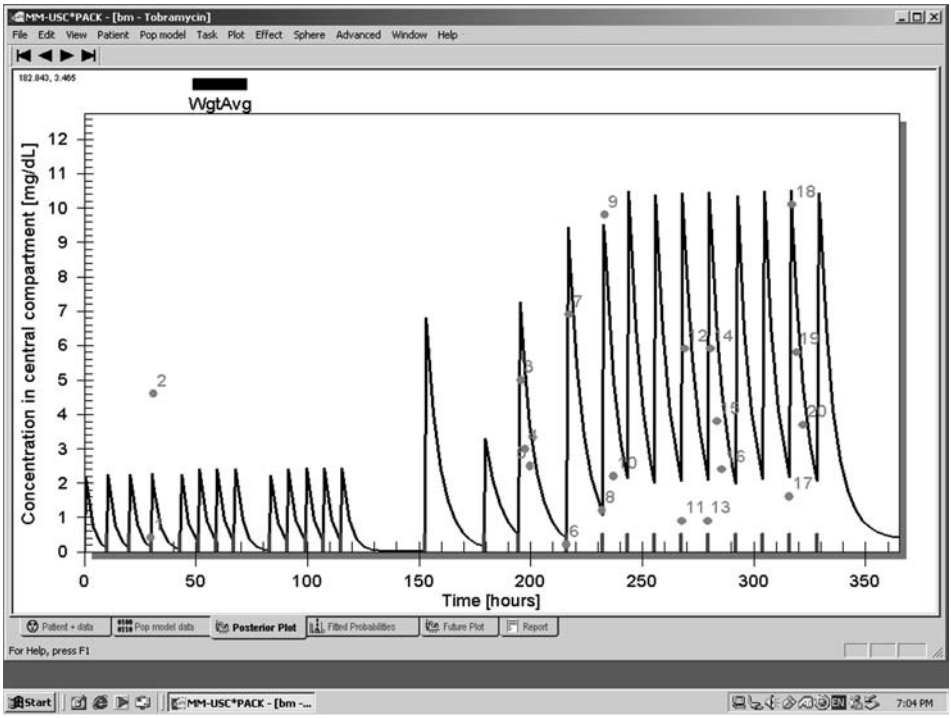

Fig. 6.17. Fit to data of the tobramycin patient described earlier, analyzed with the MM Bayesian approach. Note the very poor fit to the data, due to the patent's changing parameter values as her clinical status changed significantly, going from someone with a pyelonephritis before 150 hours, to someone with clearcut septic shock afterward, becoming an acutely and severely ill intensive care patient.

very similar with both the sequential MAP and sequential MM Bayesian procedures. However, the integrated total error of the sequential IMM procedure was only about one half that found with the other two [10].

6.13.3 MM clinical application

Nonparametric population parameter distributions, MM dosage design, and either MM or IMM Bayesian posterior joint densities appear to offer significant improvements in the ability to track the behavior of drugs in patients throughout their care, especially when the patients have long dosage histories, are unstable, and have changing parameter values. These approaches also permit development of dosage regimens which are specifically designed to achieve target goals with maximum precision. These new and powerful methods make essentially optimal mathematical use of all information contained in the past population data, coupled with whatever current data of feedback may be available up to that point, to develop that patient's most precise dosage regimen.

A good example of MM Bayesian adaptive control is that of a patient on gentamicin with changing renal function, as shown in Fig. 6.15.

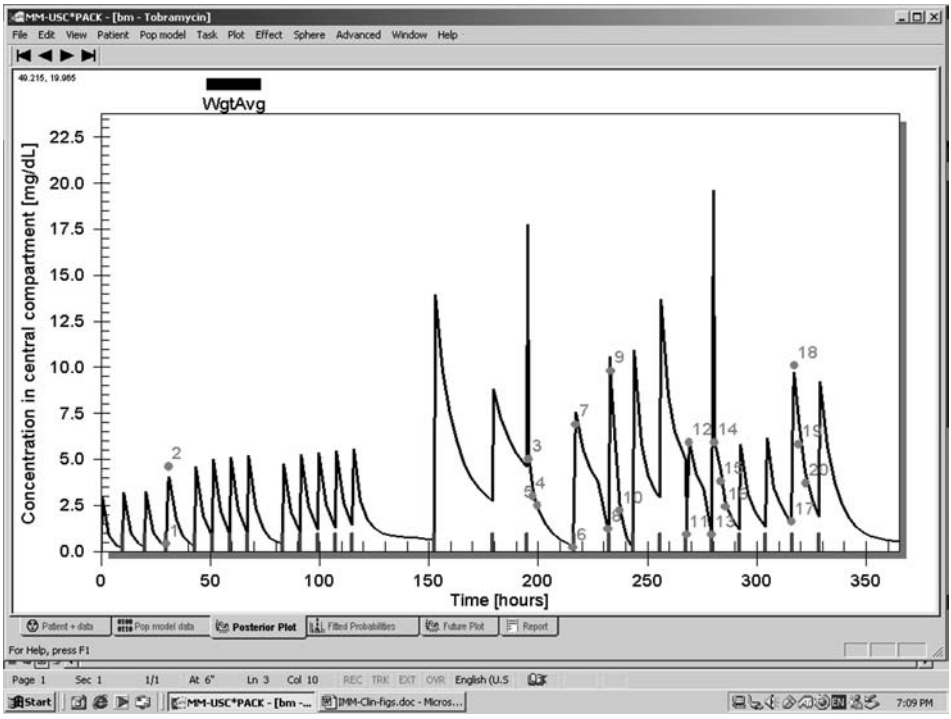

Fig. 6.18. Fit to data of the tobramycin patient described earlier, analyzed with the IMM Bayesian approach. Note the very much improved fit to the data, as the IMM approach tracks the changing parameter values taking place in this acutely ill and highly unstable patient.

Dosage for this patient was begun at 80 mg approximately every 8 hours (the first 2 doses). The first pair of serum concentrations showed a low peak but a relatively high trough. Serum creatinine was 1.2 mg/dL. Based on those results, but prior to use of this software, dosage was increased to 100 mg every 8 hours. Serum creatinine rose to 1.5 and later to 2.1 mg/dL, and creatinine clearance dropped to 27.1. A serum sample almost 15 hours after the third dose was of 100 mg was 4.1. Dosage was then cut back to 80 mg every 8 hours. The question here is what is going on with the drug in this patient, and what dosage regimen now might be indicated, for example, to achieve a peak target goal of 11.0 ug/ml and a trough goal of 0.5 ug/ml.

The suggested doses to hit a target peak of 11.0 and a trough of 0.5 μg/ml were first 95 mg, and then, after 48 hours 155 mg every 48 hours thereafter. Fig. 6.16 shows the predicted peaks of 11.2 and 10.6 μg/ml, and troughs of 1.3 and 0.95 μg/ml respectively. The Zhi model of bacterial growth and kill, for the stated MIC of 2.0 μg/ml, showed an effective result as the early doses were effective as the patient's renal function decreased, and the new regimen was also predicted to maintain the effective kill. The regimen was revised to 100 mg for the first dose and 150 mg for the second one.

6.13.4 Analyzing the changing tobramycin patient with MM and IMM sequential Bayesian methods: implementation into clinical software

The MM and IMM tools have now been implemented in clinical software, the MM-USCPACK package [32], which is in beta phase for release at this time. Fig. 6.17 shows the plot of the fit to the data from the previous tobramycin patient having the changing parameter values, when analyzed using the sequential MM Bayesian approach. The data was very poorly fitted by a single set of parameter distributions, as this highly unstable patient changed from being a general medical patient with a pyelonephritis (before 150 hours in the plot) to an acutely ill patient with severe septic shock after that time.

On the other hand, Fig. 6.18 shows the result when the sequential IMM algorithm was used to analyze that patient's data. The fit was greatly improved. The IMM algorithm was able to track the changing behavior of tobramycin much better in this acutely and severely ill, highly unstable patient.

6.14 THE FUTURE OF INDIVIDUALIZED DRUG THERAPY

The MM-USC*PACK clinical software incorporates all the strengths given by the use of nonparametric population models and multiple model dosage design, and the MM and IMM Bayesian analysis of individual patient data. Work is now under way to develop similar MM dosage designers for the large and often nonlinear models of drugs, such as Phenytoin, Carbamazepine, and many drugs used in the treatment of patients with AIDS, transplants, and cancer, for example. Nonparametric population models can be made now of large, nonlinear, interacting and multiple combination drug systems such as those found in chemotherapy of many of the above patients, where the concentrations of one drug may either compete with a metabolite, or may increase or decrease the rate of metabolism of another drug.

References pp. 166–168

As MM dosage regimens are developed for such large combination multidrug systems, it will then be possible, for the first time, to develop truly coordinated, maximally precise, combination chemotherapy for patients with such problems, maximizing effectiveness while constraining toxicity within specifically selected quantifiable target limits and values.

6.15 ACKNOWLEDGMENTS

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CHAPTER 7

Dose and therapy individualisation in cancer chemotherapy

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7.1 INTRODUCTION

Anticancer drugs, especially those with cytostatic activity, are good candidates for therapeutic drug monitoring (TDM) due to their high toxicity. However, cytostatic drugs do not necessarily have a narrow therapeutic range, which is often stated as a prerequisite for TDM. Drugs like methotrexate are administered in low doses of about 50 mg/m^2 weekly during maintenance therapy of leukaemia and also in high-dose therapy regimes with a single dose of up to 33.6 g/m^2 [1]. Similarly, cyclophosphamide is effective when given by mouth in low doses during maintenance therapy but is also often used in high-dose therapy regimens with doses of up to 100 mg/kg [2,3].

These two examples reflect important differences of cancer chemotherapy to other therapeutic areas: First, with the heterogeneity of tumours and the different stages of cancer it is difficult to define a general therapeutic goal. This means that a dose during maintenance therapy with the aim to eliminate residual cancer cells is not sufficient to reduce a tumour burden when starting a therapy with more than 10^9 cells.

Second, for many cytotoxic drugs a clear pharmacological endpoint is not defined. We actually often do not know exactly way how a cytotoxic drug exerts its antitumour effects. As an example, for anthracyclines like doxorubicin or daunorubicin different mechanisms of action were proposed [4]: intercalation of DNA with subsequent inhibition of protein biosynthesis, free radical formation and inhibition of topoisomerase II. However, it is unclear to what extent these effects observed in-vitro contribute to the ultimate cytotoxic effect in patients. It is very likely that, depending on the dose and the tumour entity, different mechanisms are important. Also, for many cytostatic agents given in low doses over longer time periods it is the effect on the vascular epithelium, not the effect on cancer cells that is critical for antitumor effect [5]. There is evidence that growth of the tumour cells is dependent on the vasculature of the tumour

References pp. 188–194

and low-dose continuous therapy with cytotoxic agents is mainly an inhibition of vascular growth [6].

Unfortunately, even for the newer non-cytotoxic targeted anticancer drugs a clear pharmacological endpoint is not defined and this was one reason for the failure of several studies with these newer drugs [7].

Although a close relationship between toxicity and individual exposure was demonstrated for several cytotoxic drugs, it appears to be much more difficult to show that there is a clear relationship between drug concentrations in plasma and the anticancer effect. This is mainly due to the fact that the therapeutic outcome is difficult to assess as this requires a longer follow-up period.

The aim of this chapter is to introduce current concepts of dose finding in oncology and to give an update on applications of therapeutic drug monitoring in oncology. For a general overview of therapeutic drug monitoring in oncology, the reader is referred to the excellent reviews written by McLeod [8] or Canal and co-workers [9]. The pharmacokinetic concepts for TDM of anticancer drugs are nicely described in two recent reviews of Roussau and coworkers [10,11]. Also, the article written by Veal et al. gives a short overview of the concepts of TDM in cancer chemotherapy [12].

7.2 CONCEPTS OF DOSE FINDING IN ONCOLOGY

For the last twenty years, the concept of cytotoxic therapy was that there is an increase in therapy outcome with increasing doses. With the availability of growth factors of the haematopoietic system like granulocyte-colony-stimulating factor, the primary toxicity of a number of cytotoxic drugs can be overcome and higher doses can be administered. The idea of increasing the response rates by increasing the doses is based on in-vitro data where there is often a log-linear relationship between cell kill and the concentration of the drug. In the late 1980s and during the 1990s high-dose therapy with stem cell transplantation became very popular for treating leukaemia or solid tumours. However, in most of the controlled studies high-dose therapy with stem cell transplantation failed to show superiority over standard therapy regimens [13]. Only for certain forms of leukaemia (ALL), neuroblastoma and Ewing sarcoma does high-dose therapy appear to be justified by well controlled clinical studies.

The consequence of the disappointing results of high-dose therapy trials is that for every drug there must be an optimal dose and schedule for every cytotoxic agent in a given combination and a certain tumour entity. The optimal dose appears to be far below the maximal tolerated dose (MTD) which is defined as the highest dose which can be administered with acceptable toxicity. Up to now, the MTD in man has to be determined for new cytotoxic drugs during clinical drug development. To assess the optimal dose for a cytotoxic drug and to adjust the dose for a certain patient, individualisation strategies are required. The strategies applied for dose finding in oncology are summarised in Fig. 7.1 and are explained in the following paragraphs.

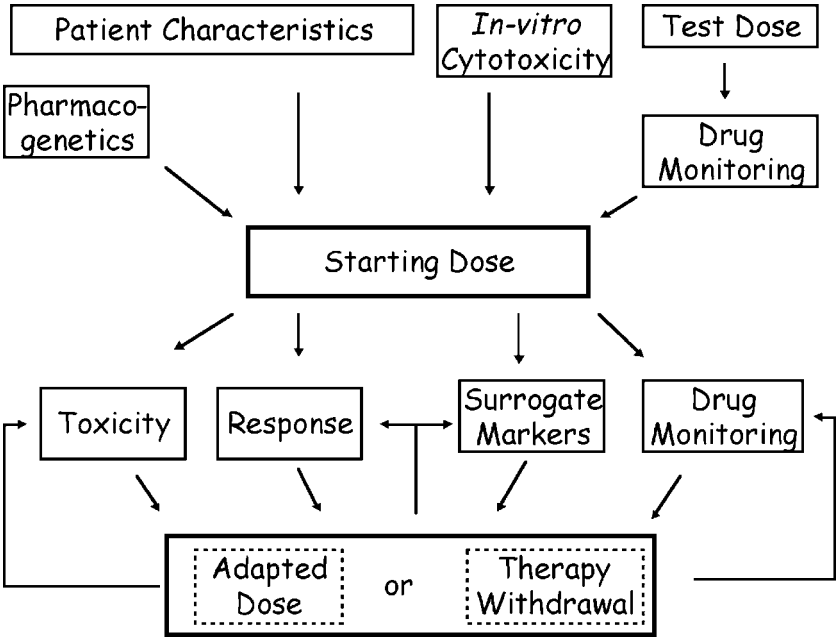

Fig. 7.1. Schematic representation of different dosing schemes in oncology.

7.2.1 Dosing based on patient characteristics

For many cytostatic drugs, dosing based on body surface area (BSA) is used in clinical routine. BSA is calculated according to the formula of Du Bois and Du Bois: $BSA = W^{0.425} \cdot H^{0,725} \cdot 0.007184$ [14].

The Du Bois formula is often applied to adults and the basis for the nomograms often used in clinical routine. However, the formula underestimates BSA in children less than 0.7 m^2.

For adults, the concept of BSA dosing was criticised by several investigators [15]. It is important to know that BSA was introduced as a tool for species scale-up, i.e. to calculate doses for humans from animal data [16,17]. Recently, Sawyer and Ratain pointed out that BSA is not able to predict liver or renal function and suggested that BSA should be tested as a covariate for every anticancer drug during drug development [18]. A recent review of Sparreboom and colleagues suggested that BSA should only be used for drugs with a low volume of distribution as BSA strongly correlated with the blood volume [19]. In a randomised cross-over trial the same group compared BSA-dose versus a fixed dose for paclitaxel in adults. Surprisingly, they found that even in a relatively homogeneous group of 12 patients with BSA ranging from 1.46 to 2.10 m^2 BSA-dosing reduced the inter-individual variability in individual exposure (AUC of unbound paclitaxel) by 53.3% [20]. Therefore, for paclitaxel BSA-dose is clearly justified. However, a retrospective analysis of phase I studies with 33 investigational

agents in 1650 adults recently showed that for only five drugs plasma clearance correlated significantly with BSA [21].

With the much greater heterogeneity of the patient populations in paediatric oncology BSA-dosing in children is still justified, because for many drugs alternative dosing strategies are lacking. In clinical routine, BSA in children is usually calculated using the formula:

$$BSA = \sqrt{\frac{W \cdot H}{3600}}$$

with W weight [kg] and H height [cm] [22].

This formula differs slightly from the original formula of Du Bois and Du Bois. Especially for smaller children and infants with a body weight of less than 10 kg, clearance better correlates with weight according to the $\frac{3}{4}$ power law:

$$Cl_i = Cl_{st}\left(\frac{W_i}{W_{St}}\right)^{\frac{3}{4}}$$

with Cl_i individual clearance; Cl_{St} standard clearance; W_i individual weight; W_{St} standard weight (70 kg) [23]. In infants, many protocols reduce the calculated BSA-dose to 2/3 for safety reasons, although this procedure is not justified by pharmacokinetic investigations. Pharmacokinetic studies in infants are rare due to the patient's burden from repeated blood sampling. Therefore, studies with improved methodologies like capillary electrophoresis to reduce the required blood volume and population pharmacokinetic methods to reduce the required number of blood samples per patient are warranted for this patient group.

7.2.2 Dosing based on pharmacodynamic parameters

In general, dose individualisation based on pharmacodynamic (PD) parameters is preferable to pharmacokinetic dose adjustment, because the same plasma concentrations in different patients can produce different therapeutic effects due to the variability in pharmacodynamic parameters (e.g. variations in E_{max}, EC_{50}, in a E_{max}-model) or variable distribution to the effect site. As mentioned above, the problem in cancer chemotherapy is the time delay in the therapeutic effect, requiring longer follow-up periods to assess the ultimate effect, i.e. response and overall survival. Therefore, the dose is often adjusted to side-effects like the drop in white blood cell count (WBC). However, also this side effect appears with a remarkable time delay making this approach difficult to conduct. This is illustrated in Fig. 7.2.

Dose adjustment based on WBC is often applied, for example in maintenance therapy of acute lymphoblastic leukaemia (ALL) in children [24]. However, this method has the major drawback that dose adaptation can only be done in intervals of several weeks. In between, the dose of the two drugs methotrexate and mercaptopurine may be inadequate with a higher risk of relapse with underdosing and a high risk of infections when the

patients are overdosed. An example is shown in Fig. 7.3. From the graph it is apparent that increasing the dose of the cytotoxic drugs does not necessarily result in reducing white blood cell (WNC) count as other confounding factors like non-compliance to therapy or co-administration of other drugs can play an important role.

7.2.3 Therapeutic drug monitoring of anticancer drugs

Jelliffe et al. in Chapter 6 of this book describe the concept of dose individualisation based on plasma concentration measurements in detail. As explained above, applications of therapeutic drug monitoring with dose individualisation in cancer chemotherapy are rare. Most of the studies were done in children, as this group of patients is highly heterogeneous and the pharmacokinetic variability in this patient group is high.

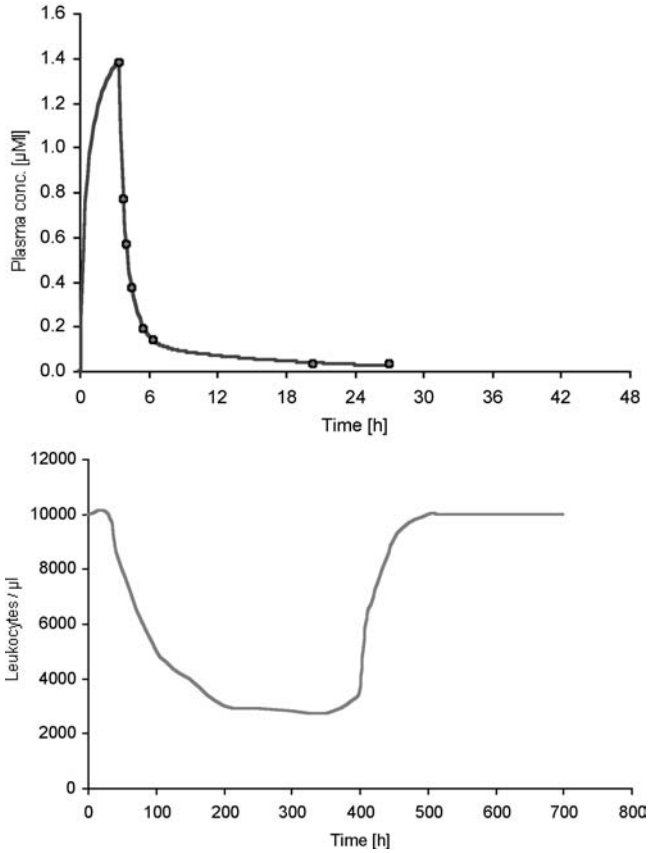

Fig. 7.2. Plasma concentration of a cytostatic drug (paclitaxel 3h-infusion of 100 mg/m^2) and drop in leukocytes (schematic representation).

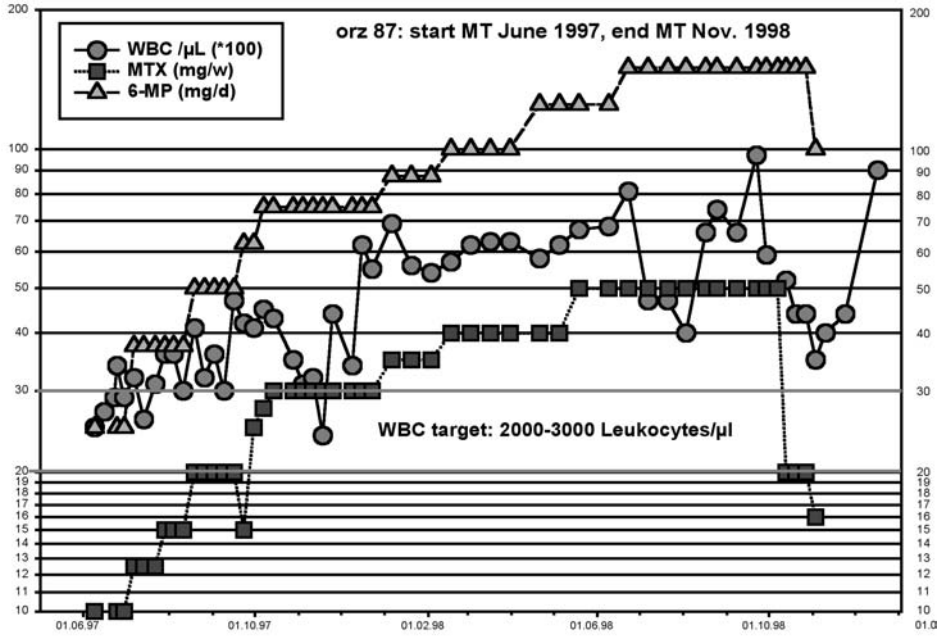

Fig. 7.3. Methotrexate and mercaptopurine dose during maintenance therapy and white blood cell count in a patient with ALL.

7.2.3.1 Analytical aspects

Only for methotrexate, is an immunoassay commercially available. The most commonly used commercial MTX assays are a fluorescence polarization immunoassay (FPIA; Abbott Laboratories) and the enzyme-multiplied immunoassay (EMIT; Behring Diagnostics) [25,26]. Because of the ease to run this assay, most clinical laboratories and hospital pharmacies prefer this method over HPLC assays. However, when using the data from the immunoassay one has to keep in mind that the immunoassay cross-reacts with the metabolites of methotrexate. The drug is mainly metabolised to 7-hydroxy-methotrexate and DAMPA (Fig. 7.5). Especially, the latter substance displays a high cross-reactivity resulting in an overestimation of the methotrexate plasma concentration. This is especially important if carboxypeptidase is applied in case of intoxications or reduced renal clearance of methotrexate [27]. This enzyme cleaves methotrexate to its metabolite DAMPA (Fig. 7.5) which is pharmacologically inactive. Therefore, data from the EMIT assay after application of carboxypeptidase are misleading.

An analytical challenge is the analysis of alkylating agents, which are mostly aliphatic compounds lacking sufficient UV absorption to detect them with a UV detector. For busulfan, the first HPLC method described was based on a precolumn derivatisation with diethyl-dithiocarbamate [28]. This derivatisation and plasma preparation procedure was modified and improved by other investigators [29–32].

Others used 8-mercaptoquinoline for derivatisation to yield fluorescent compounds [33]. Although fluorescence detection is superior to UV from a theoretical point of view with regard to sensitivity and selectivity, in practise this method has no clear advantages over derivatisation with diethyl-dithiocarbamate.

An alternative is derivatisation resulting in 1,4 diiodbutane and postcolumn photolysis. The released iodine can be detected by UV-detection [34]. Another possibility is a precolumn derivatisation using 2,3,5,6-tetrafluorothiophenol as used by Grochow et al. [35], a derivatisation procedure originally developed for gas chromatography (GC). The latter method was the first procedure enabling us to quantify busulfan in the lower μg/l-range [36]. Also, derivatisation, for example conversion into 1,4-diodbutane, is necessary for GC to convert busulfan into a thermally stable compound [37]. Detection in GC for busulfan is carried out either by electron-capture detection or mass spectrometry.

With the availability of cheaper mass spectrometric detectors, HPLC-MS is the method of choice for the determination of busulfan and other alkylating agents in biological fluids. Several validated assays with limits of quantification in the lower μg/l-range were reported [38,39]. However, LC-MS is still a method not available in most hospitals.

When drug metabolites and the parent compound have to be analysed simultaneously, HPLC is the method of choice. A recent overview of chromatographic methods to analyse anticancer drugs can be found in Ref. [40]. In cases where only a limited sample volume is available capillary electrophoresis is preferable, for example in paediatrics or for microdialysis experiments [41].

A special situation is the analysis of platinum compounds where flameless atom absorption spectroscopy is the method of choice. Although differentiation between parent compounds and metabolites is not possible with this technique, there are several investigations demonstrating the clinical significance of platinum concentrations in plasma [42].

7.2.3.2 Mercaptopurine

Mercaptopurine is a prodrug requiring activation by conjugation to the respective nucleotide catalysed by the enzyme hypoxanthine phosphoribosytransferase (HPRT, Fig. 7.4).The monophospate can be converted to the respective triphosphates which can subsequently be incorporated into DNA or RNA [43]. Another route catalysed by xanthine oxidase leads to inactive metabolites. Further, mercaptopurine is methylated at the sulfur leading to products which can also catabolised to the respective nucleotides. These methylated metabolites also display a weak cytotoxic activity and their contribution to the overall effect of the drug is not completely understood [44]. The enzyme responsible for the methylation, thiopurine methyltranferase (TPMT), displays a genetic polymorphism with 0.3% of patients showing very low activity of this enzyme. This problem is further discussed in Chapter 13.

Mercaptopurine is used in the maintenance therapy of ALL. Patients receive the drug as a daily oral therapy for several months. The peak plasma concentrations and the AUC of mercaptopurine during maintenance therapy showed a predictive value for the occurrence of relapse in a smaller group of 22 children [45]. In contrast, a bigger

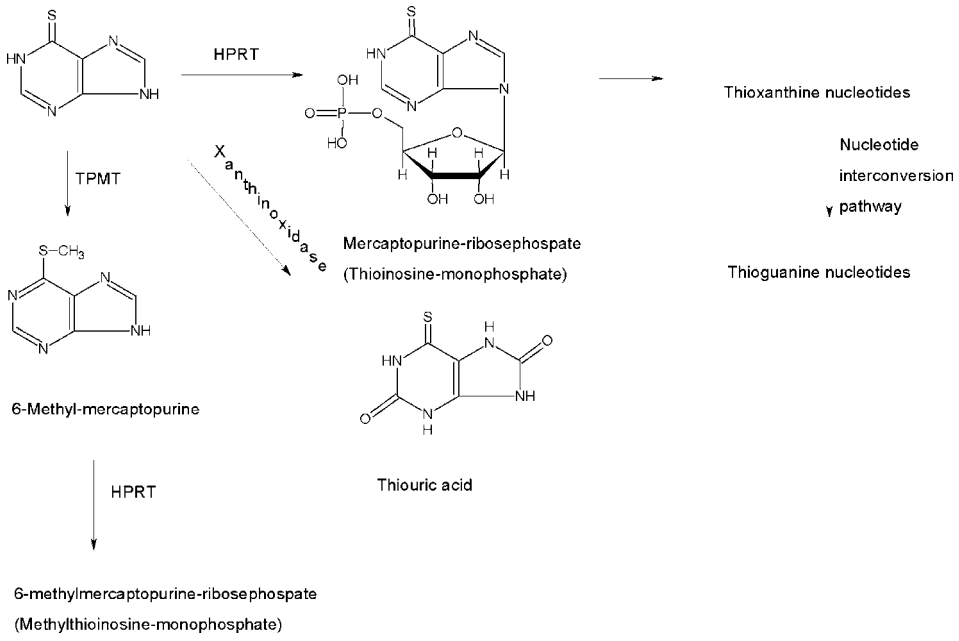

Fig. 7.4. Catabolism/metabolism of mercaptopurine.

prospective evaluation in 89 children with ALL showed no significant difference between the AUCs of patients who stayed in remission in comparison to the patients who relapsed [46]. This can be due to the high intrapatient variability in the AUC of mercaptopurine reported from several investigators [47]. As the plasma concentrations quickly decline after oral administration, intense blood sampling shortly after administration is necessary to get a useful estimate of the individual exposure, quantified as AUC. Therefore, due to the high clearance of the drug plasma concentration measurements of mercaptopurine after oral administration are not feasible for dose individualisation in clinical routine.

Dose adjustment based on WBC is the standard procedure for controlling the dose of mercaptopurine and thioguanine, the latter drug belonging to the same group of anticancer agents [48]. This pharmacodynamic dose individualisation is useful as the white blood cell count has been shown to have a predictive value for the occurrence of relapse. However, as pointed out in Section 7.2.2 of this chapter, the time delay is substantial. Therefore, if patients experience neutropenia due to overdosing the therapy has to be stopped for some weeks. These therapy breaks can results in an overall reduced therapy intensity leading to a higher risk of relapse in those patients [49].

Thioguanine nucleotides in red blood cells (E-TGN) can be used as a surrogate for the individual exposure to mercaptopurine therapy. Lennard et al. found a correlation between E-TGN and the neutrophil count 14 days later in 22 children [50]. The advantage of measuring E-TGN instead of plasma concentrations is that a single sample is sufficient to get a measure of the individual exposure to the drug and a possible

overdosing can be detected much earlier than with pharmacodynamic monitoring of WBC's.

In a recent study conducted in the north European countries Schmiegelow et al. attempted to individualise the dose of mercaptopurine and methotrexate during maintenance therapy of ALL in 538 children [51]. Patients were randomised to receive either dosing of mercaptopurine and methotrexate adjusted to E-TGN, methotrexate polyglutamates in red blood cells (E-Mtx) and WBC or WBC only. Surprisingly, girls in the pharmacologically controlled group had a worse outcome than girls in the control arm, i.e. with dosing based only on WBC. One possible explanation for this finding is that patients in the pharmacologically controlled group received a higher dose intensity leading to more episodes of therapy withdrawal. Another possible explanation the authors suggest is that higher concentrations of methylated metabolites occur in the pharmacology arm, leading to senescence of the leukaemic cells.

In conclusion, the failure of improving mercaptopurine therapy by dose individualisation based on drug or metabolite concentration measurements is possibly due to the complex mechanisms of action with several groups of metabolites contributing to the ultimate effect. However, as pointed out in Chapter 13, mercaptopurine is the first example where pharmacogenetic therapy individualisation has entered clinical routine in some centers. It remains to be seen if this can translate into improved therapeutic outcome.

7.2.3.3 Methotrexate

One of the oldest, but still very important cytostatic drugs is methotrexate. It is mainly used in the treatment of childhood ALL, osteosarcoma in children as well as for non-Hodgkin-lymphoma and breast cancer. Methotrexate, together with mercaptopurine and some other minor important drugs, belongs to the group of antimetabolite cytostatics as they were developed to replace endogenous substances necessary for cell metabolism, resulting in subsequent inhibition of proliferation. The drug is an inhibitor of dihydrofolate reductase, an enzyme responsible for the formation of tetrahydrofolate, which is the carrier for the transfer of carbon units. Inhibition of dihydrofolate reductase results in reduced thymine production necessary for DNA synthesis.

The metabolism of methotrexate is shown in Fig. 7.5. In this respect, it is important to note that the drug is mainly excreted unchanged through the kidneys. The main metabolites found in plasma are 7-Hydroxy-methotrexate and 4-amino-4-deoxy-N^{10}-methylpteroic acid [52]. When entering the cell, methotrexate is conjugated with two to seven glutamate moieties to methotrexate polyglutamates and can be stored in this form. The polyglutamates also display pharmacological activity. Similarly, 7-Hydroxy-methotrexate can be polyglutamated and stored inside the cell.

With its specific mechanism of action, methotrexate is the only cytotoxic drug where a specific antidote, folinic acid, is available. This fact allows dose escalations beyond the usually lethal dose of the drug by administering folinic acid at least 36 h after starting the methotrexate infusion. Using this approach, doses of up to 33.6 g/m^2 have been administered [53]. In fact, for certain tumour entities like osteosarcoma, a positive correlation between dose intensity and therapeutic outcome has been found in

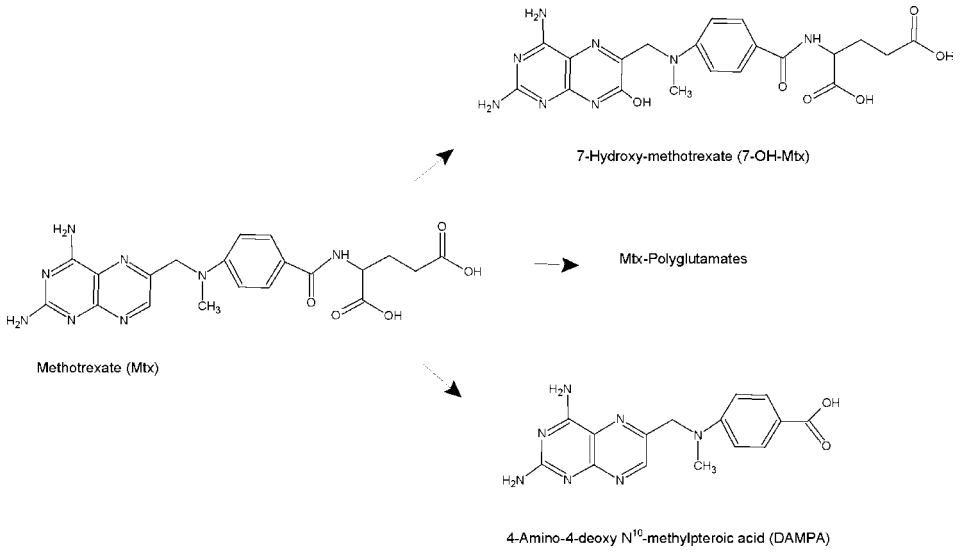

Fig. 7.5. Metabolism of methotrexate.

retrospective analyses [54]. Graf et al. found a better outcome for patients with peak plasma concentrations above 1000 μmol/l in patients with osteosarcoma in comparison to those with lower peak plasma concentrations [55]. A prospective study in 127 patients with localised osteosarcoma receiving either 0.75 g/m^2 over 30 min. or 7.5 g/m^2 as a 6 h infusion as preoperative (neoadjuvant) therapy showed a higher disease-free survival rate in the group receiving the higher methotrexate dose [56]. However, the difference between the two groups was not statistically significant. Therefore, and based on in-vitro and in-vivo data, the therapeutic value of high-dose methotrexate has been questioned [57]. Further, randomised studies showing a clear benefit of high-dose methotrexate (>1 g/m^2) with folinic acid rescue vs. low or intermittent dosing are lacking. Also, non-haematological toxicity is apparent with high-dose methotrexate like neurotoxicity, elevation of liver enzymes as well as nephrotoxicity.

Another concern is that the high doses of folinic acid (leucovorine) administered may protect cancer cells in subsequent methotrexate administrations [58]. The standard dose of 15 mg/m^2 folinic acid given every 6 hours for four days exceeds the usual supply of folic acid for one year. In this context, one has to keep in mind that in contrast to other water-soluble vitamins folic acid derivatives can be stored inside the cell as polyglutamates.

The folinic acid rescue is routinely adjusted based on the plasma concentration of methotrexate [59]. Therefore, methotrexate is the only anticancer drug where plasma concentration measurements are done on a routine basis.

Osteosarcoma patients receive 12 g/m^2 methotrexate as a 4 h infusion. The plasma concentration is assessed 4 h, 12 h, 24 h, 48 h and every 12 hours until 72 h using an

immunoassay. Patients receive the standard folinic acid rescue of 15 mg/m^2 every 6 hours if the 24 h plasma concentration of methotrexate is less than 8.5 μmol/l. Otherwise, the folinic acid-dose is escalated and patients receive the rescue therapy every 6 hours until methotrexate plasma levels fall down to 0.4 μmol/l. A similar scheme is used in the treatment of ALL, although the applied doses are lower with 1 to 8 g/m^2 administered as a 24 h-infusion with a loading dose of 10% given in the first 30 minutes [60]. Also, 36 hours after the start of infusion, folinic acid rescue is administered.

Attempts to individualise the dose of methotrexate based on plasma concentrations were done mostly in leukaemia patients. In a group of 108 ALL patients receiving 1 g/m^2 methotrexate patients with steady-state concentrations below the median of 16 μmol/l had a higher probability of relapse than patients with higher methotrexate concentrations [61]. In accordance with these findings, a study of 42 children receiving 6–8 g/m^2 methotrexate as a 24 h infusion showed the effects of methotrexate pharmacokinetics on the outcome were statistically significant with clearance per cycle being a predictor for event-free survival. However, the authors found a high intra-individual variability in methotrexate clearance. This finding makes the value of dose individualisation based on individual pharmacokinetic parameters questionable [62]. Instead, dose individualisation for methotrexate has therefore to be individualised in every cycle. This approach was utilised in the investigation of Evans et al. [63]. They were the first showing the benefits of an individualised chemotherapy regimen in comparison to conventional dosing in a randomised trial. In a group of 182 children with acute lymphoblastic leukaemia (ALL) patients were randomised to either BSA-dosed post-remission regimen with 1.5 g/m^2 methotrexate, teniposide and cytarabine or a regimen with the same drugs with dosing based on plasma concentration measurements in every cycle, i.e. taking intra-individual pharmacokinetic variability into account.

In the subgroup of 143 patients with B-lineage leukaemia, those receiving individualised therapy had a better outcome than patients given BSA-dosed therapy. Patients in the individualised group received higher doses in comparison to the control group. Analysis of the individual exposure of the three drugs indicated that methotrexate exposure is a predictor for the outcome of therapy whereas teniposide and cytarabin exposure had no or only a weak effect on the clinical outcome.

It is worth mentioning that the authors have not used this approach in the following studies and, to our knowledge, currently no leukaemia or osteosarcoma study group applies individualised methotrexate dosing in an active protocol.

In a study with 24 children with relapsed ALL patients received methotrexate 500 mg/m^2 over 1 h as a loading dose. Subsequently, during the following 23 hours, dose adaptation to reach a target concentration of 65 μmol/l was applied by repeated plasma concentration measurements and a Bayesian algorithm. The resulting doses ranged from 2864 to 6700 mg/m^2. In comparison to historic controls, the authors found reduced toxicity as these heavily pre-treated patients are especially susceptible to experience toxicity due to reduced clearance. No efficacy data were analysed in this study [64].

In our opinion, the reasons why dose individualisation based on plasma concentration measurements for methotrexate has not entered clinical routine are:

- the high intra-individual variability in methotrexate clearance. Plasma concentrations measurements have to be done in every cycle and dose adjustment has to be done during continuous infusion.
- the lack of a general relationship between plasma concentration and effect. A target exposure has to be defined for every tumour entity and every risk group separately.
- the idea that pharmacokinetic variability does not play a role for the outcome when higher doses (>5 g/m^2) are applied [65].
- the wide therapeutic range of methotrexate when administered with subsequent folinic acid rescue.

However, the latter point does not mean that methotrexate cannot cause life-threatening toxicity. Special precautions have to be taken when administering high-dose methotrexate. Besides controlling the plasma concentration, urine alkalisation is extremely important to prevent precipitation of methotrexate and its metabolites in the kidneys. In addition, according to a recent investigation, elevated serum creatinine is a better predictor of delayed methotrexate elimination than the plasma concentration at the end of infusion [66].

7.2.3.4 Etoposide/Teniposide

Etoposide (Fig. 7.6) is a podophyllotoxin derivative showing activity in both solid tumours and leukaemia. It exerts its cytotoxic action by inhibiting topoisomerse II. Teniposide is another podophyllotoxin derivative with a thiophene ring on the benzyl moiety [66]. Both drugs have to be administered in a formulation containing polyethylene glycol, Tween 80, ethanol and benzylalcohole due to their low solubility. These excipients can increase the toxicity of intravenous infusions. Recently, etoposide

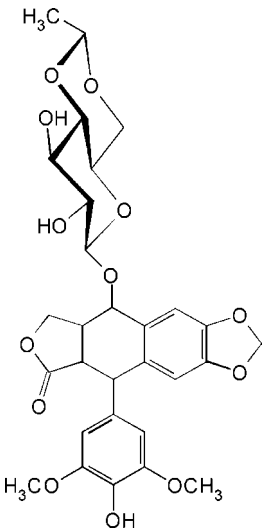

Fig. 7.6. Etoposide.

phosphate was introduced to improve the solubility of etoposide [67]. The drug can be administered without addition of these excipients. Etoposide phosphate is a prodrug which is immediately hydrolysed in plasma to form etoposide.

The elimination of etoposide is highly dependent on renal function and serum creatinine and ^{51}Cr-EDTA clearance can be used to predict the renal clearance of etoposide [68]. Consequently, previous cisplatin therapy, which affects renal function, may reduce the clearance of etoposide. Etoposide and teniposide are highly bound to proteins in plasma. As protein binding varies between individuals and is dependent on disease status, the measurement of the free fraction of etoposide in plasma is preferable. Studies in adults and children showed that the free fraction better correlated with toxicity than total etoposide [69]. The AUC of etoposide was correlated with the incidence of neutropenia in several studies. Also, the length of exposure above a threshold concentration was correlated with haematological toxicity [70,71].

Adaptive control was applied to adjust the dose of etoposide in a 72 h schedule by using a plasma concentration measurement after 24 h. However, in a randomised crossover study including 31 adults with several different malignancies there was no advantage with regard to therapeutic effect of the individualised scheme [72]. The authors conclude that their model has to be improved by including pre-dose pharmacodynamic measurement. In a subsequent study the same group applied a refined model including white blood cell count and could show they could safely increase the dose intensity of etoposide in 45 patients [73]. However, an advantage in therapeutic outcome of this approach could not be demonstrated. Probably, the patient population was too heterogeneous and optimal plasma concentrations might differ depending on the tumour entity.

In a study in 67 patients with acute myeogenous leukaemia the dose of etoposide and cytarabine was individualised to achieve plasma concentrations of 30 μmol/l etoposide given as a 96 h continuous infusion (initial dose 500 mg/m^2 per day) [74]. For cytarabine, a target plasma concentration of 1 μmol/l was aimed for during a 120 h continuous infusion. An improvement in therapeutic outcome was not achieved in comparison to historic controls. The most probable explanation for this finding is that the protocol, which also included daunorubicin and amsacrine, was too toxic so that many patients experienced infections, resulting in therapy interruptions. Neutropenia was severe during the cycles and six patients died of the toxic effects of therapy.

A smaller study in 9 children attempted to individualise etoposide to a target AUC between 4.6 and 8.2 g/l*min [75]. In this study, only feasibility of this procedure could be demonstrated without showing any benefits of this schedule. Ciccolini et al. recently proposed and validated a limited sampling strategy for 5-day continuous infusion of etoposide in 25 patients [76]. However, as pointed out by McLeod a few years ago, for etoposide as for many other anticancer drugs, the role of TDM has to be evaluated in randomised trials comparing conventional vs. individualised dosing [8]. In a randomised study individualising methotrexate, cytarabine and teniposide in children with ALL, teniposide systemic exposure appeared to have no effect on the risk of developing relapse [63]. In conclusion, TDM of etoposide or teniposide appears to be useful to control therapy intensity. Up to now, an advantage with regard to therapeutic outcome could not be demonstrated. Investigations using the unbound fraction of etoposide might

be more feasible. As pointed out by Lowis et al., pharmacokinetically guided dosing of etoposide (as well as teniposide) is likely to be of most benefit in patients with abnormalities of renal or hepatic function, or in children with prior exposure to cisplatin [75].

7.2.3.5 Carboplatin

Platinum complexes like cisplatin and carboplatin (Fig. 7.7) exert their antitumour effects by forming DNA adducts and subsequent inhibition of DNA replication and transcription. They have an important role in the treatment of several solid tumours. Besides myelosuppression, nephrotoxicity and ototoxicity are dose-limiting for cisplatin, whereas thrombocytopenia is dose-limiting for carboplatin. Cisplatin is extensively bound to plasma proteins and clear pharmacokinetic-pharmacodynamic correlations have been identified for the ultrafiltrable, i.e. unbound fraction of the drug in plasma and toxicity as well as therapeutic effect [77].

As carboplatin is preferentially eliminated through the kidney, clearance can be predicted using parameters of the renal function. Calvert et al. proposed and validated a formula to calculate the dose of carboplatin from the desired AUC and the glomerular filtration rate calculated from ^{51}Cr-EDTA-clearance [78]. As this method to calculate renal clearance is not applied routinely, other investigators used serum creatinine, weight, age and sex to predict carboplatin clearance [79]. This formula was also prospectively validated [80]. For children, modified formulae have been developed [81,82]. These formulae to calculate the carboplatin dose are today widely applied in clinical routine. They can reduce the variability in the AUC to about 25% in comparison to an up to fourfold variability in AUC when the dose is adjusted to BSA [83].

With this relatively low pharmacokinetic variability achieved TDM is limited to special situations because the variability can only be slightly reduced to a value of about 15%. To individualise dosing based on plasma concentration measurements, several limited sampling procedures were suggested [84]. These approaches can be useful in high-dose therapy where variations in the AUC of 25% could be very critical [85].

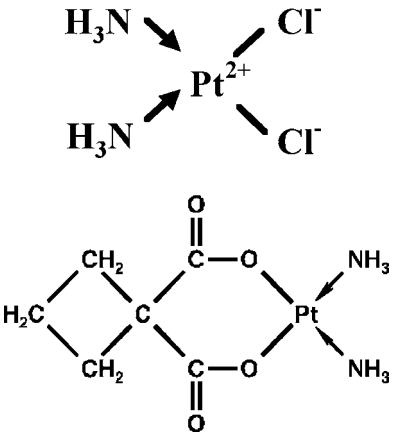

Fig. 7.7. Cisplatin and Carboplatin.

Furthermore, as with almost all cytostatic drugs, drug monitoring is desirable in patients with reduced renal function or other special situations which might influence the pharmacokinetics of carboplatin.

7.2.3.6 Busulfan

The alkylating agent Busulfan (Fig. 7.8) is part of many conditioning regimens before autologous or allogenic stem cell transplantation for the treatment of leukaemia and certain nonmalignant haematological disorders. Since the 1950s, the drug was used for the treatment of chronic myeloic leukaemia in low doses. Today, it is mostly used in combination with cyclophosphamide in high-dose therapy regimens [86].

The standard dose in the conditioning regimens is 1 mg/kg every 6 h for 4 days. Fig. 7.9 shows a typical plasma concentration time curve of a child receiving this regimen. From the graph, one can see that substantial intra-individual variability is apparent. Until recently, only an oral formulation of busulfan was available due to the high lipophilicity of busulfan. In 1999, an i.v.-formulation containing dimethylacetamide as a solvent was approved in the US and is now also available in other countries.

The drug is metabolised in the liver, mainly by the enzyme glutathion-S-transferase, by forming an sulfonium ion and subsequent oxidation resulting in inactive products

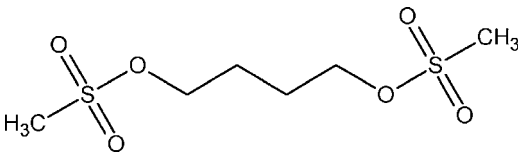

Fig. 7.8. Busulfan.

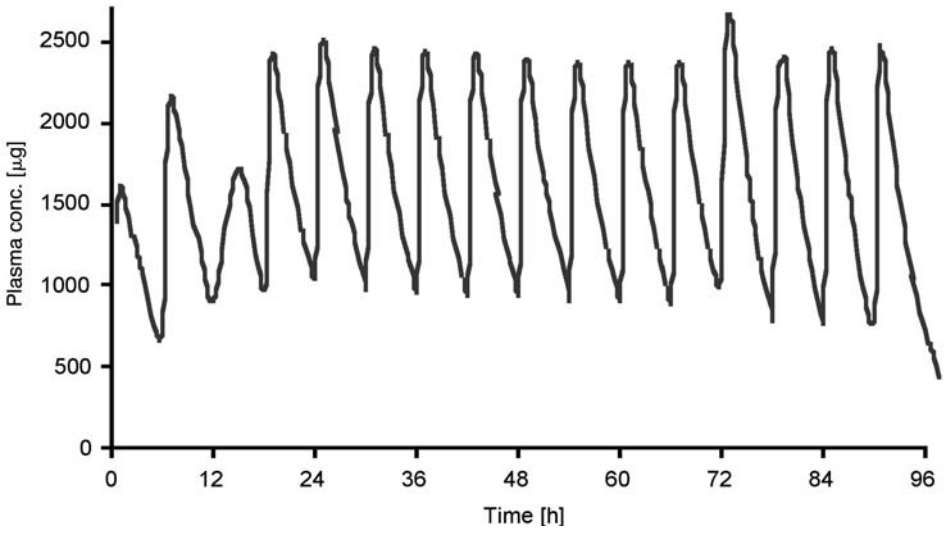

Fig. 7.9. Plasma concentration time curve after administration of busulfan 1 mg/kg every 6 h for 4 days. The line represents the posthoc estimates of a population pharmacokinetic model describe in Ref. [103].

TABLE 7.1
PHARMACOKINETIC PARAMETERS OF BUSULFAN IN CHILDREN AND ADULTS.

Age (years)	Number of patients	V.F (l/kg)	$t_{1/2}$ (h)	CI/F (ml/min/kg)	Reference
4–14	11	1.0 ± 0.5	2.3	4.4 ± 2.2	89
2–14	25	1.0 ± 0.4	2.9	4.5 ± 1.5	90
1.3–14	9		2.4	4.9 ± 2.2	91
1.3–13.5	19	0.73 ± 0.15	2.4	3.6 ± 1.1	92
< 3.6	14	1.4 ± 0.8	1.6	8.4 ± 4.3	93
< 2.7	33	1.6 ± 1.3	2.8	6.8 ± 3.0	94
> 18	28	0.6 ± 0.5	2.3	2.5 ± 1.4	95
> 18	16		2.7	2.6 ± 0.6	91

[87]. Only a few percent of the applied dose are excreted unchanged in urine [88]. The pharmacokinetic parameters of busulfan in different patient populations are summarised in Table 7.1.

As the protein binding is very low, the drug displays a volume of distribution comparable to body water [96]. In smaller children with an age < 3 years, a higher volume of distribution and a higher clearance was observed in relation to body weight [89,90]. Consequently, dosage according to body surface area (BSA) in children has been shown to produce AUC values similar to those observed in adults [97,98]. In contrast to these findings, dosing based on body weight is still used in many treatment protocols for children. A circadian rhythm in busulfan clearance has been observed with higher trough concentration in the morning [99]. The influence of obesity on the pharmacokinetics of busulfan is questionable.

Besides myelosuppression, the hepatotoxicity is dose-limiting with veno-occlusive disease (VOD) occurring with an incidence of 20%, according to an older investigation [96]. VOD is lethal in a substantial number of patients. Other severe side effects are convulsions, and interstitial pneumonia.

Several investigations have demonstrated a relationship between pharmacokinetic parameters and side effects or activity. In adults, an area under the curve (AUC) of more than 1500 μmol/l*min has been shown to be associated with an increased risk for the development of VOD [100]. Plasma trough concentrations higher than 500 μg/l (2.03 μmol/l) appear to be necessary to prevent graft rejection [101]. In accordance to this, McCune et al. found an inverse correlation between the trough concentrations of busulfan (in that paper termed C_{ss}) and the graft rejection with a risk of less than 12% with busulfan C_{ss} of more than 600 μg/l (2.43 μmol/l) [102].

Busulfan displays substantial inter-individual variability in its pharmacokinetic parameters. According to two recent population pharmacokinetic investigations the variability in the apparent clearance is about 25% and in the apparent volume of distribution 30%. However, most of the inter-individual variability after oral administration was found in the absorption constant k_a with values of more than 100%

[103,104]. In both investigations, intra-individual variability in all parameters was substantially lower. Thus, an important prerequisite for dose individualisation is fulfilled.

Consequently, in some specialised centers dose individualisation of busulfan is done on a routine basis. A problem to apply drug monitoring of busulfan in clinical routine is the difficult analytical methodology, as described in Section 7.2.3.1. Furthermore, due to the oral route of administration, a reliable estimate of the pharmacokinetic parameters is only achieved with collection of several plasma samples. For example, Krivoy et al. took as many as 10 plasma samples after the first dosing to calculate the AUC and to adjust the dose at the fifth administration [105]. However, the authors used non-compartmental pharmacokinetic analysis. With more adequate compartmental analysis and Bayesian forecasting the number of samples required can be substantially reduced.

Bolinger et al. also applied a test dose of 0.5 mg/kg in 32 children and collected seven plasma samples to calculate the pharmacokinetic parameters [106]. They adjusted the dose to achieve trough concentrations of 600 to 900 μg/l (2.43–3.65 μmol/l). In comparison to historic controls, they found an increased engraftment (as high as 94%) but no reduction in toxicity.

As mentioned by Jelliffe and co-authors in Chapter 6, Bleyzac et al. have established a dose individualisation procedure for patients receiving busulfan [107]. Patients receive a test dose of 0.5 mg/kg busulfan and three plasma samples are collected. From the measured busulfan plasma concentrations the pharmacokinetic parameters of the patient can be calculated with great accuracy. The target was an AUC between 4 and 6 mg/l*h (985–1478 μmol/l*min). In a matched-pair analysis 29 patients with individualised dose had a much lower incidence of VOD and a higher VOD-free survival than patients with standard dose while overall survival did not differ between the two groups.

In another investigation, using a limited sampling procedure with four plasma samples after the first dose of 40 mg/m^2 busulfan the following doses were adjusted to a target of 1300 μmol/l*min in a group of 38 paediatric patients [108]. As most children aged between 1 and 5 years required dose increments and older children dose reductions, they recommend age-adapted BSA-dose with the highest dose of 45 mg/m^2 given to the group aged between 1 and 5 years and lower BSA-doses for both younger and older patients. The authors critically discuss the value of dose adjustment for children based on AUC calculation of the first dose due to intra-individual factors like co-adminstration of inducers of metabolism like phenytoin and occasional delayed absorption in some patients.

However, even with expensive GC-MS equipment and specialised personnel the costs of TDM of busulfan were calculated to be less than 1% of the total costs of a haematopoietic stem cell transplantation [109]. Therefore, given the encouraging results of several independent groups, TDM of oral busulfan should be applied in every center for bone marrow transplantation.

Small pharmacokinetic studies have demonstrated that i.v.-administration of busulfan is able to reduce the variability in individual exposure [110]. However, the variability is still substantial [111]. Therefore, i.v.-busulfan in combination with therapeutic drug monitoring should be the best way to improve safety of this drug with very serious toxicity [112].

References pp. 188–194

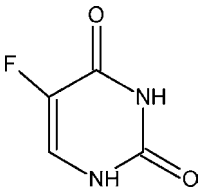

Fig. 7.10. Fluorouracil.

7.3 5-FLUOROURACIL (5-FU)

One of the most important drugs for the treatment of colorectal and head and neck cancer is 5-FU (Fig. 7.10) [113]. The drug requires anabolism to the respective nucleoside 5-fluoro-2-deoxyuridine 5′-monophosphate (FdUMP) to exert its cytotoxic activity. Inactivation is mainly determined by the enzyme dihydropyrimidine dehydrogenase (DPD) reducing the double bond. This enzyme displays genetic polymorphism with an incidence of about 3% of the population with markedly reduced activity [114]. 5-FU is often combined with folinic acid to improve inhibition of thymidylate synthase by FdUMP.

The drug is administered as a short bolus infusion or as a prolonged continuous infusion over several days. From a pharmacokinetic standpoint, the latter approach appears more logical as 5-FU has a very low half-life of 8 to 20 min [113]. With bolus injections, the drug displays nonlinear kinetics.

There are investigations demonstrating a relationship between the AUC of 5-FU and both response and toxicity, indicating that dose individualisation based on plasma concentration measurements should be able to improve therapeutic outcome [115,116]. Therefore, dose individualisation based on pharmacokinetic measurements appears to be justified [117]. An investigation was conducted in 208 patients receiving either 1300 mg/m^2 5-FU as a 8 h-infusion plus folinic acid vs. dose-adapted 5-FU to reach target concentrations of 2 to 3 mg/l. Patients in the individualised arm received a higher dose intensity (1800 mg/m^2), had higher response rates but also higher toxicity [118].

In 122 patients with head and neck cancer, treatment of 5-FU was either adjusted to body surface area (4 g/m^2) or the dose was adjusted to the AUC determined in the first cycle according to a scheme including lower threshold levels for white blood, neutrophils and platelet counts. Patients in the standard arm received a higher dose intensity and, consequently, had significantly more severe toxicity. Response rates were not significantly different in both arms [119].

In conclusion, the role of individualised dosing of 5-FU is currently unclear. An increase in response rates with individualised dosing cannot be expected. More realistically, the aim should be a decrease in the incidence of side-effects.

7.4 IN-VITRO-CYTOTOXICITY AS A TOOL FOR THERAPY INDIVIDUALISATION

The idea of in-vitro cytotoxicity determination and subsequent therapy individualisation is more than 20 years old [120]. In principle, the concept is as follows: at diagnosis,

patient tumour cells are collected from biopsy material, blood or bone marrow and are cultured and exposed to cytotoxic drugs. Subsequently, the viability or the percentage of living cells are quantified using methods like clonogenic assays, differential staining cytotoxicity assays (DiSC), the 3-(4,5-dimethylthiazol-2-yl)–2,5-diphenyl tetrazolium bromide (MTT)-assay or fluorimetric microculture cytotoxicity assay (FMCA) [121]. The idea is to select the cytotoxic drug depending on the results of the assays by excluding drugs if the patient's cells have shown resistance to these drugs. Other authors focus on the predictive value of these tests and want to use the results for stratification, i.e. scoring the patient to a certain risk group [122].

Under optimal conditions, this approach is able to avoid unnecessary or ineffective therapy for patients. However, the method is not able to optimise anticancer therapy with regard to improving clinical outcome for high-risk patients. Furthermore, the predictive value of the assays is often questionable as the cells are cultured under conditions which only partially reflect the situation in-vivo. Therefore, this approach cannot replace TDM in the future.

7.5 DOSING BASED SURROGATE MARKERS

Surrogate markers are defined as biomarkers which are able to predict clinical outcome [123]. In cancer chemotherapy, an easy to measure parameter which is predictive for disease-free or overall survival would be desirable. These markers should be prospectively validated in clinical trials. For example, prostate-specific antigen in plasma has been suggested as a surrogate marker for the treatment of prostate carcinoma [124].

In cancer research, they will become increasingly important because of the difficulties and the long time intervals required to assess clinical outcome in cancer therapy [125]. Currently, this approach is mainly used during drug development in clinical studies for new drugs [126]. In the future, they can hopefully be used for therapy individualisation in clinical routine. Several approaches are currently being developed where meaningful biomarkers will become available to control or individualise therapy. Vascular cell adhesion molecule1 (VCAM-1) or vascular endothelial growth factor (VEGF) are candidates for a surrogate marker of an anti-angiogenetic therapy in cancer [127]. In the future, it might be possible to use this parameter for dose adjustment and drug selection.

7.6 CONCLUSIONS

To summarise the role of TDM in cancer chemotherapy, one can conclude that TDM with dose individualisation is clearly indicated for busulfan. For other drugs like 5-FU, 6-MP or methotrexate, the role of TDM is not clearly defined at the moment. Drug monitoring with the aim to identify patients with reduced clearance and to adjust folinic acid rescue is necessary in high-dose methotrexate therapy. Monitoring of cytotoxic drugs should, however, be applied much more frequently than it is done today to prevent

over- or underdosing, especially in patients with liver insufficiency, reduced kidney function or ascites. Another drug where drug monitoring is definitely beneficial to patients is asparaginase, an enzyme used in the treatment of ALL. Patients may develop antibodies inactivating the enzyme. In this case, therapy has to be modified by applying asparaginase from another biological source, which does not cross-react with the antibodies, to ensure sufficient therapy intensity [128].

In the future, more pharmacokinetic studies in special populations like infants are warranted. Also, pharmacokinetic data of older patients with cancer are necessary as this is an important patients group where pharmacokinetic data are lacking. This studies may provide a rationale for dose adaptation or individualisation in this patients.

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CHAPTER 8

Rationale and utility of therapeutic drug monitoring for the optimization of antibiotic therapy

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8.1 INTRODUCTION

Central to the basic principles of therapeutic drug monitoring (TDM) is optimization of drug therapy to maximize clinical outcomes. When it comes to antibiotic therapy, agents that are often considered for TDM are those thought to possess a narrow therapeutic index where the window for either safety or toxicity is fairly small. TDM with the two primary sets of antibiotics, vancomycin and the aminoglycosides, are the most commonly encountered in clinical practice. However, as our knowledge of antimicrobial agents continues to evolve, our growing understanding of the pharmacodynamics of these drugs is rapidly expanding and shaping new concepts for potential future applications of TDM. Pharmacodynamics may soon justify the need for TDM among agents not typically monitored such as the beta-lactams, macrolides, quinolones, and carbapenems. Although these agents have remarkable safety and efficacy profiles which generally precludes them from conventional rationale for TDM, in select patient populations such as the critically ill and immunocompromised, maximizing bacterial kill through optimal drug exposure can provide a dramatic impact in the therapeutic efficacy and may therefore justify the future utility of TDM for these agents. The escalation of antibiotic resistance further suggests the need to optimize therapy not only from a safety and toxicity standpoint, but also in an attempt to maintain the long-term potential clinical usefulness of currently available agents. Pharmacodynamics can thus redefine the future applications of TDM and go beyond to force a broader perspective in terms of resistance and optimization of drug exposure for both clinical and microbiological success.

References pp. 212–219

8.2 VANCOMYCIN

8.2.1 Background

Since the discovery of vancomycin in 1956 from *Nocardia orientalis* (formerly *Streptomyces orientalis*), vancomycin has received a resurgence of interest due to the emergence of methicillin resistant organisms in the 1970s [1]. Owing to its ability to inhibit polymerization of polypeptides on the surface of Gram-positive bacteria, the glycopeptide exhibits potent activity against *Staphylococcus, Streptococcus, Enterococcus, Corynebacterium* and *Clostridium* species including penicillinase producers and methicillin resistant pathogens (i.e. methicillin resistant *Staphylococcus aureus* or MRSA and methicillin resistant *Staphylococcus epidermidis* or MRSE).

Reports of vancomycin-induced nephrotoxicity and ototoxicity at high serum concentrations have prompted the careful monitoring of vancomycin [2]. Earlier formulations of vancomycin, nicknamed "Mississippi mud", contained impurities that may have been responsible for the magnitude of reported toxicities associated with its former use [3]. This then gave rise to the monitoring of vancomycin serum drug concentrations with peak and trough traditionally associated with both drug related toxicity and efficacy, respectively. However, there is still a lack of substantive evidence in the current literature to correlate vancomycin's peak serum concentrations with toxicity and/or clinical outcomes despite years of experience with this agent. Like the beta-lactam antibiotics, vancomycin displays a time-dependent bactericidal activity in which bacterial killing is maximized at time above the minimum inhibitory concentration (MIC) of the organism at 40–50% of a given dosing interval [4,5]. As a result, the need to monitor peak concentrations has been questioned by many while others remain skeptical of the interpretation and even the benefit of monitoring vancomycin serum concentrations.

8.2.2 Pharmacology

Vancomycin acts by binding to the D-alanyl-D-alanine precursors thereby inhibiting cell wall synthesis [6,3]. It is cleared primarily via renal route and has a half-life of 5–9 hours. The drug has a volume distribution (Vd) of 0.7 L/kg (0.5–0.9 L/kg) and pharmacokinetically exhibits a two or three compartment model. Vancomycin is poorly absorbed orally and is therefore given by intravenous infusion for systemic infections. It is commonly dosed at 1 g every 12 hours or 15 mg/kg using the patient's actual body weight [7,8].

8.2.3 Drug concentrations and clinical efficacy

There is very little information correlating vancomycin serum concentrations with clinical outcomes. Vancomycin's peak serum concentrations (target: 20–40 μg/ml) have been typically associated with toxicity while troughs (target: 5–15 μg/ml) reflect

clinical efficacy. Although these established target ranges have existed for many years, few studies have examined the scientific relevance of these targets [9–11].

Standard doses of vancomycin produce concentrations well above the MICs of susceptible organisms such as *Staphylococcus aureus* (*MIC range* 0.5–2 μg/ml) and *Streptococcus pneumoniae* (*MIC range* 0.12–0.5 μg/ml) [12]. In a study by Healy et al., they found that a 1 g dose given every 12 hours produced trough serum concentrations of about 7.9 μg/ml (5.9–15.2 μg/ml) in healthy adults. At 500 mg every 6 hours, serum trough concentrations were approximately 11.2 μg/ml [13]. Based on the pharmacodynamic characteristic of this drug where the duration of drug exposure is most critical for optimal efficacy, standard doses should be sufficient to maintain serum concentrations above the MIC in excess of the generally accepted 50% of the dosing interval for susceptible pathogens. Peak or high drug concentrations of vancomycin are of less importance since further increasing drug concentration will not result in more bacterial killing [14,15]. Moreover, since vancomycin shows a multicompartment distribution profile, clinicians generally experience a great difficulty in adequately timing and subsequently interpreting concentrations obtained during peak sampling times. As a result of this distribution profile, peak drug concentrations can drastically differ when drawn even minutes apart and thus may lead to falsely elevated values. In a clinical scenario, obtaining an accurately timed peak concentration is impractical and almost impossible to get in the general practice setting. For these reasons, peak concentrations are not routinely monitored in many centers. Reproducible trough concentrations, on the other hand, may be more easily obtained; although they appear unnecessary for the majority of patients, some clinicians advocate this serum sampling technique for TDM of vancomycin.

Several studies have investigated the clinical association of vancomycin serum concentrations. Schaad et al. reported that serum bacterial titers (SBT) of 1:8 (approximately >12 μg/ml) were associated with clinical cure among pediatric patients [16]. Failure to achieve this resulted in 3 treatment failures in another study [17]. For meningitis, higher troughs showed faster clinical recovery compared to lower troughs [18]. However, many of these studies were retrospective analysis with a small sample size and numerous confounding factors (i.e. co-morbidities, no baseline measures, etc.). No studies to date have provided convincing evidence on the relationship between serum drug concentration and clinical outcome. Many believe that a typical dose of vancomycin is sufficient to achieve serum concentrations in excess of the MIC to eradicate susceptible pathogens. For the reasons stated above, many clinicians feel that monitoring vancomycin concentrations should be reserved only for patients with rapidly changing renal function, significantly altered kinetics (i.e. burn patients, morbid obesity) and deep-seated infections (i.e. endocarditis, meningitis, and osteomyelitis).

8.2.3.1 Concentrations and toxicity

Although vancomycin's potential to cause toxicity (notably nephrotoxicity) is well documented in literature [19–23], studies have failed to correlate vancomycin serum concentrations with drug toxicity. Impurities from the earlier formulations may account for many of the reported adverse reactions since its introduction in the 1950s.

Recent data show that vancomycin-induced nephrotoxicity occurs in about 5% of patients [19]. Nephrotoxic effects are often reversible after discontinuation of the agent. When combined with other renally toxic agents, the incidence of nephrotoxicity increases. Farber and Moellering found a higher incidence of nephrotoxicity (22–35%) with the combined use of aminoglycosides [19,24]. Other studies have found similar results [25,26]. Drug-induced renal dysfunction has been associated with high serum concentrations $\geq$30–40 μg/ml as well as low concentrations of vancomycin ($<$10 μg/ml) [3,27]. What concentrations constitute an increased risk for vancomycin acute toxicity remains unclear, since it appears to occur at any observed concentration. Others speculate that the duration of exposure rather than peak concentrations is the main causative factor for the development of nephrotoxicity [21,24].

Dose-related ototoxicity (i.e. tinnitus, dizziness, hearing loss) occurring at levels $>$80–100 μg/ml had been reported by Geraci and colleagues. The incidence of auditory toxicity is low ($<$2%), and cessation of drug often leads to its resolution. It is important to note that most reports relating to vancomycin ototoxicity lack baseline auditory testing on the patients studied. In addition, the definition of ototoxicity varies among the different studies [20,28].

There are other adverse-effects associated with vancomycin use. The infusion-related "red-man's syndrome", is due to the rapid drug infusion causing histamine release [29]. Decreasing the infusion rate usually prevents or minimizes the occurrence of this reaction. There had also been reported neutropenic effects seen with vancomycin use [30,31].

8.2.4 Monitoring serum drug concentration

If serum concentration monitoring is desired, vancomycin serum concentrations should be obtained on the second or third dose of the agent following initiation of therapy or dosing adjustments. This is to assure that the concentrations obtained accurately reflect a steady-state concentration of the drug. Trough concentrations are collected approximately 30 minutes before the next dosing interval. The target for troughs can range from 5–15 μg/ml with a goal of maintaining serum concentrations above the MIC of the infecting organism for at least 50% of the dosing interval. Although not commonly done, peak concentrations are drawn 1–2 hours post-infusion and kept in the range of 20 to 40 μg/ml. As discussed previously, obtaining vancomycin peak concentrations are often unnecessary primarily because the drug's efficacy is time-dependent while its toxicity is primarily correlated with the duration of exposure and not peak concentrations. If the duration of vancomycin therapy is $<$72 hours, drug levels are not needed. Empiric dosing of vancomycin can be determined using the following guideline (Table 8.1) [32–37].

Patients with normal renal function can be monitored on a weekly interval unless renal function changes or if initial target concentrations have not been met. A rise in serum creatinine (SCr) of $\geq$0.5 mg/dl may indicate a decline in renal function and may call for appropriate dose adjustments to avoid drug accumulation and the possible risk of toxicity. Because drug elimination is reduced during renal impairment, a decrease in

TABLE 8.1
EMPIRIC DOSING GUIDELINE FOR VANCOMYCIN DOSING.

Estimated Creatinine Clearance (ml/min)	Dose (mg/kg)	Dosing Interval (hours)
>85	14	12
66–85	11	12
41–65	14	24
26–40	11	24
16–25	14	48
≤15	14	random

dose or increase in the dosing interval is an option for adjustment. In these cases, random levels of the vancomycin are obtained to make certain that the drug concentration does not fall below the MIC of the organism being treated. For renal dialysis patients, random levels every 3–4 days (or even longer depending on patient specific kinetics) is reasonable to ensure maintenance of adequate trough concentrations. Monitoring patient's daily serum creatinine, adjusting the dose accordingly and monitoring or avoiding concomitant use of other nephrotoxic agents should help optimize therapy [36,38,39].

Patients who have altered kinetics with normal renal function have different dosing requirements. This includes patients who are morbidly obese, intravenous drug abusers, and burn patients. These patients have increased volume of distribution and faster drug clearance rates [40]. In morbidly obese patients, vancomycin clearance is 2.5 times greater compared to patients with normal weight. Shorter dosage intervals may be needed to maintain trough concentrations when using regular doses of the drug [36,41]. Periodic trough levels and dosing adjustments are recommended to assure adequate doses are being administered to meet the specific needs of these patients for therapeutic success.

There are several other published nomograms for vancomycin including the Bayesian models [42], Sawchuk-Zaske method [43], Matzke et al. [44], and Moellering et al. [24]. These are usually derived from a small subset of patients and are often designed to achieve a certain target level. For example, Matkze's dosing nomogram targets a steady state level of 15 μg/ml for patients with renal dysfunction. Several nomograms are available formulated for specific patient population including burn patients, pediatrics, and dialysis patients. It is important to remember that these nomograms are approximations based on estimated and/or predefined patient parameters and should therefore be used only as general guide for dosing adjustments.

8.3 AMINOGLYCOSIDES

8.3.1 Background

Since the first discovery of streptomycin in 1944, aminoglycosides remain an important antibiotic for the treatment of Gram-negative infections. Although their nephrotoxic and

ototoxic potential have historically limited their use, studies on the pharmacodynamics and pharmacokinetics of aminoglycosides have prompted a new understanding and approach to maximize the effectiveness and minimize the toxicity of this class of antimicrobials.

Early published literature with commonly utilized agents such as gentamicin and tobramycin provided the basis for the prevailing belief that high doses of aminoglycosides cause toxicity (at trough levels >2 μg/ml and peaks >12 μg/ml). On the basis of these early observations, conventional dosing strategies (also known as intermittent dosing) were designed to maintain peak concentrations of 4–10 μg/ml and produce trough of <1–2 μg/ml. However, recent data suggest that these agents are equally, if not less likely, to cause their noted toxicities when the total daily dose is administered in a single daily injection [45–49]. Moreover, the aminoglycosides display dose-dependent activity where the maximum serum concentration to MIC ratio (C_{max}:MIC) best correlates with the efficacy of these agents. The implementation of this pharmacodynamic observation has given rise to the dosing strategy commonly referred to as once-daily aminoglycoside (ODA) therapy [50–52].

There are several agents within this class, all of which exhibit similar pharmacokinetic and pharmacodynamic properties. Gentamicin, tobramycin and amikacin are most commonly used agents for bacterial infections while streptomycin is primarily used as an antimycobacterial agent. Hence, the driving force for aminoglycoside TDM is the prevention of its toxicity and optimization of its bactericidal effects.

8.3.2 Pharmacology

Aminoglycosides display concentration-dependent killing in which their bactericidal effect increases with increasing peak concentrations at 10–12 times the MIC of the organism and results in maximal kill of these agents [14,53]. Aminoglycoside pharmacokinetics exhibit wide-interpatient variability and can be significantly altered depending on the patient's disease state (i.e. cystic fibrosis and burn patients clear these agents at a faster rate), volume status, and renal function. The volume of distribution for these agents is 0.25–0.3 L/kg. They are cleared primarily via renal mechanisms. Aminoglycosides possess a half-life of approximately 2.5 hours but with an extremely long terminal elimination phase of 100–150 hours in healthy adults. Since these agents possess little if any systemic bioavailability when given by the oral route, they are administered intravenously and less frequently by intramuscular injection. Neomycin is the only clinically utilized agent given orally and is used routinely for gut decontamination prior to surgery [50,51,54].

8.3.3 Intermittent versus once-daily aminoglycosides

Although the usage of intermittent dosing strategies dominated clinical practice in earlier years due to fears of drug-related toxicities, numerous clinical trials and meta-analysis studies have evaluated the efficacy and safety of high dose, once-daily aminoglycosides (ODA). In animal studies, the efficacy of ODA was demonstrated to be

equal or greater compared to conventional dosing regimen, alone or in combination, in various types of infection models such as endocarditis, pyelonephritis, and pneumonia [55–58]. In clinical practice, the safety and efficacy with ODA are extensive and encompass a broad range of patient population including patients with Gram-negative bacteremia, bone and soft tissue infections, endocarditis, intra-abdominal infections, and cystic fibrosis patients [59–64]. In a randomized study by Prins and colleagues, the efficacy of ODA versus thrice daily regimen of gentamicin was investigated and found that 91% of patients using ODA responded to therapy within 72 hours compared to 78% of patients in the thrice daily dosing. They concluded that no statistically significant difference in efficacy was seen between the two regimens [65]. In the study by Raz and colleagues involving 124 patients, they found significantly higher cure rate with ODA among patients with Gram-negative bacterial infections, $p < 0.05$. Similarly, patients with higher C_{max}:MIC ratios of 8 had a 91% cure rate compared to only 12.5% among patients with a ratio of ≤ 4 [66]. Other reports correlating higher cure rates with higher concentrations have been documented in the literature [67]. Nicolau and colleagues evaluated the safety and efficacy of ODA in one of the largest clinical reports involving 2,184 patients treated with gentamicin or tobramycin. Clinical responses among these patients were successful. Nephrotoxicity and ototoxicity occurred in only 1.2% and 0.1% of patients, respectively. In comparison, the historically reported incidence of nephrotoxicity with conventional dosing ranged from 3–5% at the same institution [68].

In special populations such as children, pregnant women and neutropenic patients, studies on the efficacy and safety of ODA have been evaluated and demonstrated effective cure rates with a trend towards reduced toxicity [69–74]. In the pediatric population, studies using 20 mg/kg ODA of amikacin in children undergoing bone marrow transplantation or with Gram-negative infections concluded no difference in efficacy or toxicity compared to standard multiple dosing regimens [75,76]. The meta-analysis by Hatala et al. demonstrated the equivocal efficacy of ODA in neutropenic patients compared to conventional dosing regimens [74]. However, information on the use of ODA in specific patient population such as pediatrics, pregnant women, and neutropenic patients are still limited.

Pharmacoeconomic analysis comparing ODA versus standard regimens showed that ODA consumes fewer resources and requires less monitoring than intermittent dosing intervals. As a result of the ODA dosing program, Hartford Hospital was able to achieve annual savings estimated at more than $100,000 in 1995. Savings were largely the consequence of a 40% reduction in requests for gentamicin and tobramycin serum concentration determinations. During the first phase of the ODA program, random sampling of serum concentrations and the use of the nomogram were used to accomplish therapeutic drug monitoring. In the second phase, serum drug concentration testing was eliminated for patients with normal CrCl. Despite the reduction in monitoring activity, the incidence of nephrotoxicity was 1.2% in the first phase of the program and 1.3% in the second phase; with conventional dosing patterns, the occurrence is around 3% to 5% [68].

Continuous exposure to aminoglycosides has been known to promote the down regulation of receptors responsible for drug transport of aminoglycosides in Gram-

negative organisms. The mechanism of adaptive resistance decreases drug penetration thereby diminishing the bactericidal activity of these agents. This persistent exposure to aminoglycosides has been compared and paralleled to the constant drug exposure seen with conventional dosing regimens. Studies have revealed that actually allowing a drug free interval hinders the development of such resistance. Since aminoglycosides generally exhibit a long post-antibiotic effect (PAE) lasting 1–8 hours that follows a linear relationship with increasing drug concentration, high dose administration of aminoglycosides permits this drug free period [77–81]. This concept of PAE further supports the use of once-daily aminoglycosides. Therefore, it is purported that the less frequent, high dose strategy has a distinct advantage lending to its continued efficacy and wide clinical acceptance [82,83].

8.3.4 Side-effects

Nephrotoxicity and ototoxicity are the two prominent adverse reactions seen with aminoglycosides that have prompted their cautious usage. Previous reports of these toxicities indicated that high drug concentrations are correlated with such. However, recent findings suggest that their toxic potential is associated with drug accumulation and duration of drug exposure [84,85]. This is consistent with the fact that aminoglycoside toxicities have been reported even at normally targeted concentrations. In addition, because aminoglycosides exert cytotoxic effects via a saturable process in both the kidneys and vestibulo-cochlear systems, administration of either high doses as in ODA or the conventional lower dosage regimens can both be equally nephrotoxic or ototoxic [84–87].

The exact mechanism of aminoglycosides' ability to cause nephrotoxicity is unknown. It is hypothesized that drug accumulation within cellular lysosomes causes phospholipidosis to occur. Following reuptake of aminoglycosides in the proximal renal tubules, aminoglycosides accumulate within the lysosomes until they burst causing the release of lysosomal enzymes and phospholipids leading to cell death [87]. Nephrotoxocity (usually defined as an increase in serum creatinine (SCr) ≥ 0.5 mg/dl from baseline following treatment) is often reversible following drug discontinuation [68]. The risk of kidney damage increases with concurrent use of other nephrotoxic agents such as vancomycin and amphotericin B.

The incidence of aminoglycoside ototoxicity ranges from 2–25% and increases with prolonged treatment. Unlike nephrotoxicity, ototoxicity is frequently irreversible. Ototoxicity can be manifested as vestibular or cochlear injury and may have unilateral or bilateral involvement. It can also occur during therapy or days after drug discontinuation [88,89]. Cochlear damage, defined as a baseline increase of 15dB at two or more frequencies, is estimated to occur between 5–15% of patients on therapy and is often associated with cotton-ball ear sensation, loss of high frequency distinction, tinnitus, and hearing loss. On the other hand, vestibular toxicity affects the equilibrium and is commonly manifested with nausea, vomiting, vertigo, and difficulty with gait. It is recommended to perform baseline audiometric tests prior to starting aminoglycosides especially in patients who will be receiving them for > 10 days [90]. Risk factors for

ototoxicity include the aminoglycoside agent used, length of therapy, total dose, concomitant use of ototoxic agents, advanced age, and previous renal dysfunction [91]. Similar to nephrotoxicity, ototoxicity is postulated to be due to saturable mechanisms and drug accumulation. Their tight binding at the receptor site and long half-lives also attribute to this adverse effect. Aminoglycosides bind to phosphoinositides, inhibit decarboxylase, and cause mitochondrial dysfunction leading to cell death [92].

It has also been observed that the toxic potential of aminoglycosides may be related to circadian rhythm since toxicity is observed more in patients receiving the drugs during sleep hours [93]. There are rare occurrences of neuromuscular blockade associated with the aminoglycosides [94,95].

8.3.5 Dosing strategies

CONVENTIONAL DOSING: Conventional (intermittent) dosing is often the preferred regimen in patients with rapidly changing renal function because it permits rapid dosing adjustments especially in the critically ill. This is because a more individualized, pharmacokinetic-based regimen can be designed based on the patient's own specific parameters. The conventional dosing schemes of aminoglycosides for patients with normal renal function (CrCl $\geq$ 90 ml/min) are listed on Table 8.2 [50,96,97].

Steady state concentrations for aminoglycosides are generally achieved by the third dose. Peak concentrations are usually drawn 30 minutes following intravenous infusions of the third dose while troughs are often collected prior to this drug administration. Monitoring peak concentrations will ensure effective target concentrations. Troughs will serve as indicators of drug accumulation and hence the potential for toxicity (although toxicity may still present in the face of acceptable peak and trough concentrations). In stable patients with normal renal function, periodic monitoring of trough or peak concentrations every 3–5 days is sufficient. However, in patients with rapidly changing

TABLE 8.2
CONVENTIONAL DOSING REGIMENS AND TARGET CONCENTRATIONS FOR THE AMINOGLYCOSIDES.

Agents	Loading Dose (mg/kg)	Maintenance Dose (mg/kg)	Target Levels (μg/ml)
Gentamicin	1–2	1.7–2 Q8h	Peak: 4–10 Trough: < 1–2
Tobramycin	1–2	1.7–2 Q8h	Peak: 4–10 Trough: < 1–2
Netilmicin	1–2	2 Q8h	Peak: 4–10 Trough: < 1–2
Amikacin	5–7.5	5 Q8h 7.5 Q12h	Peak: 15–30 Trough: 5–10
Streptomycin	5–7.5	5 Q8h 7.5 Q12h	Peak: 15–30 Trough: 5–10

renal function or altered kinetics, more frequent monitoring may be needed. It is imperative that the dosing and draw times are properly documented.

There are also nomograms available to aid in dose adjustments. The most widely accepted nomogram for conventional intermittent dosing is that of Sarubbi and Hull. Using this method, a loading dose (based on ideal body weight) of 1–2 mg/kg of gentamicin or tobramycin or 5–7.5 mg/kg of amikacin is administered to patients with renal dysfunction. Different maintenance doses (based on a percentage of the initial loading dose) can then be selected depending on the desired dosing interval of either 8, 12, or 24 hours [98].

ONCE-DAILY DOSING: The pharmacodynamic concept of concentration dependent killing has given birth to the concept of ODA. As mentioned previously, ODA takes advantage of the high bactericidal activity in accordance with its pharmacodynamic profile and possesses a relatively long PAE allowing for drug free interval thus decreasing the risk for toxicity and adaptive resistance. In addition, ODA requires less monitoring since only trough and not peak concentrations are generally evaluated thereby consuming less clinical resources with fewer associated costs [65,68].

The range of once-daily dosing currently in practice is 3–7 mg/kg/day for gentamicin, tobramycin, and netilmicin. Amikacin dosages range from 11–15 mg/kg [50,99]. To start the initial dosing regimen, the following guideline has been proposed by Nicolau et al (Table 8.3). Drug serum concentrations should be monitored on a weekly basis (i.e. trough or random concentrations) unless the patients have altered kinetics from baseline status (i.e. renal dysfunction, change in volume load, etc.). In patients with renal impairment, there is currently no consensus as to how to adjust ODA in this population. Some advocate decreasing total daily dose of aminoglycosides whereas others advocate maintaining the same dose while increasing the dosing interval. This latter approach maintains a high C_{max}:MIC ratio thereby optimizing the pharmacodynamic profile of the aminoglycosides.

There are several nomograms available to aid the clinician when ODA is the desired dosing regimen. These nomograms are established based on a specified dose and on a specific patient population. It is important that a clinician utilizing such nomograms is aware of their specifications. One of the more widely used nomograms, the Hartford Nomogram, is based on a fixed daily dose of 7 mg/kg of gentamicin or tobramycin and was designed to obtain target peak serum concentrations of 20 μg/ml (C_{max}:MIC ratio of 10 was based on the average MIC of 2 μg/ml for P. aeruginosa at Hartford Hospital).

TABLE 8.3
EMPIRIC DOSING REGIMEN FOR GENTAMICIN AND TOBRAMYCIN ADMINISTERED USING THE ONCE-DAILY AMINOGLYCOSIDE DOSING TECHNIQUE.

CrCl (ml/min)	Dosing Regimen
≥60	7 mg/kg q 24 hrs
40–59	7 mg/kg q 36 hrs
20–39	7 mg/kg q 48 hrs
<20	7 mg/kg, then follow serial levels to determine time of next dose (i.e. level < 1 μg/ml)

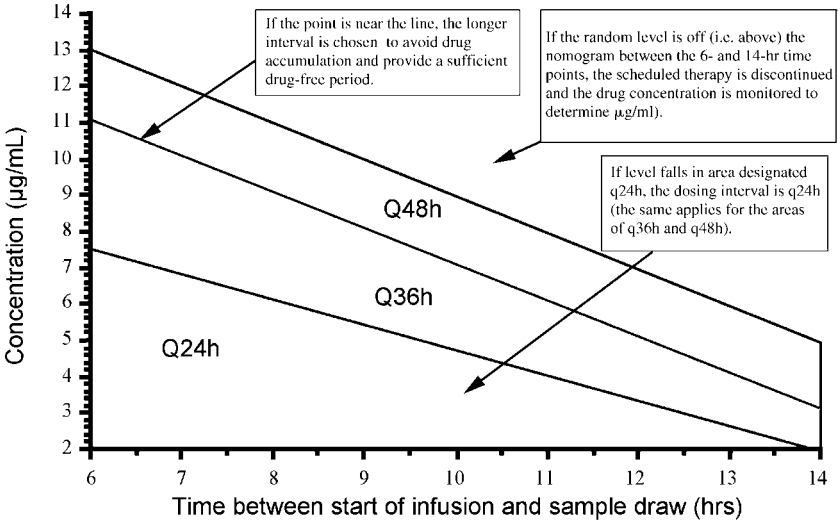

Fig. 8.1. Hartford Hospital once-daily aminoglycoside dosing nomogram.

Using this method, a random serum concentration is measured 6–14 hours after the start of the first infusion. The dosing interval is then adjusted from this concentration using a nomogram and plotting it against the time point between the start of infusion and blood draw. The graph area is designated with 24, 36, and 48 hour dosing intervals (Fig. 8.1). For example, if the serum concentration indicates 8 μg/ml drawn eight hours after the infusion, the regimen will fall under the every 36 hour dosing interval. Excluded from this nomogram include patients with ascites, burns of >20% of total body surface area, pregnancy, end-stage renal disease (including dialysis) and enterococcal endocarditis.

Other methods of adjusting aminoglycoside are also available. Nomograms created by Gilbert used 5 mg/kg of gentamicin or tobramycin [100]. With decreasing renal function, it advocated changing the dosage and/or dosing interval to maximize therapy and prevent toxicity. Similarly, Prin et al. [16] advocated decreasing total daily dose to maintain a 24 hour regimen. As mentioned earlier, there is no consensus as to the best way (whether to adjust the dose or dosing interval) to optimize therapy since there is not enough data to dispute which method is more effective to produce clinically significant outcomes [101].

8.4 ANTITUBERCULAR AGENTS

8.4.1 Background

The management of tuberculosis (TB) remains an increasingly complex and challenging clinical dilemma. While over 16,000 cases were reported in the United States in 2000 alone, TB accounts for 3 million deaths annually worldwide. Despite treatment

involving multiple agents such as rifampin, isoniazid, pyrazinamide, and ethambutol and months of therapy, therapeutic failures and relapses still occur in 2–5% of patients regardless of directly observed therapy (DOT) [102]. These failures are often multifactorial and encompass problems due to drug resistance, stage of the tubercular disease, altered pharmacokinetics, interpatient variability, and patient noncompliance.

Optimizing therapy through the use of TDM on antimycobacterial agents has the potential to maximize clinical response. In patients with modified kinetics (i.e. hepatic and renal dysfunctions) and physiologic alterations (i.e. malabsorption problems, HIV/AIDS, cystic fibrosis), subsequent dosing adjustments to achieve targeted drug concentrations can lead to a more effective therapy [103–106]. In addition, the avoidance of suboptimal drug concentrations may prevent the subsequent emergence of resistance. Drug-related toxicities and interactions can be minimized and better managed thus improving the safety, efficacy, and perhaps patient adherence with these agents. However, the use of TDM has generally been limited to patients that either have not clinically responded or have persistent positive acid-fast smears indicating a lack of pathogen eradication. This section will discuss published data and clinical experience pertaining to the use of TDM on antimycobacterial therapy.

8.4.2 Pharmacology of antimycobacterial agents

The pharmacologic activity of various antimycobacterial agents primarily consists of two main mechanisms of action: inhibition of the cell wall synthesis and RNA polymerase blockade. Cell wall active agents such as cycloserine, ethionamide, isoniazid, thiacetazone and ethambutol exhibit increased activity against rapidly growing *Mycobacterium* and display time-dependent activity. The optimal dosing regimen for these agents should be adjusted to maintain drug levels above the organism's MIC over a given dosing interval at the site of infection. In contrast, agents acting on intracellular targets such as the rifamycins, aminoglycosides and quinolones possess concentration-dependent bactericidal activity. Hence, maximizing the C_{max}:MIC ratio results to a greater killing and improved efficacy and safety profiles [107]. Table 8.4 lists the commonly utilized antitubercular agents [104,106,108,109].

The activities of these agents are also reported to have distinct phases for combating mycobacterial infections. The early bactericidal activity is defined as the ability of an agent to cause a precipitous decline of rapidly dividing mycobacteria by as much as 95% in the first few days of therapy. On the other hand, the slower eradication or inhibition of the latent, less active mycobacteria is known as the sterilizing activity. Antitubercular agents appear to inherently possess these activities. Because mycobacteria are slow-growing organisms with various stages of development, prolonged combination therapy is required for a successful clinical outcome [109].

8.4.3 Serum concentrations and clinical efficacy

There are numerous published data correlating serum drug concentrations with clinical response [103,105,110,112]. In a study by Gilljam and colleagues, clinical improvement

TABLE 8.4
COMMONLY UTILIZED ANTITUBERCULAR AGENTS AND TARGET CONCENTRATIONS.

Drug Name	Usual Dose (mg/d)	Proposed Target Serum Level 2 h post-dose μg/ml	Dose Ajustments Renal	Hepatic
Para-aminosalicylic acid (PAS)	4000	40–70	Unknown	Unknown
Ciprofloxacin	750–1000	4–7	Increase dosing interval	None
Clofazimine	100–200	0.5–2	None	None
Cycloserine	500–750	35–50	250–500 mg 3 × /week	None
Ethambutol	15–25 mg/kg	2–6	15–25 mg/kg 3 × /week	None
Ethionamide	15–20 mg/kg Max: 1g/d	3–8	None	Unknown
Isoniazid (INH)	300–450	3–5	None for most patients; dose every other day	None for most patients
Pyrazinamide (PZA)	1000–2000	20–60	15–30 mg/kg 3 × /week	Unknown
Rifabutin	300–600	0.3–0.9	None for most patients	None for most patients
Rifampin	450–600	4–8	None for most patients	None for most patients
Amikacin, Capreomycin, and Streptomycin	15 mg/kg*	34–45*	12–15 mg/kg 3 × /week	None

* Conventional given 5 × /week.

and negative sputum samples were noted following dose adjustments among cystic fibrosis patients [111]. Sahai et al. found that systemic drug exposure of rifampin and pyrazinamide among HIV-infected patients were lower by 32% and 24% compared to healthy controls, respectively. The high relapse rates found in this patient group have been attributed to poor drug absorption and low therapeutic drug levels [105]. Drug malabsorption has also been proposed as the cause of failure and relapse among immunocompetent patients compliant with therapy [112]. Moreover, the selective pressure due to suboptimal drug concentrations has historically given rise to resistant organisms and may have contributed to the development of *M. tuberculosis* resistance and subsequent drug failures [113–115]. Nevertheless, no definitive data exist to support this theory regarding resistance for antimycobacterial agents. In fact, a recent publication by Narita et al. found no correlation with drug serum levels and the recurrence of TB [116]. Whether subtherapeutic plasma concentrations promote drug resistance or increase the risk of relapse is still open to debate.

It is important to realize that serum concentrations do not always reflect intracellular concentrations and drug concentrations. Currently, the absence of an effective method to fully evaluate the drug concentrations of these agents restricts our ability to establish a definitive relationship between drug concentrations and clinical response. Measuring the individual impact of each antitubercular agent in a given therapy in a given stage of infection further complicate this dilemma. Moreover, characterizing the *in vivo* synergistic activity with various agents is very difficult to predict for these agents. While

the pharmacologic activities of antitubercular agents have been described, our understanding of the antimycobacterial pharmacodynamic profile of the frequently utilized multi-drug regimens and its application in clinical practice is still at its infancy.

8.4.4 Toxicity

Dose-dependent side effects have been observed with various antimycobacterial agents. Common adverse events reported with these agents as displayed in Table 8.5. Rifampin doses greater than 10 mg/kg have been associated with antibody-mediated immune reactions such as thrombocytopenia, influenza-like syndrome, hemolytic anemia, and acute renal failure (ARF). Flu-like symptoms have been reported to occur at doses >600 mg although others report the absence of these symptoms if serum concentrations are maintained within 8–24 μg/mL. Gastrointestinal intolerance is also common at higher doses of rifampin [108,117]. Rifabutin has been shown to cause uveitis at doses $\geq$ 1200 mg, although it is often reversible and resolves within 1–2 months following discontinuation of the drug. Dosing rifabutin less than 600 mg/d decreases the risk of this problem [118]. Ethambutol is another agent with known dose-dependent side-effects resulting to retrobulbar neuritis at doses >25 mg/kg/d. Symptoms are manifested with visual acuity impairment, central and peripheral scotoma, and red-green color blindness. Initial and periodic eye examination is recommended prior to using this drug [108,117,119]. Other agents with dose related side-effects include the aminoglycosides (nephrotoxicity and ototoxicity) and cycloserine (dose-related neurological and psychiatric impairment in 50% of patients) [108,120].

TABLE 8.5
ADVERSE REACTIONS WITH COMMONLY UTILIZED ANTITUBERCULAR.

Drug Name	Adverse Drug Reaction
Para-aminosalicylic acid (PAS)	GI upset, hypersensitivity, hepatotoxicity, sodium load
Ciprofloxacin	GI upset, hypersensitivity
Clofazimine	GI upset, skin discoloration, abdominal pain, crystal deposition leading to organ failure
Cycloserine	Seizures, neurologic toxicity (ie. psychosis)
Ethambutol	Retrobulbular neuritis, red/green color blindness, decreased/loss of visual acuity
Ethionamide	Nausea, vomiting, diarhea, abdominal pain, hepatitis
Isoniazid (INH)	Fever, rash, anemia, neuropathy, age-related hepatotoxicity, optic neuritis
Pyrazinamide (PZA)	Hepatotoxicity, hyperurecemia, rash
Rifabutin	Uveitis; orange secretions
Rifampin	Hepatotoxicity, thrombocytopenia
Amikacin, Capreomycin, Kanamycin, Streptomycin	Nephrotoxicity, ototoxicity, neuromuscular blockade
Thiacetazone	Arthralgia, myalgia, dark urine, yellowing of the skin

A caveat for TDM is that whilst established therapeutic ranges of serum concentrations may provide the best clinical response for most patients, certain patients may need a higher or lower serum targets. Some patients may experience drug toxicities at concentrations within the acceptable serum range and necessitate lower doses to achieve similar outcomes. A prime example of this involves the differences between slow acetylators and fast acetylators with isoniazid. Fast acetylators (i.e. Caucasian) have increased predisposition isoniazid's hepatotoxicity compared to slow acetylators [121,122]. Thus, the monitoring of drug levels should always follow sound judgment based on patient-specific clinical response.

8.4.5 Monitoring of drug concentrations

Antitubercular TDM should be considered if the patient does not clinically improve with therapy or in those patients who remain smear positive after 1–2 months of directly observed therapy (DOT). Serum drug concentrations are usually obtained at a minimum of 2-hour post dose for most antitubercular agents (oral or intravenous) following achievement of steady state. Steady state is typically achieved after one day following administration of loading doses agents with long half-lives or in drugs with short half-lives (1–4 hours). A second sample can also be collected approximately one half-life of the drug to eliminate any absorption issues. This is generally drawn about 6 hours post dose. For example, rifampin samples should be drawn 4 hours apart (2 hour post dose then a 6 hours post dose) since rifampin typically has a half-life of 2–3 hours in most individuals. Trough levels for most agents are not monitored since most are undetectable by 24 hours unless drug accumulation or problems with elimination are suspected [123].

As with any serum drug monitoring, proper documentation of the actual time of dose and time of blood collection is essential since inaccuracies with dosing times and blood draws can significantly alter the results and interpretation of drug concentration-time profile.

8.4.6 Dosing adjustments

Renally impaired: Streptomycin, capreomycin, kanamycin, ethambutol; and second-line agents (i.e. quinolones, and cycloserine) that are renally eliminated should have their dosing regimen modified [124]. Renal failure patients should be given standard daily doses per day but should be administered less frequently over the course of the week (Table 8.3). Hence, TDM may be useful for renally impaired patients receiving the above noted agents.

Hepatically impaired: While hepatic impairment has been found to elevate serum concentrations of INH and rifampin, it has no predictable effects for most of these agents. Thus, TDM may also be useful for hepatically impaired patients receiving INH and rifampin [125].

8.4.7 Drug interactions

There are many drug interactions associated with the use of these agents [126]. Potent inhibitors or inducers of cytochrome P450 can affect the concentration of these agents and vice versa. Examples of enzyme inhibitors include azole antifungals, protease inhibitors, and clarithromycin. Isoniazid itself is a potent inhibitor and can increase concentrations of phenytoin and carbamazepine [104]. Food interactions are also common with these agents. As a result of the multitude of potential drug interactions, TDM may be useful for the modification of the regimen in an attempt to minimize toxicity while maximizing efficacy.

8.5 ANTIRETROVIRAL THERAPEUTIC DRUG MONITORING (ATDM)

8.5.1 Introduction

The advent of highly active retroviral therapy (HAART) in the mid-1990s contributed to the significant decrease in morbidity and mortality among patients infected with HIV/AIDS. Now considered standard of care, HAART is making a dramatic impact on the longevity and quality of life for these patients. However, it is estimated that about 60–70% of patients fail HAART due to toxicity. In addition, virologic failures and the ever-changing patterns of resistance continue to occur despite aggressive therapy. As we move forward into understanding the complexity of this infection and its management, understanding the pharmacodynamics and pharmacokinetics of antiretroviral agents in relation to clinical response can provide crucial information on issues of interpatient variability, drug toxicity, drug interactions, and the development of HIV resistance. Advantages of ATDM include detection of drug-drug interactions, prevention of toxicity (i.e. indinavir and renal stones, ritonavir related to circumoral paresthesia and elevation of triglyceride levels), and optimization of effective therapy especially in patients with disease modifying kinetics (i.e. absorption, metabolism, excretion) due to hepatitis C, gastroenteropathy, and HIV/AIDS disease progression. Because of its potential impact on patient care, there is an enormous interest on the role of antiretroviral therapeutic drug monitoring (ATDM) in recent years.

8.5.2 Serum concentrations and efficacy

There are currently 3 classes of antiretroviral agents available that are being investigated regarding their potential for therapeutic drug monitoring. Nucleoside reverse transcriptase inhibitors (NRTIs) require intracellular activation to convert them to their active moiety. However, assays to monitor the intracellular concentrations of these drugs are costly, labor intensive, and are not readily available. Standardization of these assays has also not been established. Because of this, monitoring NRTI concentrations let alone establishing a relationship between clinical effect with intracellular concentrations present major obstacles for TDM [127–129].

The second class of antiretrovirals, the non-nucleoside reverse transcriptase inhibitors (NNRTIs), has had some success on predicting the occurrence of CNS-associated toxicity with serum drug concentrations. Fifty percent of patients taking efavirenz experience hallucinogenic reactions in the first 2–4 weeks of initiation. In a study by Marzolini et al., >1200 mg increase the risk and severity of effect often leading to patient discontinuation of therapy [130]. In general, however, the use of TDM on NNRTIs is limited by their low interpatient variability, early steady state, and extremely long half-lives. On the other hand, TDM with the protease inhibitors (PIs) seems the most promising. Protease inhibitors possess wide-interpatient variability, numerous drug interactions, and class specific toxicity [131]. Moreover, studies on PIs correlated drug concentrations with clinical response and side-effect profiles of these agents [132,133]. Recently, a fourth class has been added to this therapeutic list. Enfuvirtide is a novel fusion inhibitor recently approved by the Food and Drug Administration (FDA). The possible role of TDM for this new class remains to be seen.

8.5.3 Serum concentration and clinical outcomes

Trials investigating on antiretroviral therapy clinical outcomes utilize surrogate markers such as viral load, CD4 count, and appearance of drug-toxicity. Gieshchke et al. found that saquinavir (soft-gel) produces area under the plasma concentration time curve consistent with a decrease in HIV RNA and improvement in CD4 count among patients [134]. Similarly, the ADAM study found a strong relationship of saquinavir and nelfinavir plasma concentrations with the initial rate of viral clearance [135]. In the ATHENA study, 147 treatment naïve patients were randomized to physicians who either received TDM results for drug adjustments or those who did not. Results revealed that TDM in NFV and IDV containing regimens improved clinical outcome although not with RTV, SQV, or NVP. However, this study was not blinded and not all clinicians followed TDM advice [136]. Other studies have found correlations with serum concentrations and clinical outcomes [127,137–140].

8.5.4 Future of ATDM

A potentially useful tool in ATDM is viral genotyping. HIV strain mutations that render isolates resistant to certain antiretroviral agents can be identified using genotyping techniques. Genotyping analysis predicts viral resistance patterns and facilitates in determining which antiretroviral regimens are likely to fail due to such resistance. The usefulness of genotyping to aid in optimizing therapy is gaining popularity and is quickly becoming standard of practice for new infections as well as for patients with repeated drug failures and viral breakthroughs. Several studies have been conducted to evaluate its utility concurrent with a pharmacokinetic-based regimen and its impact on clinical outcomes [139,141]. The results varied and therefore cannot provide conclusive evidence on the use of genotyping with TDM.

Another potential ATDM tool is the use of inhibitory quotients. Inhibitory quotients (IQ) integrate virus sensitivity with serum drug concentrations. Drug inhibitory

concentrations (IC) are determined (i.e. IC_{90} is the concentration of drug required to inhibit viral replication by 90%) while taking into account the phenotypic characteristic of the virus. There are currently 3 different approaches in the use of these parameters: IC (C_{min}/IC_{50}), VIQ (C_{min}/Virtual IC_{50}), or NIQ (C_{min}/population-based). However, there is currently no consensus as to the optimum target concentration for these agents and further studies are needed to define its clinical utility [142–146]. The major dilemma with ATDM is that it is unclear what pharmacodynamic/kinetic parameter best correlates with the efficacy of these agents. As a result, there is currently no consensus as to which parameter (area under the concentration-time curve, C_{max}, C_{min}) best optimizes drug efficacy. Moreover, problems of interpatient variability due to viral load, immune function, virus sensitivity, resistance, and the patient's predisposition to adverse effects further add to the complexity of monitoring these agents. Currently, routine TDM with antiretrovirals is not yet recommended. In addition, its role in new agents such as the fusion inhibitors (i.e. enfuvirtide) is yet to be determined [131,145,147].

8.6 CONCLUSION

Knowledge and application of pharmacodynamic principles to dosing and administration of antibacterials can optimize clinical and bacteriologic efficacy while potentially reducing toxicity and costs associated with therapy. Within the realm of this clinical activity, the utilization of therapeutic drug monitoring may assist in reaching these goals for a broad range of antimicrobials noted in this chapter. For antimicrobials that do not readily undergo TDM, pharmacodynamic concepts can be applied to patient care based on a nomogram or dosing protocol, which attempts to provide optimal exposures. These clinical dosing protocols often incorporate a fixed dose of an antimicrobial for specific uses or bacterial pathogens. While agents such as the β-lactams are not generally regarded as requiring TDM, we are noting increasing requests for serum concentrations monitoring for this class of compounds in patients who have either not responded to conventional doses or those who are infected with multidrug resistant pathogens which now require high concentrations for eradication. At the time of writing, the authors of this chapter offer clinical assays for cefepime, meropenem and piperacillin/tazobactam, which are frequently utilized across the United States in the clinical care of the above noted patient types. While TDM is generally limited by the availability of reliable assay methods and speed at which these results can be conveyed to the treating clinician, our continuing knowledge in the area of antimicrobial pharmacodynamics may redefine the future application of TDM in an attempt to optimize drug exposure, thereby improving clinical and microbiological outcomes while minimizing potential drug related toxicities.

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CHAPTER 9

Therapeutic drug monitoring of antiepileptic drugs

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ABSTRACT

The aim of the present chapter is to discuss therapeutic drug monitoring (TDM) of the established and newer antiepileptic drugs (AEDs), based on their pharmacokinetic properties and mode of action. Analytical methods for the determination of the serum concentrations of these drugs are also briefly described.

Due to the nature of epilepsy, it remains problematic to monitor AED treatment by direct observations of clinical response in the individual patient. A reasonably good correlation between serum drug concentration and clinical effect together with large inter-individual differences in the rate of drug clearance, are factors that together with the need to identify toxicity, test compliance and elucidate possible clinical interactions would make TDM desirable.

Few studies have been designed primarily to study the serum concentration-effect relationships. As TDM is not widely practiced for the newer AEDs there are no generally accepted target ranges for any of these drugs, and for most a wide range in serum concentration is associated with clinical efficacy. Also, a considerable overlap in drug concentrations related to toxicity and non-response is reported. Further systematic studies designed specifically to evaluate concentration-effect relationships of the new AEDs are urgently needed. Measurements of some of the drugs are undoubtedly of help with individualisation of treatment in selected cases in a particular clinical setting.

There are large differences between individuals in response to particular drugs and drug concentrations, thus the primary role of TDM for both the established and newer AEDs is to identify the individual optimum concentration and thus establish a reference value in that patient.

9.1 INTRODUCTION

The pharmacological treatment of epilepsy has made considerable progress in recent years due to increased knowledge of the clinical pharmacology of the individual

antiepileptic drugs (AEDs). Furthermore, new information on factors affecting clinical response and the development of new AEDs has improved the drug treatment of epilepsy. A number of reports have been published on the kinetics of AEDs, rational prescribing, and therapeutic drug monitoring (TDM) [1–7] and recently, the Commission on AEDs of the International League Against Epilepsy has published guidelines for therapeutic monitoring of AEDs [8].

TDM has made it possible to reveal non-compliance in patients and to study the individual variations in drug utilization occurring normally and as a consequence of disease or otherwise altered physiological states. The initial pharmacokinetic studies on AEDs were started in the 1950s and early 1960s, relating serum levels of phenytoin and phenobarbital to seizure control [9]. Since then, a vast number of studies on AEDs has been published.

Serum level monitoring of the AEDs given is of major importance for optimal therapy since the relationship between serum levels and therapeutic and toxic effects has been clearly established for most drugs, and since the correlation between a dosage related to body weight and the serum level often is poor. However, the therapeutic ranges for serum levels are only guidelines and, although valid for most patients, the optimal serum level for a given patient may lie outside the therapeutic range.

Monitoring AED levels is therefore important for several reasons. For many AEDs, the therapeutic range is narrow, and the safety limit between therapeutic and toxic serum levels is small. Furthermore, treatment with more than one drug at the same time may easily result in drug interactions, changing the elimination of the drugs and the therapeutic outcome in the single patient [10–12]. There is a better possibility of evaluating the real need for combination therapy in some patients and possibly withdrawal of drug therapy in others. Illnesses other than epilepsy can also affect absorption and elimination of the drug and thereby affect the serum level. Furthermore, it will ensure that patients take their medication as prescribed.

There are many reasons related to the disorder for using TDM as a guide to an individually-tailored treatment of patients with epilepsy. This rationale for TDM in epilepsy will be valid whether new or old AEDs are used. However, the value and applicability of TDM will also depend on the pharmacological properties of the drug to be monitored. Pharmacological properties that would make TDM of a specific drug potentially valuable are:

- Pronounced inter-individual variability in pharmacokinetics
- Intra-individual variation in kinetics (due to pharmacokinetic drug interactions)
- Established correlation between the concentration of the drug and its therapeutic or toxic effects
- Narrow therapeutic range

9.1.1 What to measure?

9.1.1.1 Total versus free drug serum levels

Because it is generally assumed that free (i.e. unbound) drug in serum is the therapeutically-active fraction, the most meaningful way of evaluating clinically

significant drug concentrations would be to measure the free drug concentration. However, current analytical methods for quantification of these drugs measure the total concentration. The drug assays do not distinguish between protein-bound and free drug. Consequently, both fractions are measured and expressed as a total drug concentration, which routinely is much easier to measure.

At present the value of routine monitoring of free drug levels is doubtful. More studies of the relationship between free drug levels and clinical effect are required to evaluate this approach. Therefore, free drug level measurements should be restricted to problem cases. It is of importance in patients who clinically fail to respond to AED therapy due to altered protein binding as a consequence of drug interactions or in special physiologic or pathologic states such as pregnancy, hypoalbuminemia, or hepatic or renal failure. This is only valid for highly protein bound AEDs like for example phenytoin and valproate [4,8].

9.1.1.2 Serum levels of drug metabolites

The evaluation of TDM will be more nuanced when the active metabolites of AEDs also can be taken into account. So far, the role of metabolites is not clarified to that extent. One exception is the role of phenobarbital derived from primidone. Primidone is rapidly converted to phenylethylmalonamide (PEMA) and is more slowly converted to phenobarbital. Most physicians routinely prefer to measure and use the concentration of derived phenobarbital during primidone therapy and both primidone and phenobarbital in certain cases only. Routine determination of PEMA is then unnecessary.

Carbamazepine is also a drug with antiepileptic effect of metabolites, the main metabolite being carbamazepine-10,11-epoxide. The epoxide levels may range from 10% to 50% of the serum levels of the parent compound. However, there is no constant relationship between the concentration of the epoxide and carbamazepine, and the ratio varies during the day in the individual patient and with comedication (60, 80). It is recommended to measure both carbamazepine and the epoxide in clinical trials, but at present measuring the metabolite is not indicated for routine monitoring.

Oxcarbazepine is the keto-derivative of carbamazepine and is rapidly and almost completely metabolized to 10,11-dihydro-10-hydroxycarbazepine, and therefore, the serum level of this metabolite is measured.

More studies are needed to evaluate the relationship between clinical efficacy and serum levels of benzodiazepines and their metabolite [4,8].

9.1.2 Analytical aspects

There has been a long-standing interest in therapeutic monitoring of AEDs, and a variety of techniques have been used for measuring these drugs at biological concentrations. Some methods are developed for research purposes, and some are especially designed for routine analysis. Today most laboratories use chromatographic or immunoassay techniques. Chromatographic methods allow simultaneous quantification of several drugs and often also drug metabolites, in a single run, whereas immunoassays were developed for specific determination of one drug at a time.

Special precautions are necessary when metabolites or several drugs are present. For determination of a single AED, it is therefore, essential that the drug can be analysed in the presence of other AEDs and other medication, and that interference from metabolites can be excluded.

Most measurements of AED concentrations are made on plasma, serum, or whole blood samples. For special investigations, urine, cerebrospinal fluid, saliva, or tears may also be useful.

9.1.2.1 Comparison of methods

Several studies are available for comparison of the various techniques used for AED assays. In most cases, there is good agreement. The method of choice for determining AEDs depends on the needs of the individual laboratory.

High performance liquid chromatography (HPLC) is an alternative method that offers several advantages, including fast separation, absence of need for derivatization, good sample stability, and small sample size.

An immunoassay is the choice method in many laboratories engaged in TDM. These assays have several advantages over other currently used methods and are precise, reproducible, and rapid methods for determination of AEDs in micro samples. However, these methods do not have the same screening potential as chromatographic methods, which also can be used for determination of less common AEDs, new drugs, and their metabolites. Furthermore, the reagents used in immunoassays are more costly than those used in chromatographic procedures, but the immunoassays can be performed very rapidly. Thus, it is possible for the physician to know the drug level during examination or when rapid identification, in case of intoxication, is necessary. Small sample volumes are also of considerable importance, in both children and adults, during intensive monitoring. Capillary samples may be used, and a few microliters are sufficient for determination of several AEDs. However, it must be emphasized that the immunoassays are designed for specific determination of a single drug, and therefore, simultaneous drug analysis, including metabolites, which is an advantage of certain chromatographic systems, is not possible [15].

9.1.2.2 Quality control

Optimal analytical quality is important for an effective therapy. Otherwise, the patient can easily be mistreated and the analytical technique, as well as the laboratory, can become discredited. Therefore, participation in both internal and external quality control schemes to ensure reliable results is necessary for any laboratory engaged in routine determination of AEDs. International co-operation on voluntary quality control schemes of AEDs has improved the analytical performance of many laboratories engaged in TDM [16–18].

9.1.3 Blood sampling time

An optimal timing of samples is important. Routinely, the sampling time for individual patients should be standardized to ensure comparable conditions. Ideally, the samples

should be taken while drug fasting in the morning; in outpatients, the morning dose can be postponed a couple of hours to ensure this. When toxic symptoms of a drug are suspected during the day, it is best to draw the sample at the time of maximal serum drug level. However, one has to keep in mind that the therapeutic ranges are based on trough levels [19].

9.1.4 The therapeutic range

For several AEDs there is a more or less well defined therapeutic serum level range (Table 9.1). This range, however, must not be strictly interpreted, because many of the underlying studies are based on patients with severe epilepsy treated with several AEDs. Controlled studies in patients using one drug only for newly diagnosed or moderate epilepsy are scarce. However, most patients are optimally treated with a drug when its steady-state serum levels are maintained within that range [20–22].

TABLE 9.1
PHARMACOKINETIC PARAMETERS OF ANTIEPILEPTIC DRUGS (AEDs).

AEDs	Protein binding (%)	Time to peak level (h)	Time to steady-state (days)	Half-life (h)	Therapeutic range (μmol/L)	Conversion factors (F) (μmol/L = F × μg/mL)
Established AEDs						
Phenytoin	90	2–8	4–8	6–60[a]	40–80	3.96
Phenobarbital	50	2–8	10–35	50–160	50–130	4.31
Primidone	0	1–3	2–5	4–12	30–60	4.58
Carbamazepine	75	4–8[b]	4–7	8–20	15–45	4.23
Valproate	78–94[a]	3–6[c]	2–4	11–20	300–600	
Ethosuximide	0	3–7	5–10	40–60	300–600	7.08
Clonazepam	82	1–3	5–10	20–60	60–220[d]	3.17
Clobazam	85	1–4	4–7	10–30	0.1–1.0	3.33
Desmethylclobazam				35–133	1–10	
Newer AEDs						
Oxcarbazepine[e]	40	6–8	2–3	8–10	50–140	3.96
Vigabatrin	0	1–2	1–2	6–8	6–278	7.74
Lamotrigine	55	1–3	3–15	15–35	10–60	3.90
Felbamate	70	1–4	3–5	14–22	125–250	4.20
Gabapentin	0	2–3	2	5–7	70–120	5.83
Topiramate	15	2–4	4–6	19–23	15–60	2.95
Tiagabine	96	0.5–2	2	4–13	50–250	2.66
Levetiracetam	0	1		7–8	35120	5.88
Zonisamide	60	4–7	5–12	50–70	45–180	4.71

[a] Concentration dependent.
[b] Plain tablets.
[c] Enteric-coated tablets.
[d] nmol/L.
[e] Monohydroxy derivative.
Abbreviations: NE = not established.

In mild epilepsy, seizure control may be attained at "subtherapeutic" levels [23–25]. Then the dose should not be increased just to reach the defined therapeutic serum levels. On the other hand, some patients with more severe epilepsy need "supratherapeutic" levels to achieve optimal effects, but then it is often difficult to balance between clinical effect and side effects. The individual patient may also develop side effects with serum levels within the therapeutic range. It is also possible that the optimal serum levels differ according to seizure type. Thus, the dose should be titrated to the "optimal" or "target" serum level for the individual patient.

9.2 ESTABLISHED ANTIEPILEPTIC DRUGS

In the following, the most common established and newer AEDs are reviewed with emphasize on their pharmacological characteristics, clinical use, and most frequent side effects pertinent to serum level monitoring. Individual characteristics are shown in Tables 9.1–9.3.

9.2.1 Phenobarbital

9.2.1.1 Mechanism of action

Phenobarbital prolongs inhibitory postsynaptic potentials by increasing the mean chloride-channel opening time and hence the duration of GABA-induced bursts of neuronal activity [26].

TABLE 9.2
PUTATIVE MECHANISMS OF ACTION OF ANTIEPILEPTIC DRUGS (AEDs).

AEDs	Sodium channel blocker	GABA-potentiation	Glutamate-modulation	Calcium channel blocker	Carbonic anhydrase blocker
Phenytoin	X				
Carbamazepine	X				
Oxcarbazepine	X				
Valproate	X	X			
Ethosuximide				X	
Barbiturates (phenobarbital, primidone)	X	X		X	
Benzodiazepines (clonazepam, clobazam)		X			
Vigabatrin		X			
Lamotrigine	X		X		
Felbamate	X	X	X		
Gabapentin		X		X	
Topiramate	X	X	X		X
Tiagabine		X			
Levetiracetam		X(?)		X(?)	
Zonisamide	X			X	X

TABLE 9.3
ENZYME-INDUCING AND NON-ENZYME-INDUCING ANTIEPILEPTIC DRUGS (AED).

Enzyme-inducing AEDs	Non-enzyme-inducing AEDs
Carbamazepine	Valproate
Phenytoin	Vigabatrin
Phenobarbital	Gabapentin
Primidone	Lamotrigine
Oxcarbazepine[1]	Levetiracetam
Topiramate[1]	Clonazepam
Felbamate[1]	Clobazam
	Ethosuximide
	Tiagabine
	Zonisamide

[1] Weak enzyme inducer.

9.2.1.2 Pharmacokinetics and drug interactions

The bioavailability is almost complete, peak serum levels are usually attained 2–8 h after dose intake. Phenobarbital is about 50% bound to serum proteins. The metabolism of phenobarbital is particularly slow, and variations in the steady-state concentration are therefore small within individual patients on a constant dosage. Approximately 20–40% of a given dose of phenobarbital is excreted unchanged in the urine, the rest is slowly metabolized in the liver. The excretion of phenobarbital is considerably higher when the pH of the urine is alkaline.

The half-life of phenobarbital in serum is 50–160 h in adults; in children it is shorter (30–70 h). The elimination follows first-order kinetics. Pharmacokinetic interactions with phenobarbital are seen frequently. Phenobarbital has enzyme-inducing properties and may thus alter the effect of other medication, and other drugs may also modify the effect of phenobarbital [27–29].

9.2.1.3 Clinical use and side effects

Phenobarbital has been used for treating partial and generalized tonic-clonic seizures, and has an efficacy comparable to that of phenytoin and carbamazepine [30]. The drug can also be used in the treatment of neonatal seizures.

Phenobarbital causes few systemic side effects, but its use is limited mainly due to the high rate of sedation and cognitive impairment. Being a barbiturate, phenobarbital can

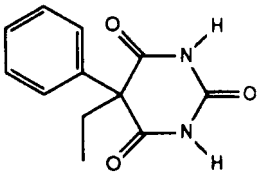

Fig. 9.1. Phenobarbital.

be associated with physical dependence, and withdrawal can produce a variety of abstinence symptoms and provoke seizures. Treatment with phenobarbital increases the risk of connecting tissue disorders [30].

9.2.1.4 Serum levels and therapeutic effect

The therapeutic ranges for the established AEDs are well recognised. For phenobarbital most patients are optimally treated with serum levels within a range of 50–130 μmol/L [1,3,4,9].

9.2.1.5 Analytical methods

A number of gas chromatographic, liquid chromatographic and immunoassays are available for determination of phenobarbital in biological samples, often simultaneous with other AEDs [15,31–37].

9.2.2 Phenytoin

9.2.2.1 Mechanism of action

Phenytoin appears to act by inducing voltage- and user-dependent blockade of sodium channels [38].

9.2.2.2 Pharmacokinetics and drug interactions

After oral administration, the absorption is rather slow and varies with the chemical form and pharmaceutical formulation employed. Peak serum levels are usually attained within 4–8 h. The serum protein binding is approximately 90%. The metabolism of phenytoin is slow, and the serum concentration is therefore relatively stable during long-term treatment, but between-patient variations do occur, depending mainly on genetic differences, but also on age, sex, and body weight.

The half-life of phenytoin in serum is usually in the order of 15–20 h, but may vary from 6–60 h. The metabolism of phenytoin, however, is limited by the fact that, in many patients, the enzyme system involved becomes saturated in the therapeutic range of concentrations. The half-life, and thus the time needed to attain steady state, increases

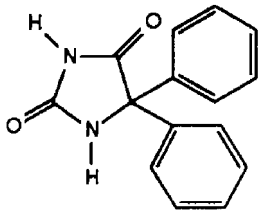

Fig. 9.2. Phenytoin.

with concentration. Therefore, stepping up the dose only slightly may lead to a large increase in the serum concentration. The metabolism of phenytoin follows zero-order (Michaelis-Menten) rather than first-order kinetics, and for this reason it would seem inappropriate to estimate any particular half-life of phenytoin. There are considerable individual differences in the phenytoin concentrations that lead to saturation of the drug-metabolizing enzyme system.

Interactions between phenytoin and other drugs occur rather frequently. A particularly important feature of phenytoin in this respect is that it has enzyme-inducing properties which can stimulate the metabolism of other drugs, resulting in lower serum concentrations and reduced therapeutic efficacy. The main interactions are probably those which lead to an inhibition of phenytoin metabolism. Since phenytoin follows non-linear kinetics by virtue of its saturable metabolism, even a slight inhibition on metabolism may lead to a considerable increase in the serum concentration and pharmacological effect [27,29,39].

9.2.2.3 Clinical use and side effects

Phenytoin is effective in the treatment of partial and generalized tonic-clonic seizures, but has no documented effect against absence or myoclonic seizures, nor in the Lennox-Gastaut syndrome [30].

Since its introduction, a long list of side effects has emerged including chronic toxicity affecting the nervous system, connective tissues, the endocrine system, hematologic effects and teratogenicity [40]. The acute concentration-dependent neurotoxicity with cerebellar signs and alteration of higher cortical functions, sometimes with exacerbation of epileptic seizures, can probably be avoided with proper drug monitoring. Idiosyncratic reactions are rare, but gingival hyperplasia can be seen in 50% of the patients. Intravenous phenytoin, and recently, also fosphenytoin, has an important role in the treatment of status epilepticus.

9.2.2.4 Serum levels and therapeutic effect

Due to the special pharmacokinetic features of phenytoin, accurate titration and use of this drug is very difficult without serum level monitoring. For most patients the optimal serum level is within a range of 40–80 μmol/L [1,3,4,9,21,22,41].

9.2.2.5 Analytical methods

Phenytoin can be measured by a variety of chromatographic methods and immunoassays [15,31–37].

9.2.3 Primidone

9.2.3.1 Mechanism of action

Primidone is rapidly metabolized to phenobarbital, but probably has an antiepileptic effect of its own [42].

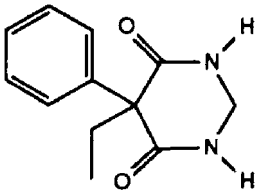

Fig. 9.3. Primidone.

9.2.3.2 Pharmacokinetics and drug interactions

Following intake of single doses, maximal serum concentrations are attained after 2–5 h, somewhat later during long-term treatment. The bioavailability relative to intravenous administration is unknown since a parenteral formulation is not available. Primidone is bound to serum proteins to a small extent only.

The metabolism of primidone follows first-order kinetics with a half-life of 4–12 h. Primidone is rapidly metabolized to phenylethylmalonamide (PEMA) and more slowly to phenobarbital. PEMA is found in serum 1–2 h after intake of primidone, and it attains maximal serum concentrations after 7–8 h. Since PEMA is not bound to proteins, the concentrations in serum and cerebrospinal fluid are equal. The half-life in serum is 29–39 h.

Phenobarbital is not found in serum until 24–28 h after intake of primidone, and the same concentrations in serum are attained as with standard doses of phenobarbital. There are no quantitative data for urinary excretion of primidone or PEMA.

Since one metabolite of primidone is phenobarbital, the same type of drug interactions as for phenobarbital can be expected. In addition, the comedication of other AEDs like phenytoin, carbamazepine, and phenobarbital may stimulate the metabolism of primidone. Instead of a reduced effect, this may give an increased effect since the long half-life of phenobarbital leads to accumulation of phenobarbital [29].

9.2.3.3 Clinical use and side effects

Primidone possesses efficacy for partial and secondarily generalized seizures [30], and is also claimed to show efficacy for juvenile myoclonic epilepsy [43], but convincing evidence is lacking for the primary generalized epilepsies. Primidone has two active metabolites, phenobarbital and PEMA, but primidone probably has an antiepileptic effect of its own, and also side effects such as dizziness and nausea [42]. In the large comparative Veterans Administration Study [30], retention was significantly worse for primidone even compared to phenobarbital, not because of lacking efficacy but the troublesome side effect profile.

9.2.3.4 Serum levels and therapeutic effect

Since primidone is metabolized to phenobarbital, most often the phenobarbital concentration is used as a guide to therapy. Primidone serum levels are usually in the range of 30–60 μmol/L [44].

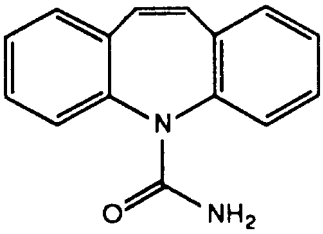

Fig. 9.4. Carbamazepine.

9.2.3.5 Analytical methods

There are several chromatographic methods and immunoassays available for determination of primidone [15,31–37].

9.2.4 Carbamazepine

9.2.4.1 Mechanism of action

Carbamazepine acts by preventing repetitive firing of action potentials in depolarized neurones through voltage- and user-dependent blockade of sodium channels [45].

9.2.4.2 Pharmacokinetics and drug interactions

Controlled-release formulations are absorbed more slowly than conventional tablets and produce more stable serum levels during the day and at night, even when given twice daily as compared with conventional tablets given three times daily. Peak serum concentrations are usually attained within 4–8 h with plain tablets. Carbamazepine is approximately 70–80% bound to serum proteins.

The metabolism of carbamazepine follows first-order kinetics and may vary considerably, resulting in a poor correlation between the dose and the serum concentration. The half-life during long-term treatment is considerably shorter (8–20 h) than following a single dose, due to autoinduction. This may result in "time-dependent" kinetics as the elimination increases gradually the first 3–6 weeks of treatment, necessitating a dose increase at a later stage to maintain the serum concentration. Autoinduction probably depends on the size of the dose, and on whether the patient is also being treated with other enzyme inducers. Carbamazepine is almost completely metabolized, the main metabolite being carbamazepine-10,11-epoxide. In animal studies this epoxide also has an antiepileptic effect of the same order as carbamazepine, and it is assumed also to contribute to the pharmacological effect in man.

Like phenytoin and phenobarbital, carbamazepine is a potent enzyme inducer influencing the metabolism of other drugs. In addition, a number of drugs may change the pharmacokinetic properties of carbamazepine [4,29,39].

9.2.4.3 Clinical use and side effects

Carbamazepine is used in the treatment of partial seizures, with or without secondary generalization, and generalized tonic-clonic seizures. The drug is not effective, and may even be deleteroius, in patients with absence and or myoclonic seizures [46].

Concentration-dependent side effects are dominated by drowsiness and cerebellar signs. Cognitive impairment is rarely a problem in non-toxic doses. Skin rash is seen in 5–15% of patients treated with carbamazepine. Haematological, dermatological, endocrinological (hyponatremia is not uncommon), hepatic and cardiac side effects, in rare instances severe, have also been reported [47].

9.2.4.4 Serum levels and therapeutic effect

Carbamazepine also has metabolites that have antiepileptic activity, however, only the parent compound is measured routinely. Common therapeutic serum concentrations of carbamazepine are in the range of 15–45 μmol/L. [1,3,4,24,48–53].

9.2.4.5 Analytical methods

Carbamazepine can be measures by a number of immunoassays and chromatographic techniques [15,31–37]. The latter also allow for determination of carbamazepine metabolites that can be of interest in certain cases.

9.2.5 Valproate

9.2.5.1 Mechanism of action

VPA probably exerts its effects through several distinct mechanisms. VPA increases the inhibitory neurotransmittor GABA by inhibition of the metabolism of GABA [54,55]. VPA does not affect GABA receptors, but glutamatergic NMDA receptors seem to be inhibited, and in addition the levels of monoamines as serotonin are affected by VPA [55]. Inhibition of voltage-dependent sodium channels which is a common mechanism for many AEDs, has also been suggested for VPA. This statement has, however, lately been questioned, and Löscher [55] concluded that at clinically relevant concentrations, inhibition of sodium currents does not seem to contribute considerably to the antiepileptic action of VPA.

9.2.5.2 Pharmacokinetics and drug interactions

The bioavailability is almost complete for all formulations in common use. Usually, conventional tablets and solutions give peak serum concentrations 1–2 h after

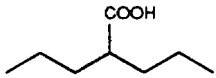

Fig. 9.5. Valproate.

administration, enteric coated tablets after 3–6 h, and sustained-release tablets after 10–12 h. Rectal application offers an alternative mode of administration when the drug cannot be given orally.

Valproate is 78–94% bound to serum proteins. The binding is concentration-dependent at serum levels above the therapeutic range. The protein-bound fraction is less in patients with renal disease, chronic liver disease, and other conditions with low albumin concentration.

Due to interindividual differences in metabolism there is a poor correlation between the dose of valproate and the serum concentration, especially in patients who are also receiving other AEDs. The half-life of valproate in serum is 11–20 h, and the substance is almost completely metabolized.

Pharmacokinetic interactions with valproate are seen frequently, and several are of major clinical significance, for instance the inhibition of phenobarbital and lamotrigine metabolism. While valproate inhibits the metabolism of a number of drugs, its own metabolism may also be stimulated be the enzyme-inducing activity of other AEDs, causing the valproate concentration to decrease. It is noteworthy that valproate does not diminish the effect or oral contraceptives, as is the case with enzyme-inducing AEDs [4,28,29].

9.2.5.3 Clinical use and side effects

Valproate is effective in patients with all types of epileptic seizures, especially in those with idiopathic generalized epilepsy [56]. Gastrointestinal side effects are seen in up to 25% of the patients, probably less if the enteric-coated formulation is used. Concentration-dependent tremor occurs in about 10% of the patients treated with valproate. Weight gain is not uncommon and hair loss [57], sometimes with regrowth of curly or differently coloured hair may occur. Valproate therapy has been associated with fatal hepatotoxicity, predominantly when used as part of polytherapy in young children, but with a rapidly decreasing incidence with age and when used as monotherapy [58]. Valproate can probably cause amenorrhea and polcystic ovary syndrome [59].

9.2.5.4 Serum levels and therapeutic effect

Most patients are optimally treated with serum concentrations of valproate within a range of 300–600 μmol/L. For valproate it is of special importance to draw drug fasting blood samples in the morning due to the wide fluctuations in the serum levels [60–62].

9.2.5.5 Analytical methods

Various chromatographic methods and immunoassays are available for measurement of valproate in biological samples [15,34,36,37,63–66].

9.2.6 Ethosuximide

9.2.6.1 Mechanism of action

Ethosuximide acts by reducing low-threshold, transient, voltage-dependent calcium conductance in thalamic neurones [67].

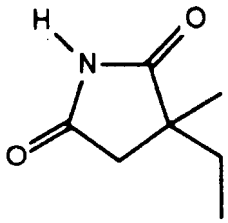

Fig. 9.6. Ethosuximide.

9.2.6.2 Pharmacokinetics and drug interactions

The gastrointestinal absorption is relatively rapid. Maximal serum concentrations are attained 3–7 h after intake during long-term treatment, somewhat earlier after a single dose. The bioavailability is similar for capsules and suspension, but the suspension is more rapidly absorbed. The protein binding of ethosuximide is insignificant. In animals the concentration in brain tissue and in serum is comparable, but this has not been investigated in man. Ethosuximide is evenly distributed to various organs and body fluids.

The metabolism of ethosuximide is slow, and the serum concentration therefore, is relatively stable even if the drug is given only once a day. The correlation between the dosage in mg/kg body weight and the serum concentration is rather good in comparable groups. The interindividual differences, however, are so great that it is not possible to predict the serum concentration following a certain dosage in a patient. The elimination of ethosuximide follows first-order kinetics, and the half-life in adults is 40–60 h, in children about 30 h. Ethosuximide is metabolized to a great extent, and only 12–20% of a given dose is found in the urine as native substance. The molecule contains an asymmetric C atom, and the commercial preparation is a mixture of the two isomers being metabolized with the same rate. The main metabolites are 2-(1-hydroxyethyl)–2-methylsuximide, 2-(2-hydroxyethyl)2-methylsuximide and a ketone derivate, 2-acetyl-2-methylsuximide. None of the metabolites have antiepileptic activity.

The excretion of ethosuximide is rather slow, and since the protein binding is minimal, this suggests that there is a considerable tubular reabsorption. During long-term treatment, about 60–80% of the dose can be traced in urine as ethosuximide and free conjugated metabolites.

Interactions with ethosuximide are rarely seen. The main interaction is with valproate which may increase the serum concentration of ethosuximide, probably by an inhibition of the metabolism which in single cases may lead to intoxication. In contrast to many other AEDs, there is no basis for ethosuximide having enzyme-inducing properties [28].

9.2.6.3 Clinical use and side effects

Ethosuximide has a clearly defined role in the treatment of primarily generalized absence seizures, typically seen in childhood absence epilepsy [68]. It has no proven effect against partial, tonic-clonic or other seizure types. Its anti-absence effect is reported to equal that of valproate [69].

The most commonly reported dose-dependent side effects, in up to 40% of the patients, are gastrointestinal discomfort, drowsiness, dizziness, and headache, but controlled trials are sparse [70].

9.2.6.4 Serum levels and therapeutic effect

In most patients, the therapeutic range of serum levels for ethosuximide is 300–600 μmol/L [1,3,4,71].

9.2.6.5 Analytical methods

A number of methods can be applied for analysis of ethosuximide [15,31–37,72].

9.2.7 Clonazepam

9.2.7.1 Mechanism of action

The mechanism of action of clonazepam involves GABA potentiation [73].

9.2.7.2 Pharmacokinetics and drug interactions

Following oral intake, clonazepam is rapidly absorbed and maximal serum concentrations are usually attained within 1–3 h, sometimes after 9 h. The bioavailability is on average 80–90%. The protein binding in serum is about 82%. Clonazepam is rapidly distributed to various organs and tissue.

As with other drugs which are metabolized to a great extent, the relationship between the doses and the serum concentrations is poor for clonazepam. The half-life of clonazepam is 20–60 h. Clonazepam is mainly metabolized by a reduction to 7-amino-clonazepam and by acetylation to 7-acetamido-clonazepam. The metabolism follows first-order kinetics as for diazepam. Hydroxy-metabolites have also been identified. None of the metabolites appears to have antiepileptic effect. The excretion of unaltered clonazepam in urine is usually less than 1% of the given dose, but the above mentioned metabolites account for about 25% as free or conjugated compounds.

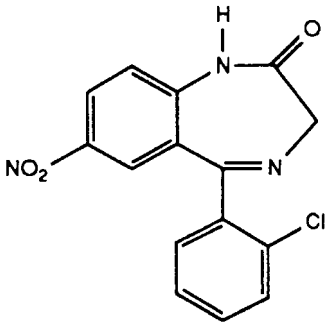

Fig. 9.7. Clonazepam.

As for diazepam several AEDs like phenytoin, phenobarbital, primidone, and carbamazepine may stimulate the metabolism of clonazepam, resulting in lower serum concentrations and a decreased clinical effect. The effect of benzodiazepines on the metabolism of other AEDs is not fully established. Clonazepam does not seem to alter the serum concentration of phenytoin, phenobarbital or carbamazepine [73,74].

9.2.7.3 Clinical use and side effects

Clonazepam has a broad spectrum of antiepileptic activity. Its efficacy has been demonstrated in double-blind studies for absences [75], atypical absences, myoclonus, and partial and tonic-clonic seizures [76]. Problems with clonazepam are a high incidence of side effects, mainly drowsiness and dizziness, development of tolerance, and frequent unwanted effects related to discontinuation of the drug [73]. These include exacerbation of seizures, insomnia, restlessness and dysphoria.

9.2.7.4 Serum levels and therapeutic effect

The documentation for clonazepam is rather poor. Development of pharmacodynamic tolerance to the sedative effects of barbiturates and also to clinical efficacy of benzodiazepines makes the interpretation of serum levels even more complicated. A therapeutic range of 60–220 μmol/L has been suggested [74,77].

9.2.7.5 Analytical methods

Various methods are available for quantification of clonazepam [4,78–80].

9.2.8 Clobazam

9.2.8.1 Mechanism of action

Clobazam is a 1,5-substituted benzodiazepine. The mechanism of action is difficult to define since the drug has various effects. Probably, the major antiepileptic effects are

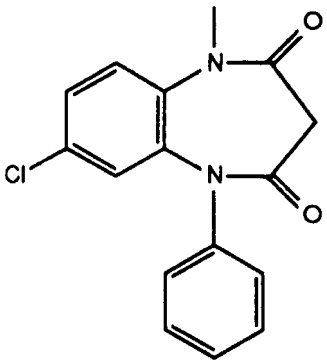

Fig. 9.8. Clobazam.

related to binding to one or more specific GABA-receptors, increasing GABA-mediated inhibition [81].

9.2.8.2 Pharmacokinetics and drug interactions

The bioavailability of clobazam is good, and after single doses peak serum levels are attained within 1–4 h. The protein binding is 85%. The mean half-life is 18 h, ranging from 10–30 h. The principle metabolite, N-desmethylclobazam, has a much longer half-life (35–133 h), and after steady-state has been reached, the serum levels of the metabolite are at least 8 times higher than those of the parent compound [82]. The elimination follows first order kinetics. It appears to be a linear relationship between the dose and the serum level of clobazam as well as of the metabolite, but with major interindividual differences.

In general, pharmacokinetic changes of major AEDs are uncommon [83].

9.2.8.3 Clinical use and side effects

Clobazam is effective in the treatment of patients with partial and generalized seizures [84]. The main metabolite, N-desmethylclobazam, also possesses antiepileptic properties with about one-quarter the potency of the parent compound.

Sedation and dizziness are the most prominent side effects, other side effects are mood changes, with occational irritability, depression, aggression, and disinhibition, especially in patients with mental retardation. Development of tolerance to the antiepileptic effect is often a problem, but can sometimes be overcome by increasing the dose, and may be prevented by giving the drug intermittently.

9.2.8.4 Serum levels and therapeutic effect

In patients treated with clobazam therapeutic serum concentrations are in the order of 0.1–1.0 μmol/L for the parent drug and 1–10 μmol/L for the metabolite, desmethylclobazam [85,86].

9.2.8.5 Analytical methods

Various chromatographic methods have been employed for analysis of clobazam [15,79,87].

9.3 NEWER ANTIEPILEPTIC DRUGS

9.3.1 Oxcarbazepine

9.3.1.1 Mechanism of action

Oxcarbazepine is the keto-derivative of carbamazepine and was developed by introducing minimal changes in the structure of carbamazepine to change the metabolism to avoid the epoxide metabolite. At least part of the antiepileptic effect of oxcarbazepine seems to be based on interactions with sodium and potassium channels [88].

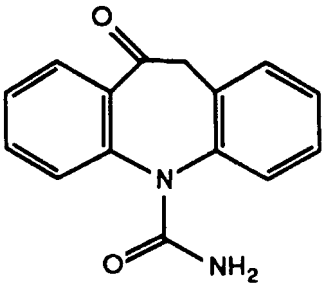

Fig. 9.9. Oxcarbazepine.

9.3.1.2 Pharmacokinetics and drug interactions

In man, oxcarbazepine is rapidly and almost completely metabolized to 10,11-dihydro-10-hydroxycarbamazepine (MHD), which has independent antiepileptic properties. Peak serum levels of the metabolite are attained at about 8 h. The half-life is in the order of 8–10 h. The protein binding of oxcarbazepine is about 67%, whereas that of the metabolite is only about 38% [4,89].

Unlike the established AEDs, oxcarbazepine and its major active metabolite are cleared from the human body mainly by non-oxidative processes undependent of the cytochrome P-450 system. Compared with carbamazepine there is less risk that oxcarbazepine will influence the kinetics of other drugs. Oxcarbazepine does not modify the anticoagulant effect of warfarin, whereas a clinically significant reduction of contraceptive drug levels and efficacy have been observed. Furthermore, in contrast to carbamazepine, oxcarbazepine does not influence its own metabolism after repeated administration [90].

9.3.1.3 Clinical use and side effects

Oxcarbazepine exerts antiepileptic effects similar to carbamazepine [91]. Severe side effects are less pronounced with oxcarbazepine compared to carbamazepine, particularly rash [92]. When prescribing oxcarbazepine in patients who have developed rash on carbamazepine, the risk of cross-allergy is about 25%. Oxcarbazepine may cause hyponatremia more frequently than carbamazepine, but this effect is usually mild and asymptomatic.

9.3.1.4 Serum levels and therapeutic effect

The target range of serum levels for the active metabolite of oxcarbazepine, MHD, has not yet been well defined. Similar concentrations have been noted in groups of patients with optimal treatment and in non-responders [93,94]. Side effects were more frequent at serum concentrations of 140 to 160 μmol/L). A retrospective analysis of sera from 947 patients treated with oxcarbazepine resulted in mean MHD serum concentration of 20 μmol/L with a wide range of 12–160 μmol/L [92], but the relation to effects and toxicity was not analyzed in detail.

A tentative target range for MHD of 50–140 μmol/L has been suggested [95].

9.3.1.5 Analytical methods

There are numerous chromatographic methods for the measurement of MHD in sera [96–99]. More recently, with the enantiomeric characterisation of MHD, enanatioselective liquid chromatographic techniques have also become available [100–102].

9.3.2 Vigabatrin

9.3.2.1 Mechanism of action

Vigabatrin, a synthetic GABA derivative, is an enzyme-activated, irreversible inhibitor of GABA-transaminase and results from a systematic search into possible ways of increasing GABA-ergic inhibition through interference with GABA metabolism. The drug is a racemic mixture, only the S(+)-enantiomer being pharmacologically active [103].

9.3.2.2 Pharmacokinetics and drug interactions

After oral administration of vigabatrin peak serum levels are attained within about 2 h. Vigabatrin enters CSF and produces dose-dependent increases in GABA concentrations in CSF. Vigabatrin is not bound to serum proteins, and no metabolites have been identified in man. The terminal half-life is between 6–8 h. Vigabatrin is excreted unchanged primarily in the urine, and thus, patients with renal impairment are likely to require lower doses. At clinical doses there is pharmacokinetic dose-linearity.

Due to the irreversible mode of action of the drug, the serum half-life bears practically no relationship to the duration of pharmacological effect. Because of the long-lasting GABA-transaminase inhibition, the antiepileptic effect of vigabatrin long outlasts its presence in serum.

No drug interactions have been observed with vigabatrin and other AEDs or other drug classes except for a decrease in phenytoin serum levels in some patients that was of no clinical significance. [104].

9.3.2.3 Clinical use and side effects

Vigabatrin has shown efficacy as add-on in the treatment of partial seizures and secondary generalized seizures [103]. The drug is also effective in children with infantile spasms.

Vigabatrin may worsen myoclonic seizures. Immunologic/allergic side effects seem to be rare, but sedation, dizziness, headache, depression and weight gain are reported. Psycosis has been reported in 3–6% of patients. Recently, it has been discovered that

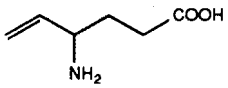

Fig. 9.10. Vigabatrin.

irreversible visual field defects occur in about one-third of treated patients [105], and this has resulted in much less prescribing of the drug.

9.3.2.4 Serum levels and therapeutic effect

In contrast to most other AEDs, monitoring of serum levels of vigabatrin is not suitable as a guide to therapy due to its mechanism of action. For checking compliance a tentative range of 6–278 μmol/L has been suggested [95,106,107].

9.3.2.5 Analytical methods

Several liquid chromatographic methods are reported for measuring vigabatrin in serum [108–114].

9.3.3 Lamotrigine

9.3.3.1 Mechanism of action

Lamotrigine, a phenyltriazine derivative, is unrelated to currently available AEDs and was developed in an effort to find antifolate drugs. Such a search was based on the hypothesis that compounds which interfere with folate metabolism may be antiepileptic. However, lamotrigine has only weak antifolate activity, and most likely acts via a use-dependent blockade of voltage-sensitive sodium channels and consequently, inhibits the release of excitatory amino acids, especially glutamate. In animal models lamotrigine shows a spectrum of activity similar to that of phenytoin [115].

9.3.3.2 Pharmacokinetics and drug interactions

Lamotrigine is readily absorbed from the gastrointestinal tract, and peak serum levels are usually attained within 1–3 h. The bioavailability is virtually complete. The serum protein binding is about 55%. In monotherapy the half-life is 15–35 h. Enzyme-inducing AEDs shorten the half-life to an average of 15 h. Conversely, lamotrigine metabolism is inhibited by valproate, prolonging the half-life to an average of 60 h.

When VPA is given in addition to lamotrigine monotherapy, the serum level of lamotrigine may be doubled or more since the half-life of lamotrigine is considerably increased as mentioned above. Regarding drug interactions, lamotrine does not appear to affect significantly the pharmacokinetics of concurrently administered AEDs, although there is conflicting evidence regarding a possible influence on the serum levels

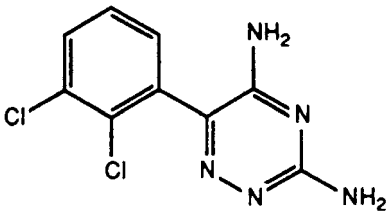

Fig. 9.11. Lamotrigine.

of carbamazepine-epoxide. If side effects occur when a patient is treated with lamotrigine as add-on to carbamazepine, these side effects will usually disappear if the dosage of carbamazepine is reduced. Lamotrigine does not interfere with the efficacy of oral contraceptive agents or warfarin [116]. On the other hand, oral contraceptives can lower the serum concentration of lamotrigine by 50% [117].

9.3.3.3 Clinical use and side effects

Lamotrigine is effective in the treatment of partial seizures with or without secondary generalization, and in generalized tonic-clonic seizures [118]. There is also growing evidence of efficacy for absence, atypical absence, myoclonic and atonic seizures and for the Lennox-Gastaut syndrome.

The side effects of lamotrigine are dominated by rash, headache, nausea and vomiting, diplopia, dizziness, ataxia and tremor [119]. The risk of development of severe dermatological hypersensitivity reactions is a problem, especially in patients who are treated with a too high starting dose, rapid dose escalation, and comedication with the enzyme inhibitor valproate.

9.3.3.4 Serum levels and therapeutic effect

Based on comparative efficacy data of lamotrigine with that of phenytoin in animal models and the lamotrigine concentrations observed during its clinical evaluation, the target range for lamotrigine was initially set at 4–16 μmol/L. However, clinical experience now suggests that this range is too low since doses and serum concentrations in excess of those achieved in the clinical trials of lamotrigine are tolerated with good clinical effect. There is growing evidence of the importance of measuring lamotrigine serum concentrations, and a target range of 10–60 μmol/L is now suggested [95,120].

9.3.3.5 Analytical methods

Many liquid chromatographic methods have been described for the assay of lamotrigine [121–125].

9.3.4 Gabapentin

9.3.4.1 Mechanism of action

Gabapentin is a new chemical compound designed as a structural analog of GABA that is effective in the treatment of partial seizures. In contrast to GABA, gabapentin readily

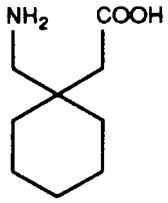

Fig. 9.12. Gabapentin.

penetrates the blood-brain barrier. In man, gabapentin has been demonstrated to increase GABA concentrations [126]. Most probably the mechanism of action is related to events modulated through its interaction with a receptor thought to be associated with the L-system amino acid carrier protein. However, the primary mechanism of action remains to be defined [127].

9.3.4.2 Pharmacokinetics and drug interactions

Gabapentin is rapidly absorbed from the gastrointestinal tract, and peak serum levels are attained 2–3 h after a single dose. The bioavailability is, however, reduced up to 24% if certain antacids are taken concomitantly. The absolute bioavailability is at least 60%. Absorption kinetics are dose-dependent, possible due to a saturable transport system. The drug is not bound to serum proteins. Gabapentin is not metabolized and is eliminated unchanged by the kidney. The elimination half-life is about 5–7 h after a single oral dose. Renal impairment reduces drug clearance and increases the serum levels of gabapentin in a linear fashion.

Gabapentin is devoid of enzyme-inducing properties. Since the drug is not protein bound and almost completely eliminated by renal excretion, gabapentin does not appear to be involved in significant pharmacokinetic interactions with other drugs. It is unlikely that gabapentin reduces the efficacy of oral contraceptives [127,128].

9.3.4.3 Clinical use and side effects

Gabapentin is effective in the treatment of patients with refractory partial epilepy, and its antiepileptic effect is probably dose-related. The doses in these studies ranged between 600 and 1800 mg/day, but in open studies doses up to 3600 mg/day have been used.

The most common side effects are somnolence and dizziness, to a lesser extent ataxia, fatigue, headache, tremor and diplopia. Psychometric testing has not revealed cognitive impairment [127,129].

9.3.4.4 Serum levels and therapeutic effect

Wide ranges of gabapentin serum concentrations have been reported to be associated with optimal seizure control. A tentative target range of 70–120 μmol/L has been suggested [95,130].

9.3.4.5 Analytical methods

Various gas chromatographic and liquid chromatographic procedures are reported for the determination of gabapentin in serum [112,113,131–133], and more recently mass spectroscopic detection has been employed with both gas and liquid chromatography [134,135].

9.3.5 Topiramate

9.3.5.1 Mechanism of action

Topiramate, a sulfamate-substituted monosaccharide, has multiple modes of action including modulation of voltage-dependent sodium channels, potentiation of GABAergic inhibition at a novel site on the GABAA receptor and a possible action at non-N-methyl-D-aspartate (NMDA) receptors [136]. The drug is also a weak inhibitor of carbonic anhydrase although this effect is unlikely to contribute to its anticonvulsant properties.

9.3.5.2 Pharmacokinetics and drug interactions

After oral administration of topiramate peak serum levels are attained within 2–4 h and are linearly related to dose, but with large interindividual variation. The binding to serum proteins is about 15%, and the half-life of is 19–23 h. Topiramate is mainly excreted unchanged in urine. No significant interactions have been reported with regard to the effect of topiramate on carbamazepine, phenytoin, or valproate, although some patients attain higher phenytoin serum levels when topiramate is added to their drug regime. On the contrary, phenytoin and carbamazepine significantly lower the serum levels of topiramate. Topiramate may reduce serum oestrogen levels in patients receiving oral contraceptives [137,138].

9.3.5.3 Clinical use and side effects

Topiramate is used in the treatment of partial seizures [139]. The efficacy in primary generalized epilepsy in adults and children, and also in Lennox-Gastaut syndrome, is promissing.

Side effects have been frequent in the first studies, but are claimed to become less if topiramate is started with a low dose and slow titration phase. Side effects are mainly fatigue, headache, weight loss, dizziness, emotional lability, thinking abnormal, depression, confusion and anxiety. The risk of renal calculi is 1–2%.

9.3.5.4 Serum levels and therapeutic effect

A wide range of doses and serum concentrations of topiramate has been associated with optimal clinical response [107,140]. Recently, Christensen et al. [141] reported the results of a concentration-response trial of topiramate treatment investigated in a triple-blind, concentration-controlled, parallel-group design, randomising 65 patients to one of

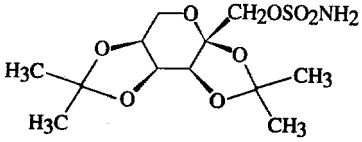

Fig. 9.13. Topiramate.

three pre-specified serum levels (low = 6 μmol/L, medium = 31 μmol/L and high- = 56 μmol/L). Patients assigned to the medium serum concentration had the best outcome with regard to seizure reduction and those in the medium and high groups experienced more adverse events than patients in the low group.

A tentative target range of 15–60 μmol/L is suggested even though most patients will probably have serum concentrations in the low to mid range with the dose regimen used today.

9.3.5.5 Analytical methods

Capillary and gas chromatographic methods have described the determination of topiramate [142–144] and also chromatographic methods with MS detection [145–148]. An immunoassay is also commercially available [149].

9.3.6 Felbamate

9.3.6.1 Mechanism of action

Felbamate is a dicarbamate derivative. Its activity is mediated by several mechanisms, including inhibition of voltage-sensitive sodium and calcium channels, reduction of glutamergic transmission through modulation of NMDA receptors, and potentiation of GABA transmission [150].

9.3.6.2 Pharmacokinetics and drug interactions

After oral administration, felbamate is readily absorbed from the gastrointestinal tract, and peak serum levels are attained with 1–4 h. The binding to serum proteins is 24–35. The elimination half-life is about 20 h when the drug is given alone, decreasing to about 14 h in patients who are also taking enzyme-inducing AEDs.

Several clinically important drug interactions are observed when felbamate is used concomitantly with other AEDs. Phenytoin and valproate serum levels can increase by

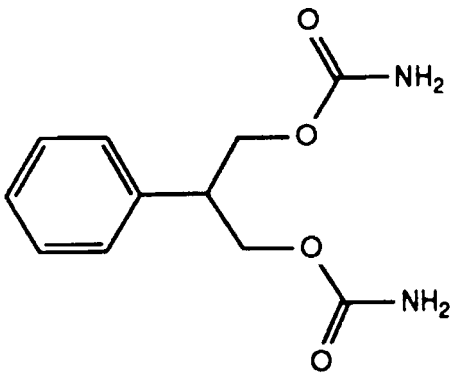

Fig. 9.14. Felbamate.

20–40% or more when felbamate is added. In contrast, carbamazepine serum levels decrease by about 20% when felbamate is added. Felbamate serum levels may be lowered by the concomitant use of other enzyme-inducing AEDs [128,151].

9.3.6.3 Clinical use and side effects

Felbamate possesses efficacy in the treatment of complex partial seizures [152] and is also effective in the Lennox-Gastaut syndrome [153].

The most common side effects are anorexia, vomiting, insomnia, nausia, and headache. Recent observations of fatal aplastic anemia and hepatotoxicity have halted a wider use of felbamate.

9.3.6.4 Serum levels and therapeutic effect

Although overall there is a lack of data from prospective studies, seizure control seems to be related to serum concentrations. Indeed monitoring felbamate concentrations may be particularly useful since serum concentrations are not easily predicted from administered dosages, and also felbamate appears to have a narrow therapeutic window. A tentative target range for optimum treatment ranging from 125 to 250 μmol/L has been suggested [95,152,154,155].

9.3.6.5 Analytical methods

A number of liquid chromatographic methods [156–159] and gas chromatographic [160] methods are available to measure felbamate in biological samples.

9.3.7 Tiagabine

9.3.7.1 Mechanism of action

Tiagabine consists of nipecotic acid linked by an aliphatic chain to a lipophilic anchor and is structurally different from other AEDs. Its mode of action is considered to be mediated by inhibition of GABA reuptake from the synaptic cleft. The lipophilic portion of the tiagabine molecule permits penetration across the blood-brain barrier. Tiagabine is a potent AED in several rodent models [161].

9.3.7.2 Pharmacokinetics and drug interactions

After oral administration the absorption of tiagabine is very rapid with peak serum levels attained within 1 h. The protein binding is very high (96%). The elimination half-

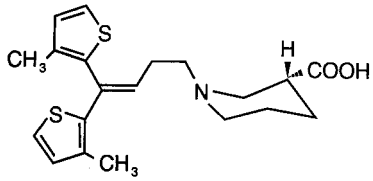

Fig. 9.15. Tiagabine.

life is quite variable, ranging from 4–13 h with an average of about 7 h and is even shorter in patients also treated with enzyme-inducing drugs. Tiagabine is extensively metabolized. There is no evidence of dose dependent kinetics.

Tiagabine has a low potential for causing drug interactions as there are no clinically relevant interactions with other drugs, including oral contraceptives. However, carbamazepine and phenytoin induce the metabolism of tiagabine [162].

9.3.7.3 Clinical use and side effects

Tiagabine is used in the treatment of partial epilepsy with or without secondary generalization [163]. The most prominent side effects are dizziness, asthenia, nervousness, tremor, diarrhea, depression and emotional lability [164].

9.3.7.4 Serum levels and therapeutic effect

Although the relationship between dose and serum concentration is linear, there are large intra-individual and inter-individual variations in serum concentration. Also, there are large inter-dose fluctuations in serum concentration (attributable to the short elimination half-life of tiagabine). However, information on the concentration-effect relations with tiagabine is scarce. A tentative target trough range of 50–250 μmol/L has been suggested [95,165,166].

9.3.7.5 Analytical methods

Some chromatographic methods have been described for determination of tiagabine [167–169]. The determination of tiagabine is quite demanding with concentrations in the nanomolar range.

9.3.8 Levetiracetam

9.3.8.1 Mechanism of action

Levetiracetam is the active, water-soluble (S) enantiomer of a racemic pyrrolodine acetamide. The mechanism of action of levetiracetam has not been determined, but it does bind exclusively to animal brain tissue. Binding studies to known receptors, however, have been negative [170].

9.3.8.2 Pharmacokinetics and drug interactions

After oral administration, levetiracetam is completely absorbed. In healthy volunteers, the time of maximum serum concentration of the drug is approximately 1 h, and the

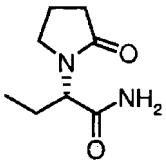

Fig. 9.16. Levetiracetam.

half-life is 7–8 h. The major metabolite accounts for one quarter of the administered dose and has been found to be inactive. Two thirds are excreted unchanged in the urine. No significant pharmacokinetic interactions with other AEDs have been identified. Its elimination is reduced in patients with renal dysfuntion [171,172].

9.3.8.3 Clinical use and side effects

Levetiracetam is effective in the treatment of partial seizures with or without generalisation to tonic clonic seizures [173]. The most frequently reported side effects are drowsiness, tiredness, asthenia, and dizziness of mild to moderate severity with a dose dependent increase in incidence [174,175].

9.3.8.4 Serum levels and therapeutic effect

The relationship between levetiracetam serum concentrations and clinical effect has not been ascertained and consequently, the value of serum concentration measurements has not been established. However, there may well be potential useful applications of drug monitoring of levetiracetam in addition to ascertaining compliance.

A tentative target range for levetiracetam of 35–120 μmol/L has been suggested [95,176].

9.3.8.5 Analytical methods

Levetiracetam can be measured in body fluids using chromatographic methods [177,178].

9.3.9 Zonisamide

9.3.9.1 Mechanism of action

Zonisamide is a benzisoxazole sulfonamide that is a relatively weak inhibitor of carbonic anhydrase which in animal models displays antiepileptic effects similar to those of phenytoin and carbamazepine [179].

9.3.9.2 Pharmacokinetics and drug interactions

Zonisamide is well absorbed from the gastrointestinal tract, and after oral intake peak serum levels are obtained within 4–7 h. The drug is extensively accumulated in erythrocytes. The serum protein binding is about 60% which decreases with increasing serum levels when the drug is given in therapeutic doses. The half-life is 50–70 h in

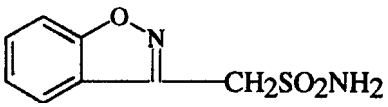

Fig. 9.17. Zonisamide.

monotherapy patients. Enzyme inducing AEDs reduce the half-life to 25–35 h. Zonisamide exhibits non-linear kinetics.

Regarding drug interactions, the metabolism of zonisamide is induced by other AEDs. In contrast, zonisamide serum levels decrease only slightly when valproate is given in addition to zonisamide [179,180].

9.3.9.3 Clinical use and side effects

Zonisamide is effective for partial seizures with or without secondary generalization and may also be effective in generalized epilepsy.

The most frequent side effects are drowsiness, ataxia, anorexia, and gastrointestinal complaints. Renal calculi is seen in approximately 2% of patients in the USA and Europe as compared to a much lower incidence in Japanese patients [180].

9.3.9.4 Serum levels and therapeutic effect

No clear relationship between zonisamide serum concentrations and clinical response has yet been found, and there is considerable overlap of serum concentrations between seizure free patients and nonresponders as well as those associated with seizure control and side effects. Thus optimal outcomes can occur both below and above the range [180,181]. Nevertheless, a tentative target range of 45–180 μmol/L has been suggested [181,182].

9.3.9.5 Analytical methods

Several liquid chromatographic methods have been described for the measurement of zonisamide in serum [183–187]. There is also a micellar electrokinetic capillary chromatographic method with diode array detection [188] and a commercially available immunoassay [189].

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CHAPTER 10

Therapeutic drug monitoring of antidepressant and antipsychotic drugs

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10.1 INTRODUCTION

Over recent decades major advances have occurred in the pharmacological management of depression and schizophrenia. Despite this, high rates of poor compliance and considerable genetic variability in metabolism have meant that the practical application of such treatments has often proven difficult. One means of minimising such problems has been the use of therapeutic drug monitoring (TDM).

10.2 RATIONALES FOR USE OF THERAPEUTIC DRUG MONITORING OF ANTIDEPRESSANT AND ANTIPSYCHOTIC DRUGS

There are four major clinical rationales for the use of TDM in the management of depression and schizophrenia: (i) achievement of therapeutic ranges (where these have been confirmed scientifically); (ii) identification of potentially toxic blood concentrations; (iii) identification of sub-therapeutic concentrations in treatment non-responsive patients; and (iv) monitoring after overdose.

10.2.1 Therapeutic ranges

For some psychotropics, therapeutic ranges have been established. The major relevant classes are some of the tricyclic antidepressants (TCAs) and the 'atypical' antipsychotic clozapine.

10.2.2 Toxicity

As many of the agents used in depression and schizophrenia have narrow therapeutic indices, the capacity to avoid serum ranges where there is a high likelihood of severe

References pp. 269–271

adverse effects or toxicity is critical in clinical practice. Serum concentrations indicative of an increased risk of toxicity have been documented for the TCAs and clozapine. Avoidance of toxicity is relevant to both mono- and combination therapies, although different issues pertain to each form of prescribing.

10.2.2.1 Monotherapy and genetic polymorphisms

There has been a rapid increase in knowledge concerning those enzymes involved in the metabolism of the psychotropic agents, and in understanding the genetic variability in the activity of these enzymes within different populations. The major focus has been on the cytochrome P450 system. CYP2D6 is responsible for the metabolism of many of the psychotropic agents, though some are also metabolized via CYP1A2, CYP3A3/4 OR CYP2C19 (see Table 10.1).

It is now clear that while the majority of the population demonstrates extensive (or normal) metabolism (EM) by these enzymes, a proportion show poor metabolism (PM), which leads to drug accumulation. Poor metabolism is usually due to an autosomal recessive trait caused by mutation or deletion of both alleles. For some enzymes, e.g. CYP2D6, a small proportion of the population also demonstrate ultra-extensive (ultrarapid) metabolism (UM), which leads to reduced drug concentrations at standard doses.

While genotyping of CYP450 enzymes has been slow to enter the clinical arena (and still has significant limitations in its sensitivity, even for CYP2D6 [1]), in the future the combination of information from both TDM and genotyping (or phenotyping where this is not feasible or affordable) should greatly facilitate identification and appropriate management of individuals prone to either excessively high or low serum concentrations of psychotropic agents [2].

TABLE 10.1
ANTIDEPRESSANT AND ANTIPSYCHOTIC DRUG SUBSTRATES OF CYTOCHROME P450 ISOENZYMES.

CYP1A2	CYP2C19	CYP2D6	CYP3A3/4
Amitriptyline	Amitriptyline	Amitriptyline	Carbamazepine
Clomipramine	Citalopram	Clozapine	Clozapine
Clozapine	Clomipramine	Desipramine	Haloperidol
Fluvoxamine	Imipramine	Fluoxetine	Imipramine
Haloperidol	Moclobemide	Fluvoxamine	Nefazodone
Imipramine	Sertraline	Haloperidol	Quetiapine
Olanzapine		Mianserin	Risperidone
		Nortriptyline	Sertraline
		Olanzapine	
		Paroxetine	
		Quetiapine	
		Risperidone	
		Sertraline	
		Thioridazine	
		Venlafaxine	

10.2.2.2 Combined therapies – Interactions and genetic polymorphisms

Toxicity may also be caused by impaired CYP450 function as a result of inhibition due to concurrent medications, i.e. there may be drug-induced PM phenotypes (phenocopying). In all cases it is the EMs that are converted to phenotypic PMs. The genotypic PMs lack sufficient enzyme to have its actions reduced further. In contemporary practice, one of the more common nongenetic causes of impaired metabolism of psychotropic agents such as the antipsychotics or TCAs is concurrent prescription of SSRI (selective serotonin reuptake inhibitor) antidepressants [3]. Those SSRIs which are most potent in inhibiting CYP2D6 are paroxetine and fluoxetine; with citalopram, fluvoxamine or sertraline having only a minimal effect on this enzyme [4]. CYP1A2 is inhibited potently by fluvoxamine, while CYP2C19 is moderately inhibited by fluoxetine and fluvoxamine. CYP3A3/4 is strongly inhibited by the 5-HT2-receptor antagonist nefazodone, and moderately affected by fluoxetine and fluvoxamine.

10.2.3 Subtherapeutic concentrations

The obverse problem is that of unacceptably low concentrations, which may be a relevant factor in either failure to respond or relapse. Low concentrations may reflect poor compliance, or less commonly, ultra-extensive metabolism (as described above). TDM should clarify whether the problem is due to subtherapeutic drug concentrations or to inefficacy of the drug in a particular patient. Another cause of subtherapeutic levels may be that of induction of relevant metabolizing enzymes. Examples of agents causing such induction include carbamazepine, St. John's wort, smoking, grapefruit juice and antiretroviral agents.

Compliance rates are frequently poor in psychiatric practice. Factors in common with other medical disorders include intolerance of adverse effects and difficulty in accepting the need for long-term treatment. Issues specific to psychiatric disorders include impaired insight (particularly relevant to the psychoses) and preference for psychological as opposed to pharmacological management (a not uncommon issue, for example, in the treatment of depression).

Numerous studies of psychotropic medications have indicated high noncompliance rates. One review [5] found that patients receiving antipsychotics took an average of 58% of the recommended medications (ranging from 24 to 90%). Patients receiving antidepressants had similarly low rates, taking on average 65% of the recommended amount (range 40–90%). A review of studies of compliance rates for patients with physical disorders demonstrated a mean rate of 76% (range 60–92%).

10.2.4 Overdose

The final rationale for TDM is monitoring of actual or suspected overdose with psychotropic medications [6].

10.3 ANTIDEPRESSANTS

While TDM has been shown to be of clinical utility for a number of tricyclic antidepressants (TCAs) – as will be discussed below – it has not been proven to be of clear value for the selective serotonin reuptake inhibitor (SSRI) antidepressants. No therapeutic concentration ranges have been demonstrated for any of the SSRIs [7] and toxicity is, in general, not relevant as these drugs have a wide therapeutic index.

As prescribing of TCAs is becoming less frequent with the growing predominance of the SSRIs and other new antidepressants [8], TDM for the antidepressants may become less relevant in clinical practice.

10.3.1 Tricyclic antidepressants (TCAs)

10.3.1.1 Clinical issues

There are a number of clinical issues which militate against the demonstration of any relationship between antidepressant concentrations and therapeutic response [9]. Depression is considered by many to be a heterogeneous disorder, with some subtypes – such as melancholia – probably responding more predictably to antidepressants. Furthermore, rates of spontaneous remission and placebo response are high in depression, confounding the demonstration of therapeutic concentrations. Despite this, such ranges have been demonstrated for a number of the TCAs. The strongest evidence for therapeutic ranges is for imipramine, desmethylimipramine (desipramine) and nortriptyline (Figs 10.1 and 10.2).

10.3.1.2 Assay methods

While TCAs can be monitored using serum or plasma, serum is preferred as it allows greater ease of extraction and involves no fibrin clots [10]. Serum TCA concentration is stable for up to one week at room temperature. A number of different collection techniques have been shown to reduce measured TCA concentrations and should therefore be avoided: heparin-containing tubes; gel separator tubes; and tris-2-butoxyethylphosphate in tube stoppers [10].

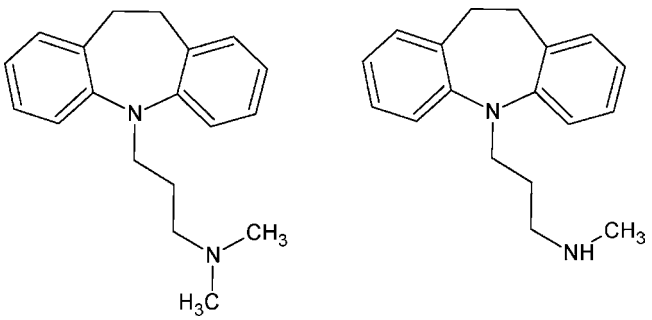

Fig. 10.1. Imipramine (left) and desipramine (right).

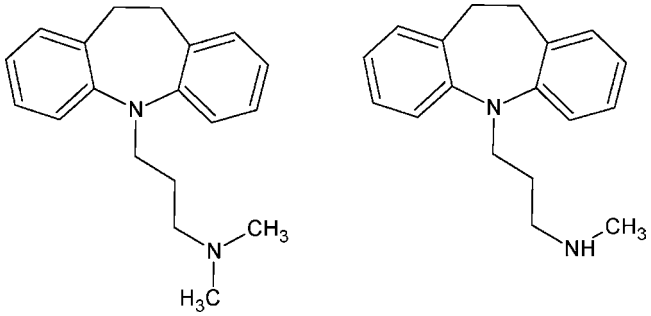

Fig. 10.2. Amitriptyline (left) and nortriptyline (right).

The methods available for quantitatively analyzing TCAs include immunoassay, HPLC and gas-liquid chromatography [10–13]. HPLC with absorbance detection is the most commonly used method. Most reversed-phase methods allow simultaneous measurement of both tertiary and secondary amines. Two main immunoassay forms are available: individual methods using the enzyme-multiplied immunoassay technique (EMIT); and fluorescence polarization immunoassay (FPIA) technology, which measures total tricyclic levels and is commonly used for uring drug screening rather than blood concentration monitoring.

The EMIT assay involves a solid-phase sample extraction followed by analysis with monoclonal or polyclonal antibodies. While this technique has a dynamic range consistent with therapeutic concentrations of the TCAs, a limitation is the considerable cross-reactivity of tertiary and secondary amine TCAs, as well as with structurally similar drugs such as the antipsychotic chlorpromazine.

Though the FPIA technique was originally developed for urine toxicology screening, its advantage is that no extraction of serum samples is involved. Compared with HPLC, there are considerable negative or positive biases for various TCAs, and there is also considerable cross-reactivity with various antidepressants and antipsychotics.

10.3.1.3 Sampling times and intervals

The half-lives of the TCAs are approximately 24 hours. The specific half-lives (and days to steady state) of the TCAs for which therapeutic ranges are accepted are: imipramine 6–28 hours (2–5 days), desipramine 12–28 hours (3–6 days) and nortriptyline 18–56 hours (4–11 days) [10]. Because of the long half-lives, most patients take a single evening dose of TCA. Blood samples are taken about 12–14 hours after dosing if the patient is on divided dose regimen. Specimens should be taken only after steady state concentrations are likely to have been achieved.

10.3.1.4 Therapeutic ranges

The American Psychiatric Association Task Force on the Use of Laboratory Tests in Psychiatry [14] concluded that there was strong evidence for plasma concentration measurements of imipramine, desmethylimipramine (desipramine) and nortriptyline,

but not other TCAs. Subsequent authorities [9,15] have come to similar conclusions. For example, Leucht et al. [16] found no evidence for therapeutic plasma concentrations of doxepin.

Studies of both nortriptyline [17,18] and desipramine [19] have, in general, demonstrated curvilinear concentration-response relationships, with optimum ranges of 50–150 ng/ml (200–600 μmol/L) and 100–160 ng/ml (400–650 μmol/L) respectively, and response rates of 70% and 59% respectively, within these ranges [9]. However, a double-blind 3-year maintenance study [20] of two fixed plasma nortriptyline concentration groups, i.e. 80–120 ng/ml (300–450 μmol/L) and 40–60 ng/ml (150–230 μmol/L), in elderly patients with recurrent depression, found no difference in recurrence rates.

While imipramine has been found to demonstrate a linear concentration-response relationship, there have been fewer studies of this TCA than with nortriptyline or desipramine. The effective threshold concentration for imipramine is 265 ng/ml (1060 μmol/L), with a remission rate of 42% at concentrations above this. There is significant risk of first degree atrio-ventricular block above 350 ng/ml (1400 μmol/L) and delirium above 300 ng/ml (1200 μmol/L).

There has been some, albeit less frequent, evidence suggesting therapeutic levels for amitriptyline (Fig. 10.2). A recent literature survey and meta-analysis [21] has reported that, when only methodologically adequately designed studies are considered, there is a therapeutic window of the sum of the serum concentrations of amitriptyline and nortriptyline of about 80 to 200 μg/L. The authors calculated a moderate and significant effect size of 0.5 for treatment with serum concentrations within this range, compared to levels outside it.

10.3.1.5 Relationship between genotype and serum concentrations

One of the most significant factors underlying the marked inter-individual variation in TCA serum concentrations is genetic variation in the activity of CYP450 enzymes, of which CYP2D6, CYP1A2 and CYP3A3/4 are particularly relevant for the TCAs (Table 10.1) [22,23].

In a recent study, Shimoda et al. [24] found significantly higher ratios of plasma concentrations of desipramine/2-hydroxy-desipramine in subjects with two mutated CYP2D6 alleles compared to those with either no or one mutated allele. The authors noted, however, that the CYP2D6 genotype only grossly predicted steady state desipramine concentrations, as within each genotype there was marked inter-individual variability.

Two studies of the effect of CYP2D6 genotype on nortriptyline levels have been reported. First, Murphy et al. [25], using oligonucleotide microarrays to detect 16 different CYP2D6 alleles, found significant correlations in a geriatric depressed population between the number of such alleles coding for decreased metabolism and nortriptyline plasma concentrations. This finding was indicative of a gene dosage effect and suggested the use of such an array to predict plasma antidepressant concentrations. Second, in a similar study, Kvist et al. [26], investigated multiple nortriptyline plasma concentrations from 20 normal volunteers receiving single oral doses, and 20 depressed patients on steady-state treatment, all of whom had been genotyped for CYP2D6. Using

non-linear mixed-effects modeling, they found that the number of functional CYP2D6 genes were able to explain 21% of the total interindividual variance in oral clearance of nortriptyline, and 34% of that of steady-state levels. This again indicated a gene-dose effect.

Dahl and Sjoqvist [27] have suggested that the main clinical value of genotyping CYP2D6 in the clinical situation in conjunction with TDM of TCAs would be to identify both: (i) poor metabolisers (with this group now being able to be determined with an accuracy of at least 95% to 99%), who would be more prone to concentration-dependent adverse reactions; and (ii) ultrarapid metabolism due to duplicated or multiduplicated CYP2D6 (accounting for between 1% to 10% in different populations), leading to potentially extremely low plasma concentrations.

Reviewing the literature on the impact of genotype on antidepressant levels, Kirchheiner et al. [28] have proposed average dose recommendations for a range of TCAs and other antidepressants based on CYP2D6 and (to a lesser extent in view of fewer studies) CYP2C19 genotypes or phenotypes. There was nil or insufficient data to provide guidance on the impact of CYP2C9, CYP1A2 or CYP3A4 genotypes. They found that while most studies distinguished between PMs (defined here by the presence of two inactive alleles) and EMs (2 active alleles), few investigations provided data on intermediate metabolisers (IMs; one active and one inactive allele) and UMs (≥ 3 active alleles). This necessitated an estimation of levels in the not uncommon IMs, thereby limiting the clinical utility of such recommendations. Nonetheless, there would appear to be clinical value in the data-based recommendations of lower doses in the PMs for both CYP2D6 and CYP2C19. While widespread clinical application may not be currently justified, there is little doubt that as more supportive data appears, genotyping is likely to have an increasing role in clinical practice, in conjunction with TDM.

Furthermore, there are an increasing number of studies of variations in metabolic disposition in different ethnic groups. For example, Shimoda et al. [29] reported a lesser clearance of clomipramine in Japanese patients compared with that in Swedish patients, which was not accounted for by any difference in body weight or concurrent medications. Such an effect may account for clinically-observed differences in tolerability of agents in various ethnic groups.

10.3.1.6 Interactions and other determinants of plasma concentrations

Tertiary amine TCAs are mainly demethylated via CYP1A2 and CYP3A3/4, while hydroxylation is via CYP2D6. Hepatic enzyme inducers such as carbamazepine, phenytoin, phenobarbital and St. John's wort (*hypericum perforatum*) (the latter via induction of CYP3A4 or drug transporters such as P-glycoprotein [30]) reduce TCA concentrations, while inhibitors such as SSRI antidepressants [3], antipsychotics or cimetidine increase concentrations, leading to complications such as impaired cardiac conduction or delirium. Furthermore, the activity of CYP1A2 is induced by cigarette smoking [31], which is more common in psychiatric populations.

In the elderly, TCA levels may be increased by reduced hepatic metabolism and blood flow, and changes in the volume of distribution. In children, increased metabolism may necessitate more frequent dosing [32], though TCAs are of limited efficacy and there are significant safety concerns in that age group [33].

Hepatic cirrhosis leads to increased TCA concentrations [34]. Renal failure has a minimal effect on the parent TCA compound or demethylated metabolites but can increase levels of the active hydroxy metabolites which require renal clearance (though these are at low concentrations and are not routinely measured).

10.3.2 Selective serotonin reuptake inhibitors (SSRIs)

10.3.2.1 Clinical issues and therapeutic ranges

TDM of SSRIs is not routine in clinical practice as: (i) no clear serum concentration: efficacy relationship has been demonstrated for any agent in this class; and (ii) the SSRIs manifest a wide therapeutic index, thereby significant toxicity is unlikely, even at high doses. In an audit of a routine clinical TDM service assaying racemic fluoxetine and norfluoxetine using HPLC (including ultraviolet light detection), no relation to outcome or adverse effects was demonstrated [35]. One of the likely explanations for the lack of a demonstrable therapeutic serum concentration is that the usual minimum effective dose leads to 70–85% inhibition of serotonin uptake, with little further effect at higher dosage. The only relevant theoretical clinical application for TDM of SSRIs would be to ensure sufficient blood levels in treatment-refractory patients. In practice though, such measurements would not be commonly employed.

Recent studies of concentrations of enantiomers of the SSRIs, such as fluoxetine and its active metabolite norfluoxetine have similarly failed to demonstrate any relationship between serum or plasma concentrations of these and therapeutic response [36].

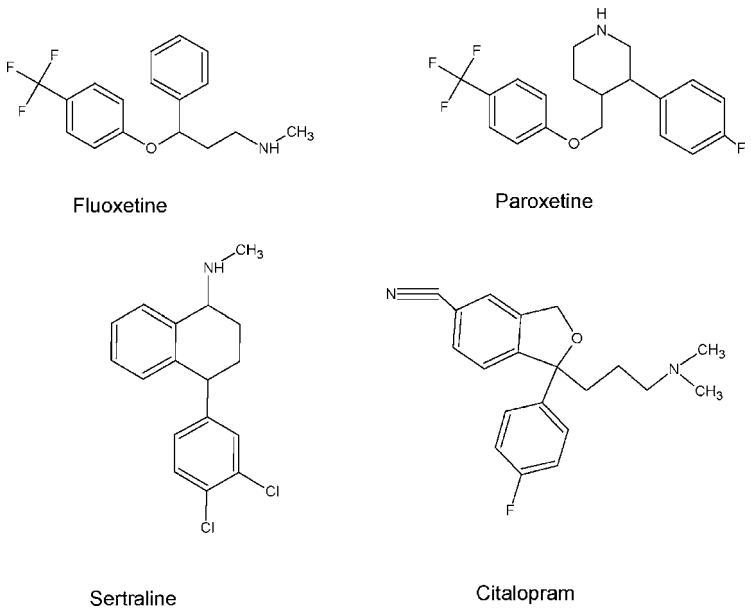

Fig. 10.3. Structural formula of SSRIs.

10.3.2.2 Assay methods

Most reported assay methodologies for determining SSRI concentrations have used gas chromatography (GC) and high performance liquid chromatography (HPLC). The specific methods used have been well reviewed by Baumann [7] and DeVane [37]. A sensitive rapid measure for the simultaneous determination of fluoxetine and norfluoxetine in plasma has been reported, using liquid chromatography/tandem mass spectrometry [38]. Stereoselective assays have also been described for separating the enantiomers of fluoxetine and norfluoxetine [39] and citalopram [40].

10.3.2.3 Sampling times and intervals

The half lives and average steady-state levels of the SSRIs are: fluoxetine 24–144 hours (90–300 ng/ml), sertraline 22–36 hours (20–200 ng/ml), paroxetine 7–65 hours (10–600 ng/ml), fluvoxamine 9–28 hours (20–500 ng/ml) and citalopram 23–45 hours (40–300 ng/ml). A recent nuclear magnetic resonance spectroscopy study [41] has demonstrated brain SSRI concentrations 10–12 times those found in plasma for fluvoxamine and fluoxetine-norfluoxetine.

10.3.2.4 Determinants of plasma concentrations

The major determinant of plasma SSRI levels appears to be dosage [36]. Other important factors are age (increasing levels) and smoking (reducing levels) [35].

10.3.3 Reboxetine

10.3.3.1 Clinical issues

Reboxetine is a selective inhibitor of noradrenaline re-uptake. It is marketed as a racemate, with the (S,S)-(+)-enantiomer being the more potent inhibitor [42]. It has a mean half-life of about 12 hours, and is predominantly hepatically metabolized, mainly via CYP3A4.

10.3.3.2 Assay methods

The major reported means of analysis have been solid phase extraction combined with reversed-phase HPLC and ultraviolet detection [43], and HPLC with column-switching and ultraviolet detection [44]. Furthermore, a technique for simultaneous determination of reboxetine and O-desethylreboxetine enantiomers using enantioselective reversed-phase high-performance liquid chromatography has been described [45].

10.3.3.3 Therapeutic levels

In the only report of the analysis of reboxetine in a clinical therapeutic drug monitoring setting, Ohman et al. [43] reported on serum levels in 38 patients prescribed long-term reboxetine at a dose of 2 to 16 mg daily. They found that the mean reboxetine concentration was "fairly" linear and dose proportional, although the variance in

concentration was large between patients, even those taking the same dosage. While they claimed that the assay was sufficiently robust to produce reliable and reproducible results, it should be noted that there have been no reports demonstrating therapeutic concentrations.

10.3.3.4 Determinants of plasma concentrations

Ketaconazole, a potent inhibitor of CYP3A4, has been found to decrease the clearance of both enantiomers of reboxetine [46].

10.3.4 Mirtazapine

Mirtazapine is an analogue of mianserin that acts upon noradrenergic and serotonergic receptors [47]. There are no published reports of either assay methodologies or concentration-response relationships for this new antidepressant.

10.3.5 Nefazodone/trazodone

Nefazodone and trazodone share the pharmacological action of antagonism of post-synaptic 5-HT2A receptors. There have been no reports of any relationship between plasma or serum concentrations of nefazodone and antidepressant response. There have, however, been two reports suggesting a relationship between trazodone levels and therapeutic efficacy. In 1989, Monteleone [48] reported a threshold plasma concentration of trazodone (650 ng/ml) for antidepressant effect in a small study of an elderly depressed population. This was replicated by Mihara et al. [49] in a trial of trazodone in 26 patients. There was a significant linear relationship between steady-state plasma concentrations and clinical response; and the proportion of responders was significantly higher in those with concentrations greater than 714 ng/ml. There was, however, no relationship between clinical response and plasma levels of the active metabolite m-CPP. These two studies in conjunction suggest that a plasma trazodone level of at least 700 ng/ml may be necessary for clinical benefit.

10.3.6 Venlafaxine

Venlafaxine is an inhibitor of the uptake of both serotonin and noradrenaline [47]. It demonstrates linear pharmacokinetics over the therapeutic dosage range and has one active metabolite (O-desmethyl venlafaxine; ODV). There has only been one report of therapeutic monitoring of venlafaxine and its metabolites in a clinical setting [50]. Using HPLC, steady-state trough samples from 635 patients were assayed. There was a wide inter-individual variability of serum concentrations on each dose level. The mean coefficient of variation of the dose-corrected concentrations was 166% for venlafaxine and 60% for ODV. No therapeutic serum concentrations have been reported.

10.4 ANTIPSYCHOTICS

In recent years there has been a dramatic shift in the prescribing pattern of antipsychotics, with a rapid increase in usage of the so-called 'atypical' antipsychotics such as risperidone, olanzapine, quetiapine, amisulpride, ziprasodone and aripriprazole. Another 'atypical' agent, clozapine (which was in fact first marketed in the 1960s), has been demonstrated to be clearly more effective than 'typical' antipsychotics (such as chlorpromazine and haloperidol) in patients with schizophrenia refractory to such agents [51].

10.4.1 Typical antipsychotics

10.4.1.1 Assay methods

The most commonly used methods for analyzing concentrations of typical antipsychotics are high-performance liquid chromatography (HPLC) and gas-liquid chromatography (GC). Methods for simultaneous assay of a number of such agents, such as fully automated sequential solid phase extraction followed by HPLC [52], have also been described. Capillary zone electrophoresis has been developed for the simultaneous determination of haloperidol and its chiral metabolites [53].

Radio-immunoassays have been developed for haloperidol and fluphenazine. A biological assay is the radioreceptor assay (RRA) in which a neuroleptic drug and its dopamine blocking metabolites compete with titrated spiroperidol (or haloperidol) for dopamine (D_2) binding sites on preparation of membranes from rat striatum [54]. The therapeutic value of RRA has yet to be proven.

10.4.1.2 Therapeutic ranges

On the basis of current data, it is reasonable to agree that 'the routine plasma-level monitoring for most of the antipsychotics remains experimental at this point' [55]. This is some evidence of a biphasic or curvi-linear relationship between antipsychotic concentrations and clinical effects [56]. Antipsychotics demonstrating such a response include haloperidol, butaperazine, fluphenazine, perphenazine and thiothixene; though for most agents the evidence is inconsistent [57]. The most frequently studied agent has been haloperidol, with some studies indicating a therapeutic window from 5 to 12 ng/ml (13.3–31.8 μmol/L) [54] or 5.5–14.4 ng/ml (14.6–38.2 μmol/L) [58]. The major uncertainty about such a 'window', however, is whether the upper cut-off point represents a true worsening of psychosis or a greater likelihood of adverse effects such as extrapyramidal symptoms of dysphoria (either of which would in turn diminish any subjective therapeutic response).

10.4.1.3 Relationships between genotype and serum concentrations

Most antipsychotics are metabolized to some extent by CYP2D6. The CYP2D6 genotype has been shown to be one of the major determinants of plasma haloperidol and reduced haloperidol concentrations [59,60]. Consistent with this, the CYP2D6 genotype

has been demonstrated to predict significantly the oral clearance of the 'typical' antipsychotics perphenazine and zuclopenthixol [61]. Conversely, the degree of impairment of CYP2D6 activity has been shown to be related to the severity of sedation [62] or extrapyramidal side-effects during neuroleptic treatment [63,64]. Since these drugs are used long-term there may be a case for phenotyping or genotyping patients at the start of such therapy. This would avoid the interpretive problem that EMs are converted to PMs by phenocopying during long-term therapy. At present, there is no data indicating that phenotyping or genotyping for CYP450 enzymes can be used to predict an optimal dose range of antipsychotic agents [62]. However, genotyping may play a role in complementing plasma concentrations when either poor or ultrarapid metabolism is suspected.

10.4.1.4 Interactions and other determinants of serum concentrations

In two Japanese studies of factors affecting haloperidol clearance or plasma concentrations, body weight (reduced levels with higher weight), concomitant use of anticonvulsants (reduced levels) and age (higher levels in older patients) were found to be significant determinants [65,66]. Haloperidol levels have also been found to be significantly lower in smokers [59], perhaps due to induction of glucuronyl transferase.

10.4.2 Atypical antipsychotics

10.4.2.1 Assay methods

HPLC methods for analyzing risperidone [67], olanzapine [68–71] and clozapine [72] have been reported. An assay for simultaneous determination of plasma concentrations of risperidone and 9-hydroxyrisperidone using liquid chromatographic/electrospray tandem mass spectrometry has been described [73].

With regard to clozapine, other assay methods reported include solid-phase microextraction [74], liquid chromatography/electrospray tandem mass spectrometry [73] and reversed phase liquid chromatography [75]. Plasma levels of clozapine are assayed as serum levels have been found to underestimate concentrations, as benchmarked against myeloperoxidase activity [76].

10.4.2.2 Therapeutic ranges

The one antipsychotic for which data concerning a therapeutic range is sufficiently substantive to justify clinical utilization is the atypical agent clozapine [77]. Clozapine is 97% bound to plasma proteins and has a mean half-life of about 12 hours (range 6–33 hours). A number of independent studies have now demonstrated a therapeutic threshold concentration of between 350 and 420 μg/L (1070–1290 μmol/L) [77–81]. Furthermore, in a follow up study of one sample [77], Miller et al. [82] found the concentration-response relationship to be consistent over a 2.5-year period. In addition, five of seven previous nonresponders became responders when plasma clozapine concentrations were increased to above 350 μg/L (1070 μmol/L).

Gaertner et al. [83] clinically determined optimal plasma clozapine concentrations in the first four months of treatment for 23 outpatients with schizophrenia, and then followed them up for five years. Using a threshold decrease of plasma clozapine levels of 40% as an indicator of significant reduction, it was found that after 2 years, patients whose levels reduced by more than this amount had 6 times the chance of relapse.

There is also a relationship between serum clozapine concentrations and CNS adverse effects. For example, Olesen et al. [84] found a significant correlation between clozapine concentrations and EEG changes. Concentrations above 1000 μg/L (3060 μmol/L) significantly increase the risk of confusion, delirium and generalized seizures [85]. There is, however, no evidence to suggest that clozapine-induced agranulocytosis is related to serum concentrations. It should be noted that a study has suggested that some patients may be more prone to this complication, with cells from patients with agranulocytosis being less viable than those of patients with agranulocytosis and normal controls after incubation with clozapine and horseradish peroxidase-peroxide [86].

There has been some preliminary evidence that there may be a therapeutic range for olanzapine. Perry et al. [87] examined the relationship between plasma concentrations and clinical response in one of the North American double-blind trials of patients with acute schizophrenia. They found that patients with 12-hour levels greater than 23.2 ng/mL were significantly more likely to respond than those with lower concentrations.

10.4.2.3 Relationship between genotype and serum concentrations

Clozapine is metabolized by CYP1A2 and CYP3A4 to the relatively inactive compounds norclozapine and clozapine-*N*-oxide. Response to clozapine is therefore not predicted by the CYP2D6 genotype [88]. A recent case report has highlighted the role of CYP1A2 in clozapine disposition [89]. In that case study, clozapine levels were affected by: (i) ultrarapid metabolism due to a single nucleotide polymorphism in intron 1 of the CYP1A2 gene; (ii) the CYP1A2 actions of grapefruit juice (enzyme induction); and (iii) the antidepressant fluvoxamine (CYP1A2 inhibition). Consistent with this, clozapine population pharmacokinetics are distributed similarly to the indices of CYP1A2 activity found in community populations [90].

10.4.2.4 Interactions and other determinants of serum concentrations

As would be predicted from a knowledge of the CYP450 metabolizing enzymes, the CYP1A2 inhibitor fluvoxamine increases clozapine concentrations, whereas the CYP3A3/4 inducer carbamazepine decreases levels [91]. However, smoking – which induces CYP1A2 activity – does not appear to affect clozapine levels [78]. Risperidone levels have been reported similarly to be affected by body weight and age [92]. TDM studies have found olanzapine serum concentrations to be affected by fluvoxamine (increased levels, presumably by inhibition of CYP1A2; [93,94]), CYP2D6 inhibitors (increased levels), carbamazepine (reduced levels; [95,96]) and the antiretroviral agent ritonavir (reduced levels, by induction of either CYP1A2 or glucuronsyl transferase;

[97]). Another factor determining olanzapine concentrations is smoking (reduced levels; [98]).

10.4.2.5 Overdose

Renwick et al. [99] have reported a biphasic plasma concentration-time curve after overdose with clozapine in a patient with schizophrenia with secondary peaks at approximately 36 hours post-admission.

10.5 LITHIUM

While lithium is neither an antidepressant nor an antipsychotic, it is nonetheless relevant to make some general comments about this mood stabilizer as therapeutic drug monitoring is integral to its clinical usage. More detailed recent reviews are available elsewhere [e.g. 100].

Recommended therapeutic serum concentrations for lithium distinguish between usage in bipolar disorder and unipolar depression; and for the former, the phase of the illness. For bipolar disorder patients who are acutely manic, the usual recommended range is 0.5 to 1.2 mmol/L. Interestingly, this range has been largely determined by expert clinical consensus, as there has only been one study randomly assigning patients to varying lithium concentrations. There have been more studies of serum concentrations for the maintenance phase of bipolar disorder, with the common recommendations being for a lower level of either 0.5 or 0.6, and a usual upper limit of 0.8 mmol/L. In the case of unipolar depression, lithium is most commonly used as an augmenting agent after failure to respond to an antidepressant alone. In that circumstance, studies indicate that a minimal threshold concentration of at least 0.4 mmol/L is required.

Lithium toxicity normally occurs at serum concentrations of greater than 1.5 to 2.0 mmol/L, though in the elderly toxicity can occur at lower levels. Concentrations greater than 3.5 mmol/L are potentially lethal, and haemodialysis is recommended. Toxicity may present with clinical features such as impaired consciousness, apathy, hyper-reflexia, hypertonia, muscle fasciculations, dysarthria, ataxia, coarse tremor or renal failure. Toxicity may be due to concurrent prescription of agents such as thiazide diuretics, ACE inhibitors or NSAIDs. Other causes are overdose (accidental or deliberate), vomiting, diarrhoea or fever.

10.6 CONCLUSION

In spite of the apparent advantages of TDM in the management of depression and schizophrenia, utilization in practice is poor. For example, a study in Sweden [101] found use of TDM for antidepressants to be low, with only 20% of psychiatrists (and 2% of GPs) using TDM for TCAs regularly. Although therapeutic concentrations have only been demonstrated for a relatively small number of antidepressants and antipsychotics, TDM is of significant potential clinical advantage where either toxicity or sub-therapeutic levels are suspected. Growing evidence for significant effects of differing

polymorphisms of the various CYP450 enzymes suggests that genotyping will have an increasing future clinical role – in tandem with TDM.

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CHAPTER 11

Monitoring immunosuppressive drugs

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11.1 INTRODUCTION

The measurement of immunosuppressive drugs as a guide to their optimal prescription is an integral part of clinical practice in most transplant centres. Each of the drugs we will highlight is considered to be a critical-dose drug and, for some, it is a regulatory requirement to monitor their concentration. The practice of monitoring immunosuppressive drugs has shown substantial evolution since the introduction of serum or plasma measurements of cyclosporin in the early 1980s. The changes that have occurred are summarised in a series of consensus documents that have brought together both laboratory scientists and clinicians. It is arguable that, because of the ensuing dialogue, issues such as assay methodology and drug pharmacokinetics are better understood by transplant clinicians than by any other prescribing group. However, many of the consensus documents no longer reflect current assay techniques or prescribing practice. For this reason, a Working Group sponsored by the International Federation of Clinical Chemistry (IFCC) and the International Association of Therapeutic Drug Monitoring and Clinical Toxicology has been established. Its brief is to provide guidelines on monitoring issues to those monitoring the drugs in the laboratory and those monitoring their effects in the clinic [1].

Many of the methodological problems related to the measurement of these drugs, such as the choice of sample matrix, have been resolved. Other issues, such as sample timing and the performance of new methods, are still the subject of on-going discussion and research. In this chapter we aim to summarise the methodological issues relating to the measurement of cyclosporin, tacrolimus, mycophenolic acid and sirolimus (with a brief mention of everolimus). The methodological issues will be set in the context of factors that affect the clinical interpretation of the measurements, since they form an integral part of their proper interpretation. It is not our intention to provide "method sheets" for each drug, since manufacturers' data sheets, or the original papers on assay development, are easily available. Rather, we seek to provide a balanced view on the

available methods, from which the reader can make an informed judgement on assay performance.

It is unfortunate that there are no established reference methods for the measurement of the immunosuppressive drugs, in line with IFCC guidelines [2], especially as there are substantial between-method differences in performance [3]. Immunosuppressive drug measurements are often used in support of regulatory studies for the introduction of new drugs or new galenical formulations. As a result, there has been a growing interest by individual laboratories in the validation of these assays to the standards acceptable to regulatory authorities, such as the Food and Drug Administration [4]. However, the absence of internationally agreed control samples makes the use of such assays on several sites difficult to police, leading to the need for blinded external testing of performance of laboratories participating in multicentre clinical studies [5].

Data on comparative performance are only available from research papers that set out to compare individual methods, or from the findings of proficiency testing schemes. The latter are often the most relevant, since they may document long term trends for such parameters as inaccuracy or specificity [6,7]. The schemes with the most peer-reviewed publications are those organised by ourselves, and we will use data from these schemes to illustrate individual aspects of assay performance [8].

The choice of assay method by a particular laboratory depends on a number of factors. These include – apparatus available, in-house technical expertise, clinical indications for which the assay will be used, sample load and required turn-around time. Thus, there is no single response on which method to choose and, even when a method has been chosen, changes in clinical practice or the availability of new assay methods may prompt a change in methodology. With these issues in mind, we will examine the methods available for the measurement of the major immunosuppressants in use today, for which monitoring is advised. We start with the longest established drugs cyclosporin and tacrolimus. They share a common mode of action, being inhibitors of the enzyme calcineurin [9].

11.2 CYCLOSPORIN

Although the early literature on cyclosporin documented measurements made in serum or plasma it soon became clear that the matrix was not optimal. Temperature-dependent partitioning of the drug into erythrocytes results in relatively low concentrations of the drug in plasma or serum, with the final concentration being influenced by the temperature at which the sample is separated [10]. As a consequence, from the earliest consensus panel report on cyclosporin measurement, the recommendation has been for the use of whole blood as the sample matrix [11]. It took some years to effect the transition from the use of plasma or serum to the use of a whole blood matrix, a change driven largely by the consistent message from consensus guidelines [12–17].

Despite reservations concerning antibody specificity for the parent compound the overwhelming majority of centres use immunoassay kits to measure cyclosporin [18]. The current immunoassays for cyclosporin almost all use monoclonal anti-cyclosporin antibodies with a high specificity for the parent compound. The exception is a

polyclonal assay produced by Abbott Laboratories (Abbott Park, IL, USA), that cross-reacts substantially with metabolites of the drug [19]. However, even the monoclonal antibody immunoassays cross-react, to a variable extent, with the metabolites of the drug [3,20]. Thus, for clinical samples from kidney transplant patients, the measured concentration using an immunoassay can have a bias in the approximate range 10% to 40%, compared with a chromatographic assay. This bias can be substantially higher for samples from patients with hepatic dysfunction, as cross-reacting metabolites accumulate in the blood [21].

The alternative to an immunoassay is high-performance liquid chromatography (HPLC), which is often cited as the "gold-standard" technique. For fully validated assays this may be so, but cyclosporin is not the easiest of drugs to measure by this technique if ultraviolet (UV) detection is used. The molecule does not have a strong chromophore, detectable at a selective wavelength, and interference from components of the blood matrix and metabolites is common. There is a growing use of HPLC with mass-spectrometric (MS) detection [22–26] but, overall, fewer than 6% of laboratories measure the drug using HPLC, and most of these use UV detection [7]. In general, HPLC does not perform well, with a wide scatter of results evident, even in spiked samples, as can be seen from Fig. 11.1. The median value for HPLC was in line with the weighed-in value, but the variability was high, probably due in part to the need for centres using this technique to prepare their own calibrators in-house. The data in Fig. 11.1 also highlight differences in calibration between the seven high specificity techniques in common use. Calibration differences between these assays have been noted before [6].

However, when samples from patients receiving cyclosporin are analysed the differences between a chromatographic technique and the immunoassays are very clear,

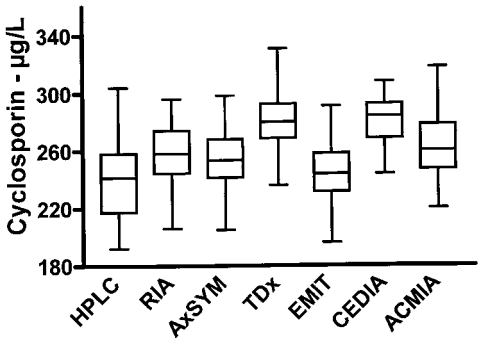

Fig. 11.1. Blood cyclosporin concentrations in a cyclosporin-free sample spiked to a nominal concentration of 250 µg/L. The data are presented as box and whisker plots, by analytical technique. HPLC – high-performance liquid chromatography; RIA – radio-immunoassay (CYCLO-Trac SP, DiaSorin); AxSYM – fluorescence polarisation immunoassay performed on the AxSYM platform (Abbott Laboratories); TDx – fluorescence polarisation immunoassay performed on the TDx platform (Abbott Laboratories); EMIT – homogeneous enzyme multiplied immunoassay (Dade Behring); CEDIA – cloned enzyme donor immunoassay (CEDIA Plus, Microgenics); ACMIA – Affinity Column Mediated Immunoassay (Dade Behring). Data – International Cyclosporin Proficiency Testing Scheme, unpublished.

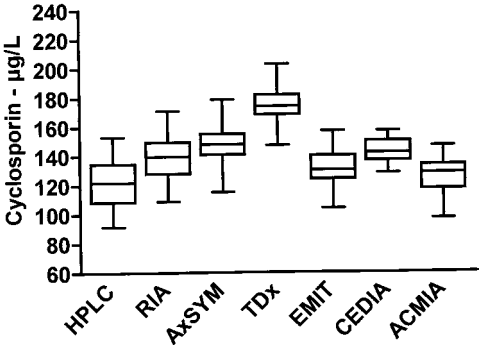

Fig. 11.2. Blood cyclosporin concentrations in a pool of blood samples from patients receiving the drug following kidney transplantation. The data are presented as box and whisker plots, by analytical technique. HPLC – high-performance liquid chromatography; RIA – radio-immunoassay (CYCLO-Trac SP, DiaSorin); AxSYM – fluorescence polarisation immunoassay performed on the AxSYM platform (Abbott Laboratories); TDx – fluorescence polarisation immunoassay performed on the TDx platform (Abbott Laboratories); EMIT – homogeneous enzyme multiplied immunoassay (Dade Behring); CEDIA – cloned enzyme donor immunoassay (CEDIA Plus, Microgenics); ACMIA – Affinity Column Mediated Immunoassay (Dade Behring). Data – International Cyclosporin Proficiency Testing Scheme, unpublished.

as shown in Fig. 11.2. For this pooled sample from kidney transplant patients HPLC gives the lowest median result whilst the Abbott Monoclonal Antibody assay gives a result more than 40% higher. The remaining immunoassays show biases of about 10–20% compared with HPLC. These differences are typical, but are not fixed. The relative differences are influenced by the proportion of metabolites in a sample and, as Fig. 11.1 shows, are altered by calibrator accuracy. Since most users of immunoassay kits use the calibrators provided by the manufacturer, changes in the standardisation of these calibrators can have a substantial influence on the results produced by a method [27]. The caveat here is that, if cyclosporin measurements are to be made as part of pharmacokinetic studies, or if measurements are to be compared over time or between clinical centres, attention must be paid to assay specificity and to longitudinal performance characteristics.

Despite experience of cyclosporin monitoring spanning 20 years, the impact of these assay differences on patient outcomes is still not clear and has been the subject of recent controversy [28]. Empirically, those applying the cyclosporin measurements to patient care have recognised the assay differences and coped with them by using assay-specific target concentration ranges [16]. The mainstay of monitoring has been the pre-dose, or trough (C_0), blood sample, in which cyclosporin concentrations are of the order 100–400 µg/L. For some time it has been known that cyclosporin efficacy is related to exposure during a dosing interval, as judged by the area under the time-concentration curve (AUC), but that the trough sample is not a good reflection of exposure [29]. Measurement of the full AUC requires the collection of several samples and is logistically difficult, even if limited sampling strategies are used [30]. As a result, AUC measurement has been adopted by few centres.

Recently, interest has focused on the use of a sample collected two hours post-dose (C2) [31,32]. The rationale for using this time point is that it is a surrogate for AUC_{0-4}, the time period during which most of the variability in the 12 hour AUC can be explained [33]. Measurement of cyclosporin in these samples has methodological implications [34]. For most immunoassays the concentrations measured in many C_2 samples are outside the calibration range of the assay. To date, only one manufacturer has introduced an immunoassay with calibrators covering a concentration range up to 2000 μg/L (CEDIA Cyclosporin Plus, Microgenics, Fremont, CA, USA). Thus, sample dilution is required, and blinded testing of laboratories has yielded some disturbing results on the accuracy of dilution techniques [35]. In addition, until recently, it was not clear what the relative differences between the immunoassays would be in C_2 samples. In fact, it has emerged that the relative differences between several of the immunoassays are smaller in C_2 samples, compared with measurements made at C_0, probably due to a smaller proportion of metabolites in the two hour sample [36]. Thus, it may not be necessary to implement assay-specific concentration ranges using a C_2 monitoring strategy. Guidelines on the implementation of C_2 monitoring have been published [37,38], and it is evident that there is substantial interest in its introduction [28]. Laboratories asked to perform such measurements should be aware that precise sample timing is important (to within ± 10 minutes of two hours) [39] and that the underlying rationale for using this sample has been validated only for the Neoral® formulation of the drug [40,41]. The introduction of this formulation has also been a spur to the investigation of pharmacokinetic modelling of cyclosporin data to predict blood concentrations using Bayesian forecasting [42,43].

Cyclosporin measurement encompasses all of the problems that are seen for the other drugs covered in this review. It is particularly complex due to the plethora of assays that have been produced and the changes that have occurred in monitoring strategies. Current assay performance characteristics of the immunoassays are well documented in a recent review [22]. However, anyone entering this minefield for the first time should ensure that the assay they use is appropriate to the transplant indications they are serving, and the sample time point they are being asked to measure.

11.3 TACROLIMUS

Tacrolimus also distributes extensively into red blood cells. Although the earliest measurements were made in plasma or serum, laboratories soon moved towards the use of a whole blood matrix. Typical concentrations in whole blood associated with efficacy are in the range 5–20 μg/L [17].

Of the drugs reviewed here, tacrolimus measurement has seen few developments in assay methodology of late. A consensus document appeared in 1995 [44] and was updated in 1998 [17], together with a review on tacrolimus monitoring [45]. Since the publication of these documents, almost all centres continue to use an immunoassay produced by Abbott Laboratories, based on the microparticle enzyme immunoassay (MEIA) technology, performed on the IMx clinical analyser. The sensitivity of this assay was improved and it was re-launched as the Tacrolimus II assay. The change was

made because, in its original format, the assay could not measure the concentrations typically achieved when tacrolimus doses were progressively lowered after the original clinical studies were published [46,47]. Because the vast majority of centres use the same analytical technique, there has been no tendency to produce assay-related target ranges.

An enzyme-linked immunosorbent assay (ELISA, DiaSorin, Stillwater, MN, USA), using the same antibody [48], was also launched, but is now used by very few laboratories [49]. Recently, an immunoassay based on the enzyme multiplied immunoassay technique (EMIT) has been launched (Dade Behring, Marburg, Germany). The assay platforms for the EMIT assay offer some operational advantages compared with the IMx analyser and, although on the market for little more than a year, there is already documented information on its use [50].

As for cyclosporin, there is a growing interest in the use of HPLC, but the actual number of centres using this technique is still very small. The only feasible detection system is MS and a number of assays can be found in the literature [51–56]. Application of HPLC/MS to the routine measurement of tacrolimus was described relatively early [52].

Immunoassay bias compared with chromatographic assays is similar to that seen with the majority of immunoassays used to measure cyclosporin. Typically, concentrations measured by the IMx (Tacrolimus II) assay are about 15% higher than those measured by HPLC, with no transplant-related difference in bias [57]. Others have noted a higher mean bias, approximately 25%, ranging to as high as 70% [58]. The cause of this bias is cross-reactivity with the 31-O-demethyl-, 15-O-demethyl- and 15,31-O-didemethyl-metabolites of tacrolimus; the 31-O-demethyl- metabolite has pharmacological activity comparable with the parent compound, but is normally found in low concentration [58,59]. Few centres have had the opportunity to investigate the extent of the bias for samples giving unexpected clinical correlates with the measured result, but one group showed a 10 fold higher result by immunoassays compared with HPLC/MS for samples from a liver transplant patient with hepatic dysfunction [60].

Since the ELISA assay uses the same antibody as that used in the IMx assay it is not surprising that it also has a bias compared with HPLC. Estimates of the bias vary, influenced by factors such as transplant indication and assay calibration, but are generally lower than those seen with the IMx assay, for reasons that are not established. Staatz et al. noted a mean bias of only 2% in samples from kidney transplant patients, but this increased to 12% in samples from liver transplant patients [61]. When MacFarlane et al. validated the ELISA against LC/MS, using samples from liver transplant patients, the mean bias was 9.5% [49]. For the same samples, the mean bias was 42% using the IMx assay. The sensitivity of the ELISA is better than that of the IMx. MacFarlane et al. suggested that the functional sensitivity was 1.0 μg/L, whereas a value of 2 μg/L seems more appropriate for the IMx assay [47]. Recent College of American Pathologists (CAP) external proficiency testing data suggest that the IMx assay has an unacceptable bias against HPLC at concentrations below 9 μg/L [62].

The only substantive publication on the EMIT assay, to date, suggested that the results produced by this assay, for patient samples from three transplant indications, were very similar to those produced by the IMx [50]. There was a small positive bias

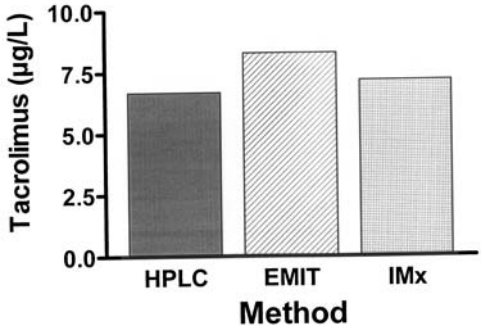

Fig. 11.3. Mean tacrolimus concentrations in a pool of blood samples from patients receiving the drug following liver transplantation. The analytical techniques were: HPLC – high-performance liquid chromatography with mass-spectrometric detection, EMIT – enzyme multiplied immunoassay technique (Dade Behring), IMx – microparticulate enzyme immunoassay technique (Abbott Laboratories). Data – International Tacrolimus Proficiency Testing Scheme, unpublished.

against the IMx assay when external proficiency testing samples were analysed. The assay uses a different antibody to that used in the ELISA and IMx assays. Independent data on the cross-reactivity of the antibody and a comparison with HPLC/MS are awaited.

Typical results for a recent challenge in our tacrolimus proficiency testing scheme show a bias using both immunoassays compared with HPLC/MS. Fig. 11.3 shows the mean data for the measurement of tacrolimus in a pooled sample from liver transplant patients receiving the drug. There was a 7.5% bias using the IMx assay, compared with 23.9% using the EMIT assay. However, data from the scheme, collected throughout 2002, suggests that, whilst spiking accuracy was good, both immunoassays over-estimated tacrolimus by about 15% compared with HPLC/MS when samples with known amounts of added tacrolimus were circulated (data unpublished). Thus, part of the immunoassay bias appears to be due to inaccuracy of the immunoassay calibrators. Data from the CAP proficiency testing scheme, collected over a period of three years, shows not only a larger bias for the IMx assay, against HPLC/MS values generated by a single centre, but also that the calibration of the immunoassay has changed over time [63].

Sample timing for tacrolimus has been, almost exclusively, pre-dose. Of late, largely kindled by the interest in cyclosporin C_2 monitoring, there has been an interest in the use of alternative sampling times. However, the absorption profile of tacrolimus is erratic and no single time point has been found to be of more clinical value than C_0. The relationship between tacrolimus exposure, as judged by the AUC, and the C_0 sample concentration is often cited as good, with r^2 values of 0.86 [64] and 0.82 [65] being quoted. However, other groups have found this relationship to be less sound [66]. Despite these concerns, trough sample monitoring for tacrolimus seems set to stay for the foreseeable future.

The question as to whether a selective measurement gives a better guide to dose adjustment than an immunoassay has not been addressed, partly because few centres have had access to HPLC/MS. Paradoxically, the most recent multicentre study that

examined parameters of efficacy and toxicity against tacrolimus concentrations, in liver transplant patients, used the ELISA assay [67]. More outcome studies, designed to assess the clinical value of tacrolimus measurements are needed, for both the IMx and EMIT assays.

11.4 MYCOPHENOLIC ACID

Mycophenolic acid (MPA) is a non-competitive inhibitor of the enzyme inosine monophosphate dehydrogenase (IMPDH) [68]. Most data on the use of MPA in transplantation are based on the prescription of the mycophenolate mofetil (MMF) formulation. This is a pro-drug formulation which uses the morpholinoethyl ester of MPA, devised to overcome the poor oral bioavailability of MPA. MMF is marketed as *Cellcept*® (Roche, Basle, Switzerland) and is also available as an intravenous formulation [69]. Following oral administration MMF is rapidly hydrolysed to MPA and is virtually undetectable in plasma. Even after intravenous infusion, MMF is not detected in plasma about 10 minutes after infusion [70]. Another oral formulation, the enteric coated sodium salt of MPA (*Myfortic*®, Novartis Pharma, Basle, Switzerland), is also in development [71]. Doses of MMF are of the order 2 grams per day, whilst those of the enteric coated formulation are about 1.5 grams per day, the difference being due to the weight of the ester in the MMF formulation. The most recent consensus guidelines on monitoring MPA were published in 2001 [72]. Almost all the drug remains within the plasma fraction in whole blood, the underlying reason for measuring MPA in plasma or serum. The drug is extensively bound to plasma proteins, principally albumin, but the free fraction can vary substantially, from as little as 1% in patients with normal renal function to as much as 7% in patients with poor renal function or low albumin concentrations [73,74].

The principal metabolite of MPA is the phenolic glucuronide, which can be present in plasma at a much higher concentration than the parent compound, especially in patients with renal dysfunction [75]. Average AUC_{0-12} values for the phenolic glucuronide can exceed those of the parent compound by a factor of more than 40 in kidney transplant patients receiving MMF 2g per day [76]. This metabolite does not possess pharmacological activity, with respect to the in vitro inhibition of IMPDH [77]. However, it can be of interest to measure this compound as it displaces MPA from its binding sites on albumin, increasing the free fraction of MPA [78]. In contrast to the phenolic glucuronide, another metabolite, the acyl glucuronide does inhibit IMPDH, in a concentration-dependent manner of similar magnitude to the parent compound [77]. In addition, a 7–0-glucoside metabolite has also been identified [79]. In terms of monitoring, this metabolite is of less interest than the glucuronides, since it has not been shown to have any pharmacological activity [77].

Of the drugs dealt with in this review, the measurement of mycophenolic acid should be the least problematic. It is measured in plasma or serum, rather than whole blood, and is present in the highest concentrations during normal therapy. Typical concentrations in pre-dose samples are of the order 1–3 mg/L [72]. Several chromatographic assays have been developed for the measurement of MPA in plasma or serum, based on HPLC with

UV detection, all with adequate sensitivity to monitor the drug as a guide to therapy, and most able to measure the phenolic glucuronide [80–84]. One of these includes validation for the two compounds in urine [84]. A method based on capillary electrophoresis, in which simultaneous quantification of MPA and MPAG can be performed, has also been published [85]. In addition, in some parts of the world, there is an EMIT immunoassay that can be performed on a variety of clinical analysers [86,87].

Results produced by the immunoassay for the measurement of MPA in samples from patients treated with the drug are generally higher than those produced by a chromatographic assay [87]. Typically, the assay bias is about 20% [88], although it varies widely, depending on factors such as the time of sampling relative to the last dose [89] and concomitant drug therapy [90]. Data from pooled samples from patients receiving the drug, circulated in a proficiency testing scheme, show broad agreement on the extent of the immunoassay bias [91]. The principal reason for the bias has been shown to be cross-reactivity with the acyl glucuronide metabolite of MPA [92]. The bias is not due to the principal metabolite, since the phenolic glucuronide does not cross-react with the antibody used in the EMIT assay [90]. The immunoassay is a convenient format to use for routine monitoring, giving acceptable precision at the concentrations associated with normal therapy [90]. However, for this assay, it has been suggested that, due to its analytical bias, the target concentrations should be higher than those used with chromatographic measurements [87].

There is another source of assay bias when using the immunoassay. The pro-drug, MMF, cross-reacts with the antibody, so use of this assay within 10 minutes of MMF infusion is not recommended [93]. In addition, MMF is not stable in plasma samples. In samples collected shortly after MMF infusion it can breakdown in vitro to form MPA, compromising the integrity of MPA measurements made in pharmacokinetic studies after intravenous infusion of MMF. This is a problem that can affect measurements by both immunoassay and chromatographic techniques. Samples can be stabilised to prevent the breakdown of MMF by the addition of phosphoric acid to yield a pH of approximately 2.5 [94].

Only one centre has published data on the measurement of the acyl glucuronide, using HPLC with UV detection [95]. The acyl glucuronide was generated by human liver microsomes and subsequently isolated. Only small amounts of the product were available, insufficient to prepare accurate calibrators, so quantitation was based on the molar extinction of the phenolic glucuronide, which has similar UV absorption maxima. Concentrations of the acyl glucuronide in pre-dose samples varied widely within the range 0.08–1.43 mg/L, median 0.48 mg/L [96]. Thus, the concentration of this metabolite can be high, relative to MPA. This factor should be considered when comparing results generated by chromatographic techniques and the immunoassay. The rationale for measuring this compound is that it may be implicated in the aetiology of the gastrointestinal adverse effects of MPA. Acyl glucuronides have been linked with a number of adverse effects [97] and the acyl glucuronide of MPA has been shown to induce the in vitro release of cytokines such as IL-6 and TNF-α [98]. In a recent case report, inflammatory symptoms in two patients receiving MMF for an autoimmune indication were associated with raised concentrations of the acyl glucuronide and IL-6 [99]. Two analytical factors inhibit research work on this compound. Firstly, there is no

commercial source of the compound and, secondly, it is unstable in plasma at alkaline or neutral pH. Unless samples are stabilised by the addition of an acid, the compound breaks down, with significant losses within a few hours at ambient temperature [95].

Direct measurement of free concentrations of MPA in plasma, following ultracentrifugation, is methodologically more challenging than measuring total concentrations using HPLC with UV detection, because of sensitivity issues. In a study involving paediatric kidney transplant patients, pre-dose concentrations of free-MPA averaged only 0.01 mg/L [100]. One group has published information on the HPLC measurement of free MPA with MS detection, easily achieving the sensitivity necessary to measure free-MPA [101], whilst another group has published a modification of the EMIT assay for the same purpose [102]. Two other approaches to the measurement of free-MPA have appeared. Nowak and Shaw used radiolabelled MPA to calculate the free fraction of MPA, using ultrafiltration and equilibrium dialysis [78]. Ensom et al used an indirect measurement of free-MPA by spiking MPA into plasma samples prior to ultracentrifugation, producing a relatively high concentration of MPA in the ultrafiltrate, against which both the total and free concentrations could be measured by HPLC with UV detection [103].

Like cyclosporin and tacrolimus, there has been interest in the measurement of MPA exposure, as reflected by AUC over a dose interval (usually 12 hours). Several studies have linked total exposure to MPA with clinical efficacy [104–106]. No single time point appears to be adequate to assess AUC, but a number of limited sampling strategies have been devised using a series of samples collected up to six hours after dosing [76,107–109]. These strategies were developed for the concomitant use of MPA with cyclosporin but, there is a pharmacokinetic interaction between cyclosporin and MPA, resulting in lower pre-dose concentrations of MPA in association with cyclosporin, compared with tacrolimus or sirolimus [110,111]. Thus, it cannot be assumed that the algorithms developed for time-points involving cyclosporin and MPA dose regimens can be transferred to other clinical settings. A limited sampling strategy to calculate MPA AUC when the drug is used in combination with tacrolimus has been published [112]. Concentrations of MPA measured in samples collected during the first two hours after dosing may exceed the calibration range of the immunoassay (15 mg/L) or of calibrators prepared in-house for chromatographic assays. Thus, it is important to follow the advice of the kit manufacturer, or to use validated dilution techniques when measuring MPA concentrations outside the calibration range.

Finally, there has been interest in measuring inhibition of IMPDH in blood samples from patients receiving MPA, as a pharmacodynamic measure of its efficacy. Whilst this is an attractive approach to monitoring the efficacy of the drug, in our experience, the original methodology was difficult to reproduce. The method involved determining the rate of catalysis of a tritiated substrate, ^{3}H-hypoxanthine [113]. One centre did report results using the same approach, again, in kidney transplant patients [114]. Others have determined IMPDH activity using the direct HPLC measurement of the product xanthosine monophosphate from IMP, in lysed mononuclear cells [115], or lysed whole blood [116]. However, it should be noted that it is now known that inhibition of IMPDH is not the only pharmacological activity of MPA that gives rise to its immunosuppressive effects. Flow cytometric techniques have established that MPA also suppresses the

expression of important lymphocyte cell surface antigens [117]. Thus, measurement of IMPDH inhibition may not give the complete picture of the pharmacodynamics of this drug.

11.5 SIROLIMUS

Sirolimus, formerly rapamycin, was licensed only recently, for use following kidney transplantation, and there are convincing data to suggest that its measurement is clinically useful. Although structurally related to tacrolimus its mode of action is different. Most data relate to the use of sirolimus in combination with cyclosporin [118,119] but it has also been used as the primary immunosuppressant [120,121] and studies are in progress using a combination of sirolimus and tacrolimus therapy [122,123]. In Europe the licence for the use of sirolimus is based on a multicentre study in which cyclosporin therapy was withdrawn from a regimen of cyclosporin, sirolimus and steroids three months after transplantation [124]. In Europe routine monitoring of sirolimus is a regulatory requirement and in other countries in which it is registered monitoring is recommended in defined clinical settings. The clinical pharmacology of sirolimus, the historical setting for its measurement and a summary of the clinical trials have been summarised recently [125,126].

Sirolimus is bound extensively within red cells; the average blood to plasma ratio is in excess of 30 [127]. Thus, whole blood is the only feasible matrix for monitoring. It is not necessary to monitor the drug as frequently as cyclosporin or tacrolimus as the drug has a relatively long elimination half-life of about 60 hours,. Currently, target sirolimus concentrations after kidney transplantation are in the range 4–12 μg/L whilst the drug is administered with cyclosporin and 12–20 μg/L in cyclosporin-free regimens.

When the early clinical and pivotal clinical studies were planned they built on the experience gained during the development of both cyclosporin and tacrolimus [126]. Sirolimus was measured using validated HPLC assays with either UV [128,129] or MS detection [130,131]. For a brief period an investigational immunoassay for the measurement of sirolimus was available [132]. The assay was based on the MEIA technology and was performed on the IMx clinical analyser (Abbott Laboratories). When immunoassay results for pooled or individual pre-dose blood samples from kidney transplant patients receiving sirolimus were compared with results produced by LC/MS the immunoassay results were higher. The mean bias was in the approximate range 20% to 40% [129,132,133] and could be attributed to the known cross-reactivity of the anti-sirolimus antibody with metabolites of the drug.

The immunoassay was withdrawn before it became a marketed product, though not because of any concerns about its technical performance. As a result, HPLC was the only technique available for the measurement of the drug, a situation that still exists. Not that there is any lack of interest in developing an immunoassay for the drug; at least two diagnostic companies are in the process of developing such assays.

Because the concentrations of sirolimus associated with therapy are relatively low and, since the molecule has a poor UV absorption, quantitation is not easy using UV

detection. The UV-based assays available at the time the drug was licensed tended to have long chromatographic run times, required relatively large sample volumes and had a lower limit of quantification close to the lower range of the target concentration range. Sensitivity was achieved by extensive sample clean-up procedures [128,134,135]. To fulfil the regulatory requirement for monitoring it was necessary to develop an HPLC/UV assay that was robust enough to cope with the demand for sirolimus measurements, in laboratories without the resources to turn to the use of HPLC/MS. The manufacturer of the drug, Wyeth Pharmaceuticals, developed and promoted a method based on a 0.5 mL sample volume, liquid/liquid extraction and a chromatographic run time of less than 15 minutes [136]. The method gave good agreement with an HPLC/MS assay that was developed for routine clinical use and a small sample volume (100 μL) [137]. Subsequently, a number of methods have been published, based on either LC/UV [138,139] or HPLC/MS [26,53,56,140]. There is a clinical demand to provide results with a short turn-around time, and an increasing use of combined therapy with several immunosuppressive drugs. With this in mind, some of these methods have been designed to explore the use of HPLC/MS to measure cyclosporin, tacrolimus and sirolimus in one sample extract, with some success [26,56]. The stability of these systems has also been exploited to allow minimal calibration of routine assays, thus increasing throughput. In this laboratory our routine method for the measurement of sirolimus [137], regularly used to measure the drug in up to 120 samples per day, was validated to require full calibration only every 6 working days. Day-to-day assay calibration is confirmed by the use of internal quality control samples. Similarly, Taylor et al. have validated a single point calibration of their assay [141].

Most assays have used 32-desmethoxyrapamycin as the internal standard. Both this compound and sirolimus exist as an equilibrium mixture of inter- converting isomers. For sirolimus they are designated as A, B, and C, the major component being isomer B. On some chromatographic systems isomers B and C are resolved. However, since equilibrium is achieved between each isomer in calibrators, controls and patient samples, it is only necessary to use the peak for isomer B for quantitation.

Mass-spectrometric detection of sirolimus is aided by the useful fragmentation pattern and abundance of a product ion, allowing the selective measurement of low drug concentrations. The superiority of this technique, compared with the use of UV detection, is illustrated in Fig. 11.4, showing chromatograms for extracts from blood samples from patients receiving the drug following kidney transplantation. With mass-spectrometric detection, using a sample size of 100 μL, it is easy to measure concentrations as low as 1 μg/L. This is more than adequate for the use of the drug following solid organ transplantation, but we have needed to develop a more sensitive assay to measure the drug following its elution from sirolimus-coated stents used to prevent restenosis following angioplasty [142].

Sirolimus is extensively metabolised by the CYP3A enzyme system. Several metabolites, including hydroxylated and demethylated compounds have been identified [127,143]. It should be noted that few centres have definitive data to show that their chromatographic assays are free from interference from metabolites of sirolimus. This is likely to be of more concern for centres using UV detection, especially in clinical settings leading to the accumulation of metabolites, such as hepatic dysfunction.

However, it must be remembered that MS detection may not be entirely free from interference from metabolites [137], and that the influence of matrix effects should also be excluded during assay validation [144].

There have been some concerns regarding the stability of sirolimus working solutions and of blood samples containing sirolimus. It is good practice to protect methanolic solutions of sirolimus from light and to freeze them at − 20°C, or lower. Once sirolimus is spiked into whole blood it has proved stable at most working temperatures up to about 30°C for about a week, and to be stable when subjected to freeze/thawing [132,145]. It is at temperatures above about 35°C that instability of the drug has been noted, with a loss of more than 10% in blood samples stored at 37°C, increasing to more than 20% within a week [132]. As a result, systems have had to be established to enable sample

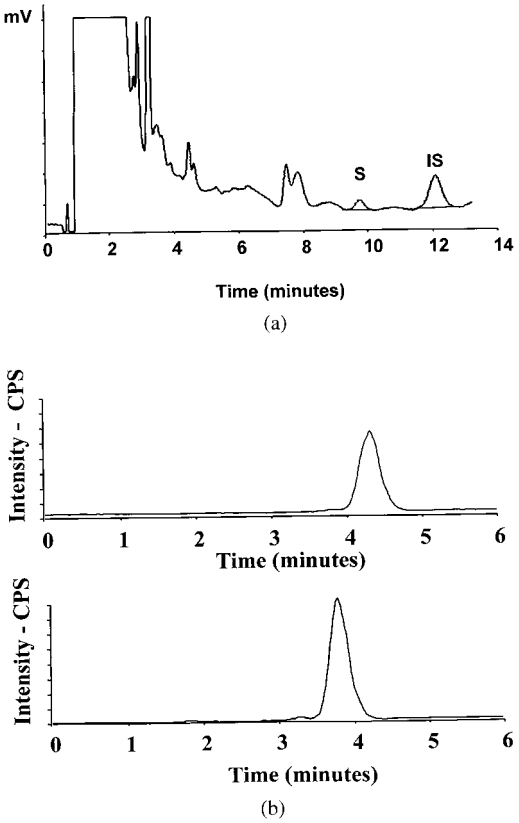

Fig. 11.4. Chromatograms for extracted blood samples from patients receiving sirolimus following kidney transplantation. a – with UV detection, using the method of French et al. [136]. S = sirolimus, IS = internal standard. Sample size 0.5mL, sirolimus concentration 6.6 μg/L. b – with mass-spectrometric detection, using the method of Holt et al. [137]; upper panel sirolimus channel, lower panel internal standard channel. Sample size 0.1mL, sirolimus concentration 5.2 μg/L. Data – Analytical Unit, unpublished.

transport at sub-ambient temperatures when shipping samples to central laboratories for analysis [5]. This has added substantially to the cost of sirolimus monitoring.

The large body of data on blood sirolimus concentrations that resulted from the early and pivotal clinical studies has had a significant impact on dosing decisions [146]. Concentration ranges for use of the drug in kidney transplantation are well established, but are less well defined in liver or heart transplantation, or when the drug is used in combination with tacrolimus. The lack of an immunoassay for use on automated clinical analysers is seen as a disadvantage to the use of the drug by some clinicians, although it has proved possible to validate central laboratories to measure the drug by chromatographic assays on a global basis [147].

11.6 EVEROLIMUS

Although not a registered product at the time of writing, this drug has reached the stage of regulatory submission. The drug is the 40-O-hydroxyethyl analogue of sirolimus. Multicentre clinical trials of this compound in kidney transplant patients, in association with cyclosporin, suggest that the concentrations consistent with efficacy are similar to those used for sirolimus; target trough concentrations are in the range 3–15 μg/L [148]. In some preliminary studies the drug was measured using an in-house immunoassay developed by the manufacturer of the drug, but the assay was never commercialised. The main regulatory studies have all used HPLC/MS, performed by a limited number of central laboratories. Whilst at least one immunoassay is in commercial development, as for sirolimus, the only technique available at present is HPLC; almost all laboratories are using MS detection. A number of HPLC/MS methods have been published [23,26,149,150].

Assuming this drug becomes a licensed product, it looks likely that routine monitoring will be required, since its route of metabolism and, hence, scope for pharmacokinetic drug interactions, is similar to the calcineurin inhibitors and sirolimus [151,152].

11.7 EXTERNAL PROFICIENCY TESTING

Participation in an external proficiency testing scheme has been a recommendation of all consensus documents on immunosuppressive drug monitoring. Proficiency testing data have highlighted several problems relating to assay inaccuracy and specificity [3,6,153]. The latter parameter is best addressed by circulating actual patient samples, although samples supplemented with authentic metabolites of the target drug can also be used [7]. Data from these schemes also provide a snapshot of current practice, useful when assessing the relative performance of new assays and changes with time that may affect assay calibration.

The two largest schemes are those co-ordinated from our laboratory at St George's Hospital Medical School and the CAP Scheme. Both have more than 400 participants for cyclosporin and over 300 participants for tacrolimus. There are other, local, schemes in Germany and Canada.

Both the pharmaceutical and diagnostics industry pay close attention to the results of these schemes. The data from our own schemes have been and are being used to monitor the consistency of assay performance at centres participating in long-term multicentre clinical trials. Our library of samples, all characterised with mean values for the available methodology, has been used in the validation of new methods.

Recent data for both cyclosporin and tacrolimus assays show that a large proportion of the variability recorded for the repeat measurement of these drugs was due to within-laboratory factors [63]. These results underline the need for laboratories to obtain frequent feedback on the performance of their assays if they are to maintain consistent performance for the measurement of critical dose drugs that are subjected to prolonged monitoring.

11.8 CONCLUSIONS

Methods for the measurement of immunosuppressive drugs, as a guide to dose optimisation, are well documented. In general, the assay most selective for the parent compound should be used. In general, there is no evidence that measurement of metabolites adds to the interpretative value of the result for these drugs. Where the measurement of metabolites is useful, it should be performed using a selective method, rather than by biasing the result due to assay cross-reactivity or poor chromatographic selectivity. That said, the choice of assay technique adopted by any individual laboratory will depend on economic and service constraints.

Service providers must realise that it is not sufficient to provide only the drug concentration in isolation. Those establishing a routine assay service should be aware of the performance characteristics of their assay technique and its limitations. They should also have some knowledge of how to place the drug concentration into its clinical context [154]. This is becoming increasingly difficult, as the number of immunosuppressive drugs prescribed to each patient expands. The complexity of dose regimens may, themselves, endanger patient compliance, of concern to many clinicians [155,156]. It is now not uncommon to combine the use of drugs such as cyclosporin, sirolimus and mycophenolate in a single immunosuppressive regimen. Whether the use of complex flow cytometric tests to assess the pharmacodynamic properties of several drugs in combination will assist in the task of choosing the optimal doses of each drug remains to be seen [157]. Similarly, assays based on the ability of immunosuppressive drugs to bind to specific binding proteins show some promise in reflecting active drug concentrations, although they remain a research tool, rather than a routine procedure, at the moment [158].

At present, there is a tendency for immunosuppressive drug assay performance to be judged against a consensus value, usually an analytical method mean. This state of affairs may, in part, be encouraged by the style of data output from proficiency testing

schemes [159]. However, there is a need to move towards judging these assays by more established rules of metrology, based on certified reference materials analysed by reference methods [160]. If this were the case, there would be an incentive for kit manufacturers to play closer attention to calibration and specificity issues.

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CHAPTER 12

Pharmacogenomics: Methodologies for genotyping and phenotyping

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12.1 INTRODUCTION

With the development of new technologies and the near completion of the human genome project, pharmacogenomics is becoming a clinically useful tool [1,2]. Genetic questions can now be addressed, as various polymorphisms, biomarkers, and genes are being identified and linked to specific diseases or drug responses. The driving force of pharmacogenomics and its ability to be used effectively partially depends on the available technologies [3]. Over the past several years, the methods for genotyping and phenotyping in pharmacogenomics have become more cost-effective resulting in the growth of genetic testing in clinical, forensic, and research laboratories [3–6]. However, the complexity, accuracy, and throughput of the methods/technologies will have to be addressed as pharmacogenomics continues to be used more clinically [7]. Selection of the appropriate technology will be based on prior knowledge of the mutation/polymorphism, sensitivity/specificity, sample requirements, and cost. Furthermore, the future volume of pharmacogenomic testing is anticipated to be large. As a result, the automation of pharmacogenomics will become increasingly important. This chapter will attempt to provide an overview of the various technologies available for genotyping and phenotyping individuals for known or unknown polymorphisms ranging from a small to large-scale basis.

12.2 METHODS FOR PHENOTYPING

Individuals respond to medications differently [8]. After taking a medication, an individual may experience the desired therapeutic effect, no effect at all, or may suffer an adverse drug reaction (ADR). These pronounced interindividual differences in drug disposition may be due to genetic variation encoding the activity of drug-metabolizing enzymes like the cytochrome P450 (*CYP*) family, or due to drug–drug interactions.

References pp. 316–319

These can cause induction or inhibition of drug-metabolizing enzymes. As a result, techniques have been developed to assess an individual's enzyme activity, since *CYP* activity can vary more than a 100-fold between individuals [2]. Before genotyping, phenotyping was one of the first methods to determine how an individual would metabolize a particular medication. Phenotyping is accomplished by the administration of a test drug, whose metabolism is known to be primarily dependent on one particular drug-metabolizing enzyme. The metabolic ratio – the ratio of unchanged drug to metabolite, is then measured in serum or urine. For example, caffeine has been used as a metabolic probe for one *CYP* isoform (*CYP1A2*). Caffeine has a number of properties that make it a good probe, such as its pharmacokinetic characteristics – complete absorption, high clearance and short half-life and its safety margin. In addition, the metabolic pathway has been well characterized in humans. *CYP1A2* is responsible for the *N1*-, *N3*-, and *N7*-demethylations that account for approximately 90% of caffeine elimination in humans [9]. *CYP1A2* is polymorphic and involved in the metabolizing of several clinically-important drugs such as clozapine [10] and theophylline [11]. N-acetyltransferase 2(*NAT2*) is another major liver enzyme that is polymorphic and involved in the biotransformation of several carcinogenic arylamines and drugs [12]. Mutant alleles of *NAT2* genes are associated with a slow or rapid acetylator phenotype. Isoniazide, procainamide and dapsone have all been used as probes for *NAT2* [13]. Table 12.1 lists some of the major polymorphic enzymes and a few selected drugs metabolized by these enzymes. Another important family of enzymes, methyltransferases is responsible for the biotransformation of anticancer drugs such as 6-mercaptopurine, 6-thioguanine, and azathioprine [14]. In particular, thiopurine methyltransferase (*TPMT*) catalyzes the S-methylation of 6-mercaptopurine [15]. *TPMT* is polymorphic and a very common genetic polymorphism results in low activity of the enzyme. Consequently, this can have life-threatening effects – myelosuppression for individuals treated with standard doses of thiopurines [15]. As a result, phenotyping to determine the *TPMT* status should be done prior to the administration of anticancer regimens using 6-mercaptopruine or azathioprine [16,17]. *CYP2D6* is another clinically important *CYP* involved in metabolizing 25% of all prescribed drugs [18]. Phenotyping for *CYP2D6* has been done using debrisoquine, metoprolol and more recently dextromethorphan as probe drugs [17,19]. Subjects defined as poor metabolizers have a metabolic ratio of the probe drug debrisoquine of >12.6. However, phenotyping is not always the best technique to use for assessing *CYP* activity. In fact, phenotyping can be potentially dangerous. Since a particular test compound/drug has to be administered, the patient may develop an adverse drug reaction. As a result, newer technologies, based on the genetic information (genotype), have been developed that predict an individual's response to therapeutic drugs. However, the predictive value of a genotyping test has to be evaluated prospectively in the clinic. These newer genotyping technologies will be described in this chapter.

12.3 DISCOVERY OF UNKNOWN POLYMORPHISMS

The human genome contains millions of single nucleotide polymorphisms (SNPs) with new ones being constantly discovered [20]. As a result, a number of techniques are

TABLE 12.1
COMMON POLYMORPHIC ENZYMES AND PROBES USED FOR PHENOTYPING

Polymorphic Enzyme	Probe drug	Selected drugs metabolized by enzyme [67]
CYP1A2	Caffeine [68] Melatonine	Clozapine [10] Olanzapine Theophylline [11]
CYP2C9	Tolbutamid Diclofenac Indomethacin	Warfarin Phenytoin Losartan
CYP2C19	Mephenytoin Omeprazole	Diazepam Celecoxib Citalopram Phenytoin
CYP2D6	Dextrometorphane Debrisoquine	Tricyclic antidepressants (Amitriptyline), Metoprolol Opioids (Codeine, Oxycodone) β-blockers
CYP3A4	Erythromycin Midazolam	Ifosfamide Nifedipine
NAT2	Caffeine Dapsone	Isoniazid Procainamide Amonafide [13]
TPMT	N/A	6-Mercaptopurine Azathioprine [16]

N/A: Not applicable-TPMT activity has been measured in erythrocytes using a radiochemical microassay [69].

available to identify new mutations. One common strategy for SNP screening requires the amplification of the gene of interest by polymerase chain reaction (PCR). Next, a conformation-based mutation scanning method is used and all positive PCR products are sequenced. Since the vast majority of individuals assayed may be negative, samples can be pooled together. This detects low frequency SNPs if a sensitive technology is used [21,22].

12.3.1 Single-strand conformation polymorphism (SSCP) analysis

One of the most commonly used methods for mutation detection is SSCP [23]. In SSCP, DNA regions with potential polymorphisms are first amplified by PCR. The PCR products are denatured and separated on a non-denaturing polyacrylamide gel. If the DNA contains a variant (single base modification, insertion, or deletion), it generally forms a different conformer that migrates differently when compared to wild-type DNA

[24]. The sensitivity of this method can be increased to 100% by coupling it with restriction enzyme fingerprinting [25] or dideoxy-sequencing fingerprinting [26].

12.3.2 Denaturing high performance liquid chromatography (dHPLC)

dHPLC detects polymorphisms by analyzing the DNA mobility of different heteroduplexes using chromatography under slightly denaturing conditions. This technique has been commercialized by two companies Transgenomic Inc. (San Jose, CA (WAVE®)), and Varian Inc. (Palo Alto, CA (Varian Helix-System®)). Both systems work on the same principle – temperature-modulated heteroduplex analysis [27]. Samples are hybridized with wild-type DNA to form a mixture of homo- and heteroduplexes. The heteroduplexes can be separated from the homoduplexes by HPLC at a temperature that partially denatures the mismatched DNA. A major advantage of this technology is that multiple samples can be pooled together for variant detection with increased throughput. These systems can also be used for several applications besides the detection of genetic variants such as, size-based double-strand DNA separation analysis, single-strand DNA separation, and analysis of DNA purification. Schwab et al. suggest dHPLC is a highly sensitive, rapid, and cost effective method for genotyping that also detects genetic variability [28].

12.4 IDENTIFICATION OF KNOWN POLYMORPHISMS

12.4.1 Gel-based methods

12.4.1.1 Restriction fragment length polymorphism (RFLP) analysis

The discovery of bacterial enzymes that cleave specific sequences in DNA has been used as a means to identify SNPs, small deletions or insertions. In general, restriction endonucleases recognize a specific four to six base long sequence of DNA and cleave it producing DNA fragments with blunt or sticky ends. Prior to enzymatic digestion, a specific region of DNA, containing the polymorphism (SNP, insertion or deletion) is amplified using PCR. The amplified PCR product is then digested with an appropriate restriction endonuclease. After digestion, the resulting products are separated by gel electrophoresis and stained for visualization. The main limitation of this methodology is that the polymorphism must alter or contain a restriction site. As a result, the polymorphism could result in the loss or gain of a restriction site that would change the DNA fragmentation (restriction digestion) pattern on the gel (Fig. 12.1). RFLP analysis has been successfully used to genotype *CYP2D6 *3* and **4* using restriction endonuclease *Msp1* and *Mva1*, respectively [29]. RFLP analysis can also be used as a genetic marker for disease, since RFLPs are more abundant when compared to polymorphisms at the level of protein [30]. In general, the gel-based RFLP analysis is relatively straightforward and can provide useful information. However, this labor-intensive methodology increases the time and cost, and is not suitable for large-scale clinical applications.

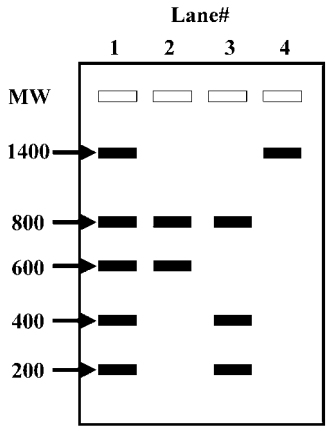

Fig. 12.1. A representation of RFLP analysis in which an amplified PCR product has been separated on an agarose gel and stained. Lane#1 contains the molecular weight markers; Lane#2 represents a wild-type PCR product that is digested once by a restriction enzyme; Lane#3 represents a PCR product that contains a SNP resulting in the gain of a restriction site; while Lane#4 represents a PCR product that contains a SNP resulting in the loss of a restriction site.

12.4.2 Fluorescent-based technologies

There are several techniques that utilize sequence-specific hybridization of fluorescent oligonucleotide probes, all of which have their unique advantages and disadvantages. In this section, selected techniques are reviewed in detail. Due to the increasing number of assays developed, others are summarized in Table 12.2.

New developments in polymerase chain reaction (PCR) equipment have made an impact on pharmacogenomic testing. The more recent technologies now have the capability to amplify and detect product formation during the PCR reaction, which is commonly referred to as real-time PCR. This ability represents a major advantage and time saving feature, since it does not require post-PCR analysis (i.e. gel electrophoresis).

12.4.2.1 Allele-specific amplification using real time PCR

One technology, the LightCycler®, Roche Molecular Systems (Pleasanton, CA), offers the real-time detection of sequence-specific SNPs. This method uses two fluorescently labeled probes that during amplification can detect specific single copy sequences in genomic DNA, or identify single-base mutations. The two probes – an anchor probe and mutation probe, each labeled with a different marker dye – hybridize in a head-to-tail arrangement to adjacent sequences on the target DNA as shown by Fig. 12.2. The two dyes can only interact when they are both bound to the target DNA in close proximity. The mutation probe, which contains the donor molecule, binds over the predicted site of the mutation. The anchor probe, which contains the acceptor molecule, produces the fluorescent signal. Once both probes bind, fluorescent resonance energy transfer (FRET) can occur between the two probes. In order for FRET to occur, the donor and acceptor

TABLE 12.2
OTHER SNP DETECTION ASSAYS

Method	Technique	Reference
MASDA (multiplex allele-specific diagnostic assay)	Utilizes oligonucleotide hybridization to interrogate DNA sequences on a solid support.	70
MADGE (microtiter array diagonal gel electrophoresis)	Modified gel electrophoresis based detection of large number of PCR reactions.	71
OLA (oligonucleotide ligation assay)	Inhibition of ligation due to mismatch at 3′ end.	72
DOL (dye-labeled oligonucleotide ligation)	OLA modified with use of fluorescence resonance energy transfer (FRET) detection monitored in real time.	73
LNA (locked nucleic acids)	Binding affinity dependent fluorescence release.	74
CFET (combinatorial fluorescence energy transfer)	Generation of unique fluorescence tag emission signatures.	75
DASH (dynamic allele specific hybridization)	Melting temperature dependent fluorescence release.	76
MD-PASA (multiple double PCR amplification of specific alleles)	PCR based haplotyping.	77
CASPA (consumed allele-specific primer analysis)	Determination of unincorporated primers upon completion of PCR reaction.	78

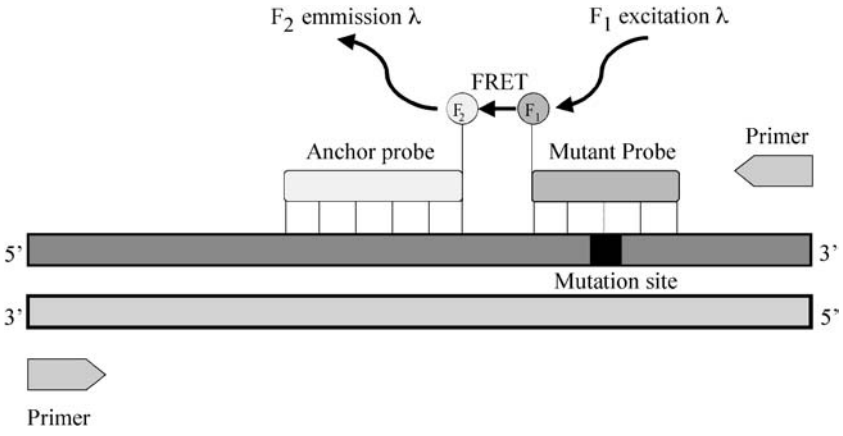

Fig. 12.2. A schematic of a single color one point mutation experiment on the LightCycler®.

molecules must be in close proximity (less than 5 bases apart) and the emission spectrum of the donor must overlap with the excitation spectrum of the acceptor. The design of the probes is relatively simple and software tools are available to help choose the probe sequences. In general, the melting temperature (Tm) of the probes is higher than the primers. The Tm of the anchor probe is also higher than the mutant probe. In addition, the binding site for the probes should be near the 3′ end of the target. This enables sequence fluorescent measurements before the primers are extended and the probes displaced by *Taq* polymerase.

The LightCylcer® can also provide sequence confirmation of the amplified product using melting curve analysis. After the PCR amplification is complete, the temperature is lowered so that the probes are hybridized to the DNA. The temperature in the reaction chamber can then be slowly increased while measuring the fluorescence at frequent intervals. Fluorescence will decrease with increasing temperature as the mutation probe is released and FRET ceases. If a SNP is present, the mutation probe has one base-pair mismatched and will melt off at a lower temperature than if the SNP is absent. This technique has been successfully applied to the identification of many SNPs in both clinical and research settings [5,31–34]. Mangasser-Stephan K et al. described simultaneous identification of both point mutations in the hemochromatosis gene *(HFE),* responsible for an autosomal recessive iron overload disease. In one PCR reaction, special fluorescently labeled oligonucleotide hybridization probes [31] are used. The usefulness of real time PCR has also been demonstrated by several groups through the detection of several *CYP2D6* polymorphisms (**3* and **4*) [5] and (**3*, **4*, **6*, **7* and **8*) [35] using melting curve analysis. The principle of discrimination between genotypes by melting curve analysis is also applied in the LightCylcer® detection kits (Roche Molecular Systems, Inc., Pleasanton, CA) for Factor V Leiden, Prothrombin, Apolipoprotein B and Apolipoprotein E. This technology has several benefits including rapid real-time analysis and a closed system to prevent DNA contamination.

12.4.2.2 Invader®

The Invader® system developed by Third Wave Technologies (Madison, WI) represents a nuclease-based approach for known polymorphism detection [36,37]. In the Invader® process, two DNA probes bind to the target to form a structure that is recognized by a proprietary enzyme (Cleavase®). The oligonucleotides (a wild-type or variant probe and an upstream Invader® probe) are designed so that they hybridize to and partially overlap a known SNP site. The binding of the upstream probe forces a small section (5′ end) of the overlapping probe (wild-type or variant probe) to remain non-hybridized (Fig. 12.3). The Cleavase® enzyme recognizes and cleaves the overlapping flap that then releases a small fragment of DNA (flap) [38,39]. The released flap undergoes a secondary reaction and forms another cleavage structure. The flap, bound to a 5′ fluorescently end-labeled probe (FRET™ cassette), has a donor fluorophore that is quenched by an internal acceptor dye. Cleavase® recognizes and cuts the FRET™ cassette/DNA flap structure resulting in a fluorescent signal. The Invader® system relies on this linear amplification of the signal rather than target amplification. Each flap (cleavage product) can generate thousands of signals per hour, yielding millions of detectable signals per target. The

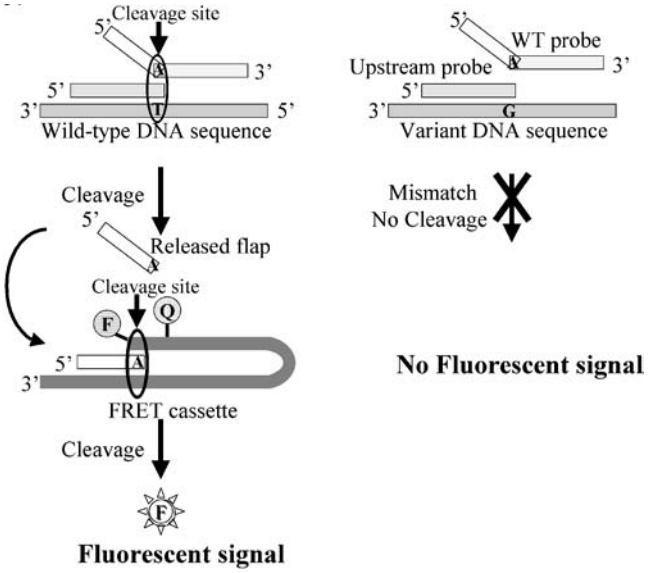

Fig. 12.3. A schematic demonstrating the principles used in the Invader® assay.

major advantage of this technology is genotyping directly from genomic DNA without PCR amplification. However, this technology has limited multiplexing capabilities and cannot detect small insertions or deletions [40].

An application analysis for high-throughput analysis was demonstrated by Mein et al. where 384 individuals were genotyped for 36 SNPs and one insertion/deletion polymorphism [41]. This study showed an average failure rate of 2.3% and an accuracy of 99.2% compared to results generated with established methods. Most of the failure was attributed to a PCR step. The major disadvantage is the need to assay each allele for a given SNP separately. A variation of the Invader® assay by Kwok et al. for high-throughput genotyping adopted a more cost effective detection method. Their modifications include identification of variation of both alleles of a SNP in a single reaction and switching the detection method from the FRET based detection to fluorescence polarization [42].

12.4.2.3 TaqMan™ DNA probes (real-time PCR)

TaqMan™ Allelic Determination is based upon the 5′ nuclease activity of *Taq* polymerase [23,43]. One pair of PCR primers is used to amplify the desired DNA region containing the SNP. In addition, two TaqMan™ probes that differ only at the polymorphic site are designed. One probe is made complementary to the wild-type allele and the other probe to the variant allele. Each probe has a different covalently attached reporter dye and a common quencher dye on the 5′ and 3′ end respectively. The probes bind to the polymorphic site of interest during the annealing phase of the PCR reaction. In the extension phase of PCR, the 5′ nuclease activity of *Taq* polymerase will

cleave the 5′ reporter dye from the perfectly hybridized probe, resulting in increased fluorescence. Only the perfectly hybridized probe (wild-type or variant) will be cleaved by *Taq* polymerase, resulting in the real-time detection of PCR products [44]. The ratio of the fluorescence from the two reporter dyes after the PCR reaction determines the genotype of the sample. The advantage of this methodology is that Applied Biosystems (Foster City, CA) has ready to use, validated assays for genotyping common polymorphisms, providing both the reagents and instrumentation. The main disadvantages are that dual-labeled probes and PCR amplification are needed for genotyping, increasing the cost of genotyping. However, this technology can easily be automated to increase throughput. Ranade K et al. reported genotyping of more than 1600 individuals for two separate SNPs, with an error rate of less than one in two thousand [45].

12.4.2.4 Depolymerization assay (ReadIt™)

The ReadIt™ SNP Genotyping System by Promega Corporation, (Madison, WI) uses the ability of READase™ Polymerase to catalyze the depolymeration (pyrophosphorylation) of DNA. In the presence of target DNA and inorganic pyrophosphate, the depolymerization reaction occurs and is driven by mass action. Under these circumstances, the READase™ polymerase catalyzes the addition of a pyrophosphate across the terminal phosphodiester bond, resulting in the release of the high-energy deoxynucleotide triphosphate (dNTP) from the 3′ end of the DNA template. The READase™ Kinase enzyme then transfers the terminal phosphate from the dNTP onto ADP forming ATP. In the presence of Luciferase, ATP formation results in light signal production. This is proportional to the amount of ATP formed in the coupled reaction (Fig. 12.4). The specificity in the ReadIt™ reaction is provided by the sequence specific hybridization of one or both oligonucleotide probes to the target DNA. The 3′ end of one DNA probe corresponds to the wild-type sequence while the other matches the variant sequence of

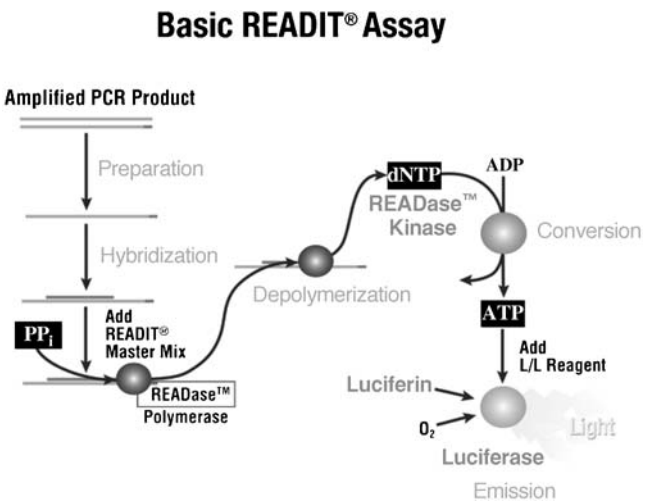

Fig. 12.4. A schematic showing the basic ReadIt™ assay for genotyping SNPs.

interest. The DNA probes must form a perfect homology at the 3′ end of the DNA sequence of interest for depolymerization to occur. The ReadIt™ Polymerase does not begin the coupled reaction unless the correct structure is formed. The ReadIt™ system can be used to analyze a wide variety of sequence variations including insertions, deletions, and SNPs. The ReadIt™ technology does require PCR amplification in which one primer contains three phosphorothioate linkages at the 5′ end to protect one strand from nuclease degradation. After PCR amplification, T7 Gene 6 Exonuclease digests the unprotected strand in the PCR product to produce single-stranded templates. The PCR product is then diluted and analyzed using the ReadIt™ reagents. Basically, the amplified target sequence is denatured, neutralized, and can then anneal to the correct sequence of the interrogation probes. In the presence of correctly paired target DNA and probes, ATP is generated and detected by using Luciferase. This technology has several advantages since it can be partially automated and be done in 96- or 284-well plates for high throughput analysis.

One recent application demonstrated that PCR-RFLP methods are readily adapted to the ReadIT™ SNP-genotyping format [46]. In this study, *CYP2C9 *2* and **3* were genotyped successfully for 23 unique samples. In fact, the ReadIt™ method correctly identified the *CYP2C9 *3* allele in two instances were PCR-RFLP required repeat testing. This methodology has also successfully been applied to identification of Factor V Leiden polymorphism by Hodges KA et al. [47].

12.4.2.5 Molecular beacons™

Molecular Beacons™ (MBs) detect single-nucleotide differences in DNA. Basically, MBs™ are hairpin shaped fluorescently labeled hybridization probes that can detect the presence of specific nucleic acids in a homogenous solution [48]. The MBs™ contain a fluorophore on one end of the oligonucleotide and a quenching moiety (non-fluorescent chromophore) on the other (Fig. 12.5). The DNA sequences on the ends of the probe are designed to be complementary to each other so that it forms a hairpin loop. The intervening loop portion of the probe is designed so that it is complementary to the target DNA sequence of interest. In the presence of target DNA, the loop of the MBs™ will undergo a conformational change as it hybridizes to the complementary target DNA. As the hairpin loop opens up, the fluorophore and quencher move apart and an increase in fluorescence is detected. However, an unhybridized probe in solution would form the hairpin structure that brings the fluorophore and quencher close together to allow quenching of the fluorophore. MBs™ have several advantages: the ability to use multiple fluorophores to detect several allelic variants in real-time, a sealed homogenous system that minimizes cross-contamination, and inherently more sensitive and specific linear probes. This is due to the stem loop formation that thermodynamically competes with the amplicon for hybridization [40,49]. These benefits have allowed this technology to be used for accurate heteroplasmy quantitation and mtDNA copy number estimation from small biopsy samples. Performing such tasks were previously not possible due to technical restrictions [50]. On the other hand sensitivity of this technique is further improved by wavelength-shifting molecular beacons, a modification that result in much brighter signal, described by Tyagi S et al. [51].

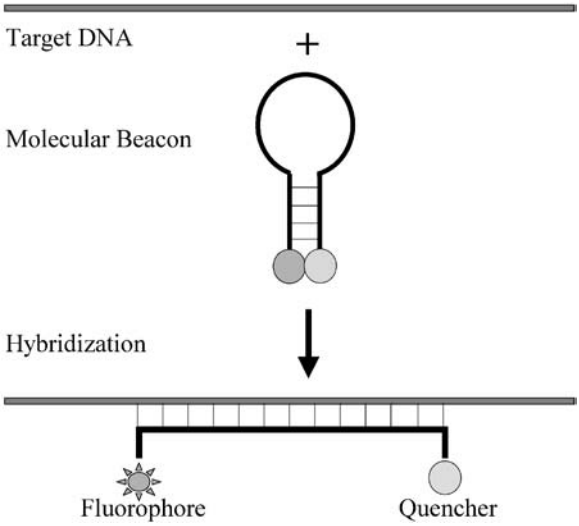

Fig. 12.5. A schematic showing the operation of molecular beacons.

12.4.2.6 Genotyping using the LCx® platform (Abbott Laboratories, Abbott Park, IL) [52]

As with all other techniques, this method requires amplification of target DNA. It is versatile in the sense that it can either utilize a standard PCR or LCR™ reaction that takes place in the LCx® platform. In the LCR™ reaction, the two primers are positioned closer to each other compared to conventional PCR. The gap between them is closed using a ligase instead of a polymerase. After purification of the genomic DNA, the sample is mixed with the assay mixture that contains the amplification reaction reagents and two probes. The amplification of the target DNA takes place in the LCx® analyzer. The probes in the assay mix are complimentary to either the wild type or variant allele of the gene of interest. Annealing of the probes is facilitated at the end of the PCR reaction in a melt-cool down cycle. Each probe contains a specific hapten that is recognized by a unique antibody, the latter is conjugated either to alkaline phosphatase or β-galactocidase. Incubation of the target/probe complex, in the presence of substrates methylumbelliferyl phosphate (MUP) and *N-(2-hydroxyethyl)–2-(7-b-D-galactopyranosyloxy-2-oxo-2H-chromen-4-yl)* acetamide (AUG) to alkaline phosphatase or β-galactocidase, respectively. This results in detection of positive signal in either MUP and/or AUG channels. Detection of positive signal in one or both channels signifies homozygosity or heterozygousity for the template. A schematic explaining the steps of this system is presented in Fig. 12.6.

12.4.2.7 Bioluminometric assay coupled with modified primer extension reactions (BAMPER) [53]

In determining the allelic frequency for a specific SNP, pooled DNA from several individuals is first subjected to PCR using biotinylated primers to amplify the target

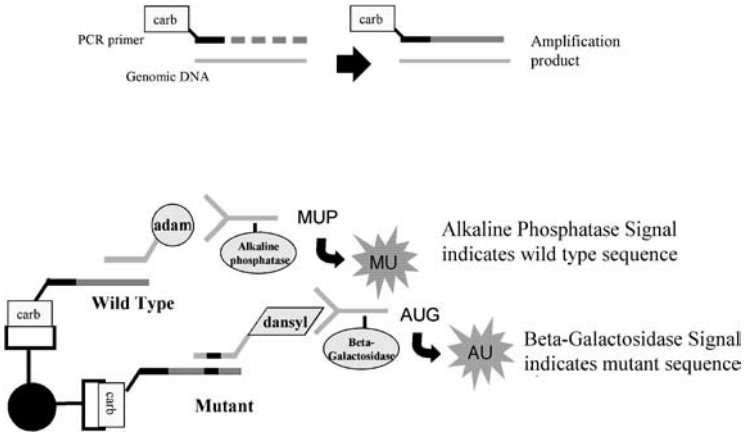

Fig. 12.6. A schematic representation of Abbott LCx® method.

region. Following the PCR reaction, single stranded DNA is obtained using bead technology and alkali treatment. The obtained single stranded DNA is treated with either one of the extension primers (wild type or mutant), and is allowed to go through primer extension. The primers contain artificial mismatches at the 3′-terminal region that improves the switching characteristics of the extension reaction. The release of subsequent PP_i from the extension reaction is coupled to the formation of ATP. Generated ATP is then detected by light production in a luciferin/luciferase reaction. A mismatch at the 3′ end will result in low or no light generation compared to wild type, correlating to the heterozygosity or allelic frequency of the target.

12.4.3 DNA sequencing

12.4.3.1 Pyrosequencing™ (Pyrosequencing Inc., Uppsalla, Sweden)

As the name implies, this technique is based on a sequencing concept. A signal is generated as dNTPs are incorporated. This method promotes the extension of the complementary sequence compared to standard sequencing reactions where terminations of DNA synthesis is monitored. Therefore, this system is considered a sequencing-by-synthesis reaction. Briefly, this system requires PCR amplification with biotinylated primers to generate the target region. Biotin incorporated during the PCR reaction is used for immobilization of the strands. Following the generation of single stranded DNA, sequencing primers and a mixture of enzymes (DNA polymerase, ATP sulfurylase, luciferase and apyrase) are added. The next step is the addition of one of the four dNTP's. If incorporation of the added dNTP is achieved, a PPi is released and coupled to the formation of ATP in the presence of adenosine-5′-phosphosulfate (APS). A visible light proportional to the amount of ATP is generated by the conversion of luciferin to oxyluciferin by luciferase activity. The light production is detected by a

charge coupled device (CCD) camera and seen as a peak in the PyrogramTM. Presence of apyrase ensures unincorporated dNTP's and ATP is degraded to switch off the signal. This process is followed one nucleotide at a time until the required sequence is obtained. Sequence information up to 30–40 bases can be obtained by this technique. Pyrosequencing has recently been shown to be an attractive alternative to SSCP analysis for the detection of N-*ras* mutations in malignant melanoma tumor samples [54]. This method has also been proven to be useful for forensic medicine by allowing a rapid turnaround time with less workload. In addition to this feasibility, application of this method has allowed sequencing of the coding region as well as the variable regions of the D-loop from mtDNA to increase discriminating power [55].

12.4.3.2 SNaPshot (Applied Biosystems)-MINISEQUENCING

One of the other basic sequencing techniques with numerous variations is called minisequencing. Common to all sequencing techniques is PCR amplification of the target DNA region containing the SNP of interest and generation of single-stranded DNA. All techniques require a solid support systems in which the PCR amplified single stranded DNA or the sequencing primers are immobilized on. The sequencing primers involved are only one base short of the polymorphism site and extension over the interrogation site is achieved. The PCR reaction contains one biotin labeled and one unlabeled primer. The amplified template can be immobilized on the solid matrix, through streptavidin-biotin interaction via the 5′ biotinylate incorporated into the PCR product. A wash step under alkali conditions forms the single stranded DNA that consequently hybridizes to the sequencing primer. In the presence of labeled dNTP's, ddNTP's and a polymerase, the genotyping reaction is achieved. Each dNTP harbors a different label; therefore for each reaction type, depending on the genotype, a different signal is obtained [56]. One such commercial system is SNaPshot™ from Applied Biosystems®. In an alternative approach reported by the Scripps Research Institute, the sequencing primers can be bound to a Teflon coated glass surface and act as individual wells. These primers then anneal to the single stranded DNA product of the PCR reaction adjacent to the polymorphic site. Single base incorporation of labeled ddNTP's is achieved via klenow fragment (3′–5′ exonuclease) and the signal identifying the incorporated base is detected. A reported improvement to the SnaPshot™ protocol is the **m**ultiplex **a**utomated **p**rimer extension **a**nalysis (MAPA) that can simultaneously genotype up to four SNPs accurately [57]. In this study, Makridakis et al. have introduced multiplexing at the initial PCR step, and/or of the primer extension reaction.

12.4.4 Mass spectrometry

Mass spectrometry based detection systems have become increasingly popular in many areas of science due to its high sensitivity to distinguish small mass differences with great accuracy. Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF-MS) techniques have been utilized in various DNA analysis; detection of PCR products, sequencing, genomic DNA characterization, single

nucleotide extensions, and others. A clinical application is the detection of cystathionine β-synthase (CBS) gene deficiency that result in homocystinuria [58]. MALDI-TOF-MS has also been used in detection of Toll-like receptor (TLR4) polymorphisms in patients to predict susceptibility to bacterial infection [59]. Most of these analyses require a purification step prior to MS analysis. The sensitivity of MALDI is increased by having a single positive or negative charge associated with the target. Sauer, S et al. has reported a modified procedure named "*GOOD ASSAY*" that uses this concept [60]. This assay has many advantages since it does not require a purification step and the reagents can be added in a single reaction tube followed by mass spectroscopy of the product. Briefly, this assay involves an initial PCR reaction where the target DNA is amplified. Prior to the primer extension reaction, degradation of dNTP's is achieved by incubation with shrimp alkaline phosphatase. However, if Tma 31 FS DNA polymerase is used for primer extension, this step can be omitted because this enzyme has a preference for ddNTP's. The extension primers containing a charged tag anneal immediately upstream of the SNP to be genotyped. Thermocycling in the presence of a specific set of α-S-dNTP and α-S-ddNTP results in allele-specific extension products containing phosphorothioate bridges. Following the extension reaction, the primer is digested by phosphodiesterase. However, the 3′ end of the primer is not susceptible to the digestion since it contains phosphorothioate bridges. Finally after an alkylation reaction, optimized only to add methyl groups to the bridge thiolates, the sample is mixed with a non-acidic matrix (cyano-4-hydroxy-cinnamic acid methyl ester) to be applied on to the target. Using this technique, SNP detection by the difference in generated mass is well over the detection sensitivity of the instrument. In the range of 1000–2000 Da the MALDI-TOF-MS has a resolution power of 4 m/z, and the smallest difference due to a SNP is 9 m/z, corresponding to an A-T change. Positively and negatively tagged products will only be detected in their corresponding mode allowing for multiplexing. Also the middle base of the extension primer harboring the amino-modification for the positive mode can be used as a mass tag location introducing an alternative for multiplexing.

The MassARRAY™ system from SEQUENOM® (San Diego, CA) is based on MALDI-TOF MS detection of a primer extension reaction that is loaded on to a chip for detection of allele specific masses. The PCR amplification uses biotinylated primers and is followed by strand separation and an extension reaction over the SNP of interest. The MASSEXTEND™ primer specific for the SNP of interest is extended by only one and two nucleotides to generate two extension product. This is controlled by the ddNTP concentrations. The denatured extension products are then added on to Spectro CHIPs™ for analysis by mass spectrometry using SpectroTYPER™ software.

A different application of SNP detection by mass spectrometry is the Survivor Assay [61] where single nucleotide primer extension (SNuPE) is detected with electrospray ionization mass spectrometry (ESI-MS). This technique utilizes single or double stranded DNA and measures the amount of unreacted ddNTP's rather than the extended primer. The conventional PCR reaction is followed by primer extension at the SNP site. The extension primers are complementary adjacent to the 3′end of the polymorphic site. A single nucleotide incorporation is allowed and the amount of remaining ddNTP's is measured using electrospray ionization mass spectrometry (ESI-MS).

12.4.5 Capillary electrophoresis

The MegaBACE™ genotyping system of Amersham Pharmacia Biotech® is a platform that provides analysis of microsatellites [62], DNA sequencing up to 1000 nucleotides, and SNP validation, by changing detection parameters and analysis software. This system can serve a dual function, capable of performing sequencing and genotyping. One disadvantage system of this system compared to a gel based system, is the requirement of highly purified template and accurate concentration determination. On the other hand, the loading of samples are performed electrokinetically so all the samples are injected simultaneously. MegaBACE™ 1000 has up to 96 capillaries. Unlike conventional sequencing reactions, termination is achieved by DYEnamic ET Terminators, modified ddNTP's harboring fluorescent labels. The excess terminators are cleaned up with ethanol precipitation. Genotyping by this method requires the amplification of target through a PCR reaction. Detection of the SNP relies on single base extension by PCR where the complementary-labeled ddNTP is incorporated at the polymorphic site, depending on the identity of the mutation. Fig. 12.7A is a schematic representing the identification of a SNP site. A homozygous will have a single peak with the corresponding dye compared to a heterozygous that will have two signal peaks corresponding to each dye. The detection system relies on excitation of the fluorecently-labeled extension reaction products, loaded on to the capillaries, through confocal scanning. The primers or the terminators utilized in the extension reactions have

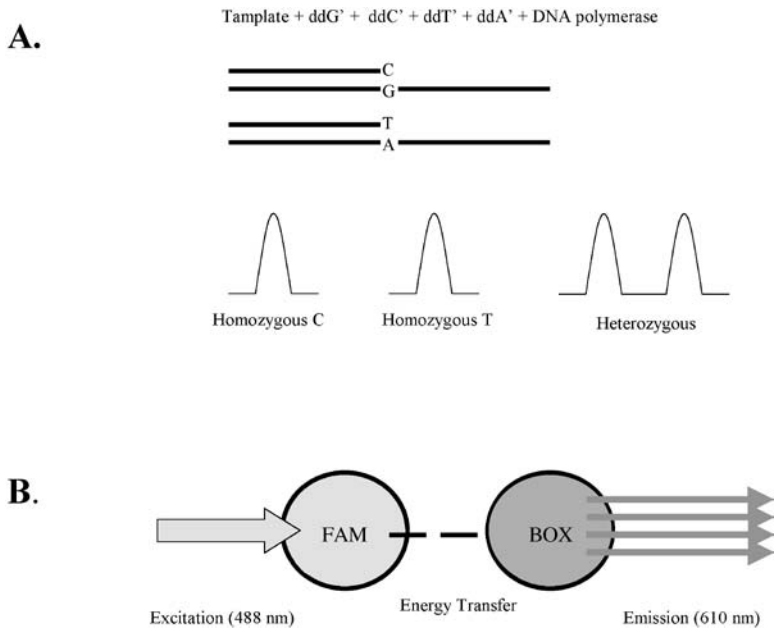

Fig. 12.7. (A) A schematic illustrating the expected signals for capillary electrophoresis. (B) A representation of energy transfer dye technology.

fluorophores and the system uses a patented energy transfer dye technology (ET). For excitation of the dyes, a blue (488 nm) and a green (532 nm) light is used. These donor dyes are incorporated into the target sequence during PCR amplification. The donor dye (FAM) is excited by a laser light, the absorbed energy is then transferred to the acceptor dye (ROX) at its characteristic wavelength (Fig. 12.7B).

An application of capillary electrophoresis in combination with ASPCR (allele-specific PCR) and ET (energy transfer) primers has been demonstrated in the identification of hemochromatosis-related mutations by Mathies RA et al. In this study, 96 reference samples were tested for three common variants, (C282Y, H63D, and S65C), by ASPCR, and products were simultaneously separated in less than 10 min. by the difference in their size and the incorporated fluorescent label [62]. Another application that can utilize any commercial available instrument, without the need for special probes, is the separation/identification of multiplexed PCR products for simultaneous detection of biallelic polymorphisms. Ulvik A et al. has demonstrated such an application by the simultaneous identification of methylenetetrahydrofolate reductase (MTHFR) C677T, and factor V G1691A along with their corresponding normal alleles [63].

12.4.6 DNA microchips

Several high-density oligonucleotide arrays or DNA chips are being manufactured and used to determine genetic expression profiles from genes or the detection of SNPs. These DNA chips contain short oligonucleotide sequences that are anchored on glass supports. One major advantage of these chips is the amount of information that can be obtained. A single chip can provide information on thousands of genetic regions. Affymetrix (Santa Clara, CA) has a DNA analysis product (GeneChip®) that combines high-density microarrays, scalable assays and automated genotype calling. The GeneChip® Mapping 10K array system can genotype 10,000 SNPs with only 250 ng of genomic DNA. Affymetrix has another product under development that will expand the genotyping capability to 100,000 SNPs in a single experiment. Currently, cancer research studies are using DNA chips to determine gene expression profiles of RNA fragments from tumor tissues and comparing them to normal tissues to determine which genes are differentially regulated. This technology allows for the large-scale screening to simultaneously detect thousands of SNPs that have clinical relevance. Furthermore, this technology can be fully automated for large-scale analysis. However, the expense of these chips is one limiting factor for the widespread clinical application of this technology. In addition, the volume of data that is generated requires huge databases and its clinical significance is still not fully understood. Although as the cost decreases and our understanding of the information provided by the chips increases, the potential use of this technology will rise.

12.4.6.1 eSensor™ DNA detection system

Currently, Motorola Life Sciences (Pasadena, CA) manufactures a less expensive and smaller scale DNA chip for detecting key SNPs in several *CYP* isoforms (*2D6*, *2C9*, and

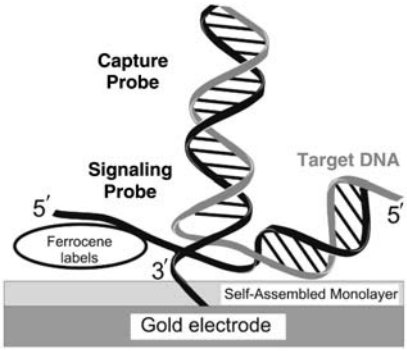

Fig. 12.8. A schematic showing the eSensor™ DNA detection system for polymorphisms.

2C19). The desired region of genomic DNA containing the polymorphism (DNA base substitution/deletion) is first amplified by PCR in a multiplex reaction. The resulting PCR product is digested with Lambda Exonuclease to form single-stranded DNA which is loaded with the signaling probes and assay buffer onto an eSensor™ Biochip cartridge. If present, the target DNA hybridizes to the complementary capture and signaling probes, bringing the specific electrochemical labels on the signaling probe near the electrode surface (Fig. 12.8). The eSensor™ utilizes alternating current voltammetry to detect the resulting positive or negative electrochemical signals from each electrode on the eSensor™ cartridge. This data is then used to determine which of the *CYP* polymorphisms are present or absent in the target DNA. Both the wild-type and variant signaling probes are labeled differently. This allows the eSensor™ system software to examine the positive electrochemical signals detected to determine whether the wild-type and/or variant sequences of a specific *CYP* gene are present in the target sequence. The eSensor™ DNA Biochip cartridges have been shown to be a simple and accurate methodology to genotype selected mutations of *CYP2D6* genes encoding the drug metabolizing enzymes [64]. This technology has several advantages: the simplicity, the ability to detect multiple polymorphisms simultaneously, and a homogenous sealed system. The main limiting factor is that a maximum of 48 individuals can be genotyped on the eSensor™ 4800. However, the hybridization and analysis time is only two hours, so multiple batches of 48 can easily be performed during a workday.

12.4.7 Flow cytometry [65]

Comparison of all the methods to detect SNPs that were out lined in this chapter reveal that most are based on primer extension, but differ in their capabilities and limitations depending on the method of detection. Yet another detection technique is the use of flow cytometry. The modification required for detection by this system is the generation of fluorescently-labeled PCR amplification products and attachment of the target-probe complex to microsphere beads that are fluorescently labeled.

12.4.7.1 GammARRAYS

GAMMArrays [65] is a combination of primer extension and mini-sequencing using micro-spheres as a solid support and flow cytometry for detection. Briefly, a mini-sequencing reaction is performed with fluorescent ddNTP's to extend a biotinylated primer that is adjacent to the mutation site. Four parallel reactions are performed; in each reaction one of the ddNTP's carries a different fluorescent label and three unlabeled dideoxy-bases. Using strepavidin- or avidin-coated microspheres, the extension reaction products are separated and sorted out by the flow cytometer to quantify the amount of incorporated label.

12.4.7.2 ZipCodes™

A combination of oligo liation assay (OLA) coupled to flow cytometry [66] detection is provided by Glaxo-Wellcome®. This system utilizes allele-specific capture probes. Each capture probe contains a unique 25 base sequence at the 5′ end, called ZipCode™. The ZipCodes vary at their 3′ ends defining each allele. The 3′ end of these capture probes are complementary to the target sequence and include one of the four bases at the interrogation site. The reaction also contains a reporter probe that harbors a fluorescent label and a phosphate at the 3′ and 5′ ends respectively. If the base at the 3′ end of the capture probe is complementary to the polymorphic site, the presence of a ligase the reporter and capture probes will be ligated. The 25 unique nucleotides identifying

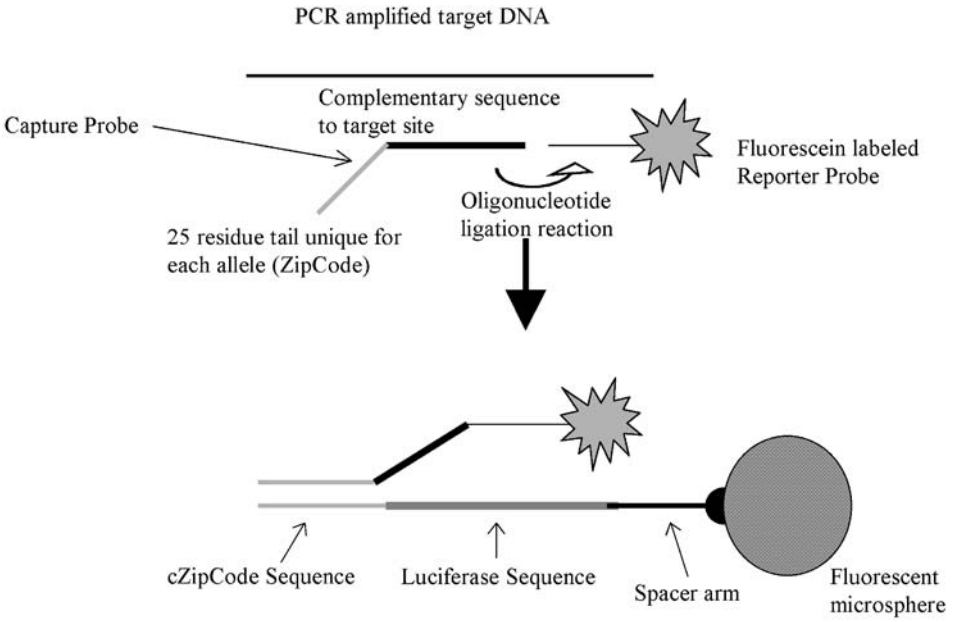

Fig. 12.9. A schematic description to illustrate the special correlation between ZipCode, cZipCode, capture, and reporter probes.

the sequence at the 5′ end are specific for each allele. Analysis of the SNP site is achieved by coupling the ligated capture-reporter probe product to fluorescent microspheres via complmentary zipcode sequences (cZipCode). cZipCode sequence contain complementary sequence to the 25 base allele specific ZipCode and is chemically coupled to the fluorescent microspheres (Fig. 12.9). The genotype is determined by the unique combination of fluorescence provided by the microspheres and the reporter probe.

12.5 CONCLUSION

A large number of genotyping technologies are available to detect known and unknown polymorphisms. The success and clinical application of pharmacogenomics depends on user-friendly, rapid, accurate techniques with good throughput. Table 12.3 lists some of the key components of an ideal genotyping system, according to Dennis O'Kane [7].

TABLE 12.3
PROPERTIES OF AN IDEAL GENOTYPING SYSTEM[7]

- High sensitivity (no false-negatives)
- High specificity (few false-positives)
- Rapid turn-around time (hours)
- Inexpensive
- High throughput
- No sequence bias in mutation detection
- No sequence information required for mutation detection

TABLE 12.4
COMPARISON OF SELECTED GENOTYPING TECHNOLOGIES*

Technique	Manufacturer(s)	Advantages	Limitations
RFLP	N/A	Simplicity	Polymorphism must alter/create a restriction site
LightCycler®	Roche Diagnostics Corp.	Rapid, real-time detection, closed system	Limited multiplexing capabilities
ReadIt™	Promega Corp	Can be automated for high-throughput	Requires PCR amplification
Pyrosequencing™	Pyrosequecing Inc.	Simplicity	Requires PCR amplification
DNA biochips-eSensor™	Motorola Life Sciences	Detection of multiple polymorphisms	Requires PCR amplification

N/A: Not applicable.
* Based on author's experience.

Each of the discussed methods above has specific advantages and can be used in a variety of settings. However, each method also has its limitations. Based on the authors' experience, Table 12.4 lists the pros and cons of some genotyping methods. Since the number of genotyping technologies is so large, it is impossible to review each one in detail. As a result, a list of other miscellaneous genotyping methods with unknown future potential has been compiled (Table 12.2). Ultimately, each laboratory will have to choose the technology that best fits their needs.

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CHAPTER 13

Applications of genotyping and phenotyping for clinically-relevant polymorphisms of drug metabolizing enzymes and drug transporters

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13.1 INTRODUCTION

More than 5% of in-patients suffer from serious adverse drug reactions, and a large fraction of these reactions could have been avoided by appropriate selection of drug and/or drug dose [1]. A main reason for such adverse reactions is the heterogeneous response of patients to standard treatment which is caused by heterogeneity in the disease(s) treated, in drug targets, in comedication, in other environmental factors, and/or in drug concentrations. Heterogeneity attributable to drug concentrations may be handled by a personalized dose adjustment in order to reach a therapeutic concentration range, with considerable improvement of the risk/benefit ratio of individual drug therapy. This may be done based on clinical response, on drug concentration (=therapeutic drug monitoring), or on factors known to predict clinical response and/or drug concentrations.

Drug metabolizing enzymes and drug transporters mediate rate-limiting steps for pharmacokinetic processes of many important drugs. The activity of such enzymes and transporters in man depends on genetic and environmental influences and usually shows a more than 10-fold variation within and between subjects [2]. Individual activity of the respective protein may have a major impact on the pharmacokinetic properties of its substrates and therefore may be of outstanding importance for therapeutic efficacy and safety of drugs [3]. Thus, for a fraction of drugs it would be desirable to take individual activity of such enzymes and transporters into account as the basis for optimizing therapy by individual dose adjustment.

Actual activity of many important enzymes in a subject may be determined by administration of a selective substrate for this enzyme and subsequent determination of

References pp. 343–353

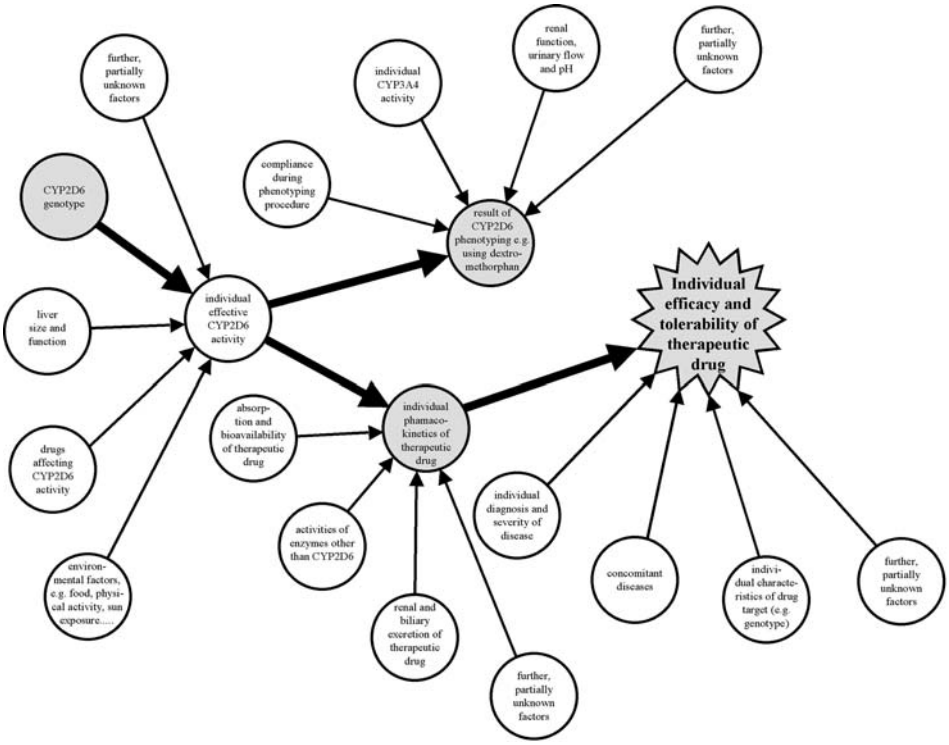

Fig. 13.1. Schematic presentation of the influence of CYP2D6 genotype on apparent CYP2D6 phenotype and on the action of a therapeutic drug metabolized by the enzyme. Thick arrows reflect a presumed major influence, shaded symbols represent processes that are accessible by direct measurement.

pharmacokinetic parameters reflecting activity of the enzyme. This procedure is called *phenotyping*. The respective metric used may be systemic clearance of a drug eliminated exclusively by this enzyme, or partial clearance for the respective metabolic pathway. Other parameters such as ratio of metabolite over parent compound in plasma, saliva and/or urine are also often used [4,5].

If a genetic polymorphism is the main reason for interindividual variation in enyzme or drug transporter activity, individual enzyme activity may be predicted by identification of the respective individual allele variants [6]. This procedure is called *genotyping*. It is potentially useful only if the functional impact of the individual alleles is known. By genotyping, other factors influencing enzyme activity such as liver disease or drug–drug interactions cannot be taken into account; on the other hand, these factors do not confound the result.

However, the existing phenotyping and genotyping methods to date have one major limitation: It is often not clear to which extent these data indeed reflect enzyme or transporter activity in vivo or whether other individual factors (e.g. renal function, activity of other enzymes) have a more pronounced impact [7]. Furthermore, even if the activity of individual enzymes can be measured or predicted accurately, it remains

to be shown that this information indeed improves the risk/benefit relation of therapy with a given drug. The relationship between genotyping, enzyme activity, phenotyping, pharmacokinetics and pharmacodynamics is explained for a CYP2D6 substrate in Fig. 13.1, showing that these aspects are closely related but need not be predictive for each other. Unfortunately, prospective studies with clinical endpoints in the field are sparse. Finally, some of the test drugs used for phenotyping have adverse effects that are not really harmful but relevant for the clinical use, e.g. sedation by midazolam used for CYP3A4 phenotyping. For the reasons described above, applications of genotyping and phenotyping for drug metabolizing enzymes and drug transporters today are limited to experimental settings with few exceptions, but there is the potential for a very broad use. The objective of this article is to describe the rationale for phenotyping and/or genotyping with respect to those drug metabolizing enzymes and drug transporters for which the actual evidence suggests that these procedures indeed may be useful to optimize individual drug therapy with its substrates. In addition to the role of diversity in these proteins for drug therapy, such enzymes and transporters are also related to carcinogenesis and other diseases; this however is beyond the scope of this chapter.

13.2 THIOPURINE-S-METHYLTRANSFERASE (TPMT)

Thiopurines like azathioprine [AZA], 6-mercaptopurine [6-MP] and thioguanine are efficient immunosuppressive and cytotoxic agents for the management of inflammatory bowel disease, rheumatoid arthritis, acute lymphoblastic leukemia (ALL) and graft-versus-host disease. However, the application of thiopurine derivates is complicated by a high incidence of severe adverse reactions, the most important of which is myelotoxicity resulting in discontinuation of therapy or even fatal outcomes in patients who were treated with standard doses. Thiopurine-S-methyltransferase (TPMT) is the main enzyme in the complex metabolism of thiopurine-based drugs converting the active compounds into inactive metabolites. While about 89–94% of Caucasian populations show a high enzyme activity approximately 6–11% exhibit an intermediate and 0.3% a low catalytic activity. In the latter cases, toxic thioguanine nucleotides are largely generated and it is well established that people who lack a high TPMT activity are prone to an increased incidence of thiopurine-induced adverse events [8,9]. On the other hand, a high TPMT activity corroborates with a higher subsequent relapse rate in children with ALL [10]. The interindividual differences in enzymatic activity are caused by genetic polymorphisms and to date, 11 mutant alleles have been identified associated with a reduction or a complete loss of TPMT activity. Epidemiologic studies have revealed the alleles *2, *3A and *3C to be most frequent in Caucasians while additional alleles have only been reported in isolated cases and there are considerable inter-ethnic differences [11]. The catalytic activity of cytosolic TPMT in erythrocytes correlates well with the hepatic activity and phenotyping methods that are based on the determination of cytosolic TPMT activity by radiochemical assays and HPLC techniques are well established [12,13]. Phenotyping is routinely applied by a number of centers like the Mayo Clinic, Rochester, MN. Genetic TPMT deficiency is highly predictive for myelosuppression due to AZA/ 6-MP-treatment with standard doses although there are

various other causes. Out of 41 patients with Crohn's disease and AZA/ 6-MP-related hematotoxicity, all four homozygous mutant patients developed bone marrow toxicity within 6 weeks after start of treatment and all 7 patients heterozygous for variant alleles showed toxic adverse events by 18 months after beginning of chemotherapy. However, variant alleles were not detected in the remaining 73% of patients with myelosuppression [14]. TPMT-genotypes especially accounted for myelotoxicity during the initial period of treatment while later cases tended to be probably caused by immunological factors. In a recent study, 2 of 5 patients with inflammatory bowel disease suffering from AZA/ 6-MP-induced toxicity were carriers of mutant TPMT alleles indicating that genetic impacts can only explain the minority of cases but are highly predictive with a specificity near 100%. In contrast, gastrointestinal adverse events like hepatotoxicity and pancreatitis seem not to be induced by TPMT deficiency [15]. Reduction of dose to one tenth of standard dosage has been shown to prevent TPMT-deficient patients from hematotoxic side effects while it upheld therapeutic efficacy [16,17].

For the vast majority of cases phenotyping and genotyping strategies are equally suitable. However, phenotyping can be affected by several factors and some cases of misinterpretation have been reported. For instance, patients with a TPMT-deficiency might misleadingly demonstrate normal enzyme activity after transfusion of erythrocyte concentrates from a homozygous wild-type individual [18,19]. Additionally, phenotyping results might be compromised by concomitant therapy with furosemide, sulphasalazine, salicylic acid and derivates, certain food and uremia [20,21].

TPMT is one of the classic examples for a promising link between pretherapeutic screening and clinical practise. Consequently, determination of the TPMT-metabolizing status should become routine usage before commencing AZA/6-MP-therapy in order to identify patients at high risk of myelotoxicity and to adjust the individual drug dosis in these cases. The method to choose – phenotyping or genotyping – is dependent of disposibility and the individual case. However, unambiguous identification of a competent TPMT metabolizing status does not preclude the occurrence of toxic side effects. Therefore, regular examination of leucocyte blood count and a permanently mindful observation of the clincal symptomatic is necessary during therapy with AZA/ 6-MP even if data on individual enzyme activity is available.

13.3 DIHYDROPYRIMIDINE DEHYDROGENASE (DPD)

Since decades the chemotherapeutic drug and pyrimidine analogue 5-fluorouracil (5-FU) has been widely used in the treatment of, e.g. colorectal, breast, head, and neck cancers [22]. 5-FU pharmacokinetics demonstrate a considerable inter- and intra-individual variability, depending on dose, route and schedule of administration, and other, partially unknown factors of influence. In humans, more than 80% of administered 5-FU is eliminated in the liver by biotransformation through DPD, the rate limiting enzyme that catabolizes 5–FU into 5-fluoro-5,6-dihydrouracil (5-FUH_2), thereby regulating its pharmacokinetics, efficacy and toxicity [23–25]. DPD is a saturable enzyme whose Km for 5-FU is approximately 5μM [26]. This characteristic explains the nonlinearity of 5-FU pharmacokinetics observed during intravenous bolus

injections, because 5-FU blood concentrations around the end of the bolus injection may equal or exceed the Km value of DPD [27,23]. Three to five percent of the Caucasian populations are estimated to demonstrate a reduced catalytic activity [28]. Application of 5-FU is restricted by a narrow therapeutic index because of severe toxicity of WHO grade III-IV including mucositis, granulocytopenia, neuropathy and death [29]. Severe side effects have also been reported for the prodrug capecitabine [30]. The enzymatic activity can be measured in peripheral mononuclear blood cells (PMBC) [31]. However, the correlation with hepatic DPD activity and 5-FU-pharmacokinetics is poor and the predictive value for severe toxicity is low so that this kind of phenotyping approach is not reliable [32].

Polymorphisms within the DPYD gene have been reported with deficiency in enzyme activity [25]. To date, 31 mutations within coding sequence and 5′-UTR have been detected in affected patients [33]. The most frequent one associated with severe 5-FU-related toxicity in cancer patients is a G > A nucleotide substitution at the 5′-splice donor site of exon 14 (1905 + 1G > A) (Fig. 13.2). According to a large investigation in 851 German Caucasians the prevalence of heterozygotes was determined at 0.94% while 24% (6/25) of selected patients who had experienced severe 5-FU-related toxicity were carriers of the so-called exon 14-skipping mutation. The mortality was 50% among the affected patients [34].

The clinical value of genotyping for the exon 14-skipping mutation or comprehensive searching for deleterious mutations of the DPD gene is a matter of an ongoing debate. In a retrospective study of 37 patients exhibiting severe toxic side effects owing to 5-FU-based therapy, 59% had a reduced DPD activity in PMBC. Direct sequencing of all exons in 14 patients allowed detection of the underlying genetic defect only in 57%. However, three quarters of the latter individuals were heterozygous for the splice

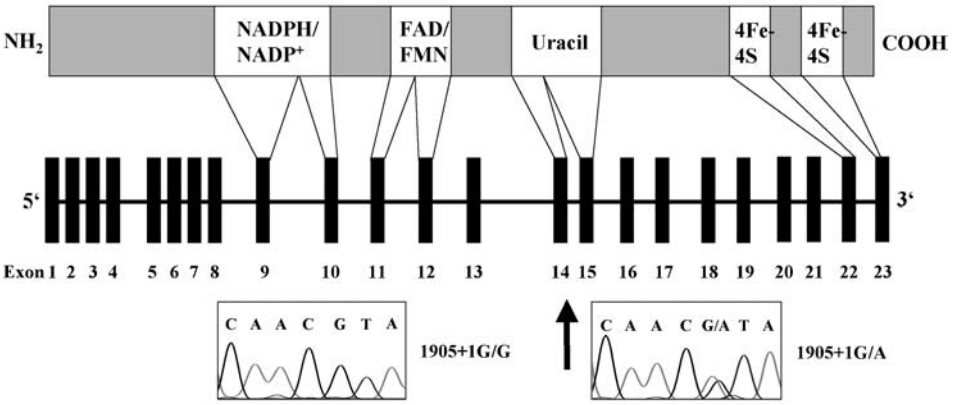

Fig. 13.2. Schematic overview of the 23 exons of the DPYD gene and a number of corresponding functional domains of the DPD protein. The uracil binding domain is encoded by the exons 14 and 15. Below, the elctropherograms of a homozygous wild-type individual for the 5′-splice donor site of exon 14 and of a heterozygous carrier of the exon 14 skipping mutation are displayed. NADPH/NADP$^+$: putative consensus binding site for nicotinamoide-adenine dinucleotide phosphate; FAD/FMN: putative consensus binding site for flavin-adenine dinucleotide/flavin mononucleotide; 4Fe-4S: iron sulfur cluster.

mutation. In the present study, screening for the exon 14-skipping mutation allowed for identification of high-risk patients with almost complete predictivity but a poor sensitivity of 25% [32]. Contrarily, selective screening for known mutations in another sample of cancer patients undergoing 5-FU chemotherapy detected patients with severe toxicity at a sensitivity of 17%. The sole carrier of the exon 14-skipping mutation demonstrated a normal catalytic activity [28].

The quantitative genotype-phenotype-correlation with regard to severe 5-FU-induced toxicity is currently subject of two major, prospective studies. Preliminary data suggest that the predictive value of the exon 14-skipping mutation is low [35]. However, there is increasing evidence that complete DPD deficiency because of occurrence of two "null-alleles" is highly prone to severe toxicity and that the exon 14-skipping mutation might often exert its influence together with undetected deleterious mutations located elsewhere within the DPD gene. Recently, two cases of combined heterozygosity for the exon 14-skipping mutation and additional mutations related to fatal outcome due to 5-FU therapy have been reported [36,37]. The risk for severe adverse events in case of presence of the exon 14-skipping mutation has been calculated to be 51.5% [38]. Following this assumption, prospective screening for the respective polymorphism and withholding of carriers of mutant alleles from 5-FU therapy might prevent 22.5% of cases of severe toxic events. Originally, on the basis of the data mentioned the German working group for internal oncology (AIO) and the society of haematology and oncology (DGHO) had given the recommendation to screen for the exon 14-skipping mutation before commencement of 5-FU chemotherapy [39]. Although since then, the clinical data available has not fundamentally changed the general advice is not upheld anymore and genotyping for DPD polymorphisms is recommended only for clinical trial settings instead [40,41]. The recent official statements are contradictory and waive the tremendous risk for fatal outcomes at least for exon 14-skipping mutation homozygous individuals.

There is ongoing controversy about the value and the demand to investigate a patient's metabolic status or genotype with respect to DPD. This is mainly because of two reasons. One reason is an undoubted but relatively weak correlation between DPD deficiency and toxicity or between genotype and DPD deficiency, respectively. The other reason is that an adjusted dose regimen according to the individual phenotype or genotype still remains to be found. Despite the conflicting and partly disappointing results of the previous genotyping and phenoytyping strategies, a novel promising approach to monitor pharmacokinetics of cancer patients treated with 5-FU and to predict drug response might be the determination of the ratio of the parent compound and its metabolite (5-FUH_2) in plasma. So far, clinical studies of small sample sizes suggest satisfactory correlation of 5-FU/5-FUH_2-ratio with therapeutic response and toxicity [42–44].

13.4 THYMIDYLATE SYNTHASE (TS)

The metabolism and efficacy of fluoro-pyrimidine-based chemotherapy provides an impressive example for a multigenic trait of drug response and the complex cooperation

of at least two genetically polymorphic enzymes involved in the metabolism of 5-FU and other antineoplastic agents. The physiological function of thymidylate synthase (TS) is the conversion of deoxy-uridine monophosphate (dUMP) to deoxy-thymidine monophosphate (dTMP) which represents the only metabolic pathway for a cell to generate thymidylate for DNA synthesis [45]. TS is inhibited by the 5-FU metabolite 5-FU-dUMP as well as by raltitrexed and the antileukaemic agent methotrexate (MTX), thereby exerting cytotoxic effects. It is well known for long that the level of TS expression is regulated by a variable tandem repeat polymorphism (VNTR) within its 5′ untranslated enhancer region [46]. Either two (2R) or three (3R) 28-base-pair repeats have been identified and the 3R allele has been shown to be correlated with higher TS expression and activity [47].

Several studies consistently indicated that TS expression is a key determinant of response to infusional treatment with 5-FU in patients with advanced colorectal cancer. For example, in a study of 50 affected patients 50% of the homozygous carriers of the 2R allele clinically responded to a 5-FU therapy while only 15% and 9% of the patients with a 2R/3R genotype and with a 3R/3R genotype were sufficiently chemosensitive, respectively [48]. Similar results have been reported by others. Furthermore, there is ample evidence that the VNTR TS polymorphism has a prognostic impact on 5-FU-based adjuvant chemotherapy and predicts survival. In a study of 221 patients with colorectal carcinoma at Dukes' stage C the short repeat allele was associated with a significantly better long-term survival. Contrarily, individuals with a 3R/3R genotype did not benefit from 5-FU treatment [49]. Overexpression of thymidylate synthase in metastatic tumor tissues was found to be an independent factor of poor prognosis in overall survival [50]. Additionally, homozygosity for the 3R allele was associated with increased expression of thymidylate synthase and poorer outcome of acute lymphoblastic leukaemia among 205 children treated with methotrexate compared with patients carrying the double repeat version [51].

Taken together, high levels of TS expression have abundantly been shown to be related to poorer clinical response to fluoropyrimidine-based therapy and less survival benefit in advanced disease patients with colorectal cancer (CRC). TS expression is normally evaluated by immunohistochemistry or RT-PCR. However, TS expression in primary colorectal cancer cells did not correspond to the level of expression in metastases and clinical outcome was only associated with TS expression in metastatic cells in some studies [52,53]. Thus, the predictive value of genotyping of thymidylate synthase for the individualization of treatment for patients with CRC or acute lymphoblastic leukaemia needs further elucidation.

13.5 N-ACETYLTRANSFERASE TYPE 2 (NAT2)

It is known since more than four decades that the rate of acetylation of clincally important drugs including isoniazide, sulfamethazine, sulfasalazine, procainamid, hydralazin and dapsone shows pronounced interindividual variation and is under control of a genetic polymorphism [54–56]. Initially, subjects have been classified as "rapid acetylators" or "slow acetylators" based on phenotype with respect to acetylation of a

substrate to NAT2. Starting in the mid-1980s, the genetic background of this polymorphism has been elucidated [57]. The enzyme involved has been termed Arylamine N-Acetyltransferase Type 2 (NAT2). It is located on chromosome 8p22 and has a single, intron-free open reading frame with 870 base pairs without for the coding sequence; NAT2 mRNA consists of the protein encoding exon and a second, non-coding exon. The protein has 290 amino acids and a molecular weight of 34 kD. Currently, 17 SNPs have been found in the coding sequence (2 of which silent), 8 SNPs in the 5′-flanking region and 19 SNPs in the 3′- flanking region. A wide range of 35 resulting variant alleles have been described for NAT2, including NAT2*4 (=wild type), NAT2*5[A-I], NAT2*6[A-E], NAT2*7[A-B], NAT2*10, NAT2*11[A-B], NAT2*12[A-D], NAT2*13, NAT2*14[A-G], NAT2*17, NAT2*18 and NAT2*19 [57,58]. Presence of at least one wild-type allele (termed NAT2*4) or a high-activity variant allele (NAT2*12) is related to overall high NAT2 activity (rapid acetylator), whereas slow acetylators have two low-activity variants. Obviously, both phenotype groups are heterogeneous, and some researchers also discriminate intermediate acetylators, i.e. subjects with one high-activity NAT2 allele), but these have considerable overlap with rapid acetylators with two high-activity NAT2 alleles, although current evidence indeed suggests a gene-dose effect with co-dominant regulation of NAT2 activity.

The fraction of individuals with at least one highly active NAT2 allele (=rapid acetylators) varies markedly between populations. The highest frequency occurs in Eastern Asia (58 to 90%). Studies in other parts of Asia and in Caucasians have reported lower frequencies between 32 and 43%. The lowest frequencies have been found in two small African studies [59,60].

The appropriate procedure for genotyping depends on the population to be investigated. For instance, testing for only 6 SNPs (G191A, C282T, T341C, C481T, G590A, A803G, G857A) can identify >99.9% of all slow acetylators in Central Europeans. Phenotyping most frequently is carried out using a secondary metabolic step of caffeine. Urinary metabolite ratios reliably identify slow and rapid acetylators and match phenotyping results if advanced analytical techniques are used [61].

Isoniazid is the NAT2 substrate for which most information with regard to the effect of enzyme activity is available. Numerous studies have demonstrated that the metabolism of isoniazid varies widely among different individuals, with a mean apparent elimination half-live of 80 minutes for rapid acetylators and 180 minutes for slow acetylator [62]. Despite these marked differences in isoniazid elimination, it seems that in general rapid and slow acetylators respond equally well to standard daily isoniazid treatment for tuberculosis. However, early studies showed that not only isoniazid concentrations but also therapeutic efficacy and tolerability in the treatment of tuberculosis may be different between slow and rapid acetylators. This difference had been assessed e.g. for the combination of streptomycin and isoniazid. It was most pronounced for weekly treatment (efficacy observed 76% vs. 56% of patients, respectively), but was still present in the case of initial daily treatment for 13 weeks (100% vs. 85%) [63]. These studies based on NAT2 phenotype have been completed by more recents genotyping studies. In a trial in 60 patients with tuberculosis, the apparent elimination rate constant and the area under the isoniazid concentration-time curve over the interval 2 to 6 h after oral isoniazid were determined, and NAT2 genotype was

determined in 47 patients. Three eliminator phenotypes could be distinguished, and concordance between the phenotype and the genotype of the individual could be demonstrated. Slow acetylators had 4–6 fold higher isoniazide concentrations than rapid acetylators, and concentrations in intermediate acetylators were in between, prompting the authors to propose an individualized isonaizide dosing regimen [64]. Adverse effects of isoniazid therapy also appears to depend on NAT2 activity. Most but not all studies suggest that hepatic toxicity is observed more often and is more severe in slow acetylators, neurotoxic effects that may be overcome by vitamin B6 supplementation are more pronounced in slow acetylators, and there is circumstantial evidence that systemic lupus erythematodes occurs more frequently in this group of patients [65]. In a recent study conducted in Japanese in-patients with pulmonary tuberculosis, the relative risk for isoniazid + rifampicin-induced hepatotoxicity was 4.0 (95% CI 1.94–6.06) for intermediate and 28.0 (95%CI 26.0–30.0) for slow acetylators compared with rapid acetylators as predicted according to genotype [66]. Accordingly, the authors propose NAT2 genotyping prior to onset of therapy. Despite this body of evidence, isoniazid is administered at standard doses of 5 mg/kg body weight for daily administration and of 15 mg/kg body weight when given two to three times weekly, and NAT2 genotyping or phenotyping is neither used in clinical routine, nor has it been evaluted in prospective studies. However, taking the emerging problem of multi-drug resistance in *mycobacterium tuberculosis*, an individualized treatment with the chance to increase the isoniazid dose in rapid acetylators may become increasingly important.

NAT2 activity also appears to be clinically relevant in the treatment with sulfonamide drugs. Das et al. [67] reported a significant association between total serum sulfapyridine concentration and toxic symptoms, including cyanosis and hemolysis, and 86% of the patients with side effects from sulfasalazine were slow acetylators. Serum sulfapyridine concentrations greater than 50 μg/mL have been associated with toxicity. Thus, slow acetylators may be at risk for toxicity from accumulation of sulfapyridine. The authors recommended that the total dose of sulfasalazine not exceed 2.5 to 3 grams/day in slow acetylators and 4 to 5 grams/day in rapid acetylators [68]. A more recent retrospective study was conducted in 144 patients with rheumatoid arthritis (RA) who had been treated with sulfasalazine, another NAT2 substrate. Sixteen patients (11.1%) had experienced adverse effects from SSZ, the most common being allergic reactions including rash and fever. Patients without NAT2*4 haplotype had experienced adverse effects significantly more frequently (62.5%) than the fast acetylators with at least one NAT2*4 allele (8.1%). In 25% of the slow acetylators, the adverse effects were so severe that the patients were hospitalized. The authors concluded that NAT2 genotyping before administration of SSZ is likely to reduce the frequency of adverse effects in Japanese patients with RA [69]. A study conducted on infants receiving cotrimoxazole (100 mg/kg b.w. daily), adverse effects such as rash, granulocytopenia with anaemia or liver impairment were more frequent in children with mutations of the NAT2 encoding gene [70].

Hydralazine undergoes first-pass metabolism after oral ingestion depending on acetylator phenotype. Indeed, rapid acetylators required an 60% greater dose of hydralazine monotherapy than slow acetylators for adequate blood pressure control [71]; this difference was not seen, however, when hydralazine was combined with other

antihypertensive drugs [72]. More importantly, slow acetylators have been shown to have a higher risk for developing systemic lupus erythematosus and related laboratory findings with hydralazine [73,74]. Therefore, it has been recommended that if patients require chronic hydralazine therapy, acetylator phenotype should be assessed in order to predict the risk of developing drug-induced systemic lupus erythematosus, and slow acetylators should be treated with alternative agents.

The antiarrhythmic agent procainamide is acetylated to form the active metabolite N-acetylprocainamide. Rapid acetylators have a significantly higher metabolite/procainamide ratio than slow acetylators, and high concentrations have been related to junctional tachycardia and intraventricular conduction defects. Furthermore, acetylator phenotype may also be related to the development of systemic lupus erythematosus during procainamide therapy [65].

In summary, the current evidence suggests that NAT2 genotype and/or phenotype should be taken into account for the treatment with isoniazid and several others of its substrates. However, the appropriate procedure for dose adjustment and the benefit for patients must be addressed in additional prospective clinical studies.

13.6 CYTOCHROME P450 2D6 (CYP2D6)

The cytochrome P450 enzyme CYP2D6 is responsible for the metabolism of about one quarter of clinically important and frequently prescribed drugs, such as several antiarrhythmics, classical neuroleptics, tricyclic antidepressants, selective serotonin reuptake inhibitors, and β-blockers [75]. CYP2D6 exhibits tremendous interindividual differences in enzyme activity and the metabolism of a large variety of drugs. The catalytic activity as measured by the respective metabolic ratio can vary 10,000 fold between individuals [76], thus determining the dose that is needed for efficacious therapy and that is tolerated with acceptable adverse effects. For example, the therapeutic dose of nortriptyline may vary between 10 mg and 500 mg between two patients (Fig. 13.3) [77]. The genetic basis for this enormous phenotypic variability has been extensively investigated so that CYP2D6 pharmacogenetics is plainly the classical example for the large interindividual differences in metabolic status and drug response. To date, at least 43 different CYP2D6 alleles are known [78].

The majority of Caucasian populations carries two functional CYP2D6 alleles, metabolizes model drugs, such as dextromethorphan, debrisoquine or sparteine, extensively and is therefore classified as extensive metabolizers (EM). About 5–10% of Caucasians are poor metabolizers (PMs) lacking substantial CYP2D6 activity because of deleterious polymorphisms in both alleles. Poor metabolizers are supposed to have an increased risk for toxic effects with standard doses of drugs inactivated by CYP2D6 [76]. In contrast, about 1–7% of the Caucasian population and up to 20% of Northern African populations demonstrate increased CYP2D6 activity (ultrarapid metabolizers, UM) and may suffer from failure of drug therapy with administration of routine dose [79–82].

Comparative studies indicate that the CYP2D6 genotype predicts the PM phenotype with high accuracy. The most prevalent PM alleles in the white Caucasian population are CYP2D6*3, *4, *5, and *6 which predict approximately 95% of all PM phenotypes

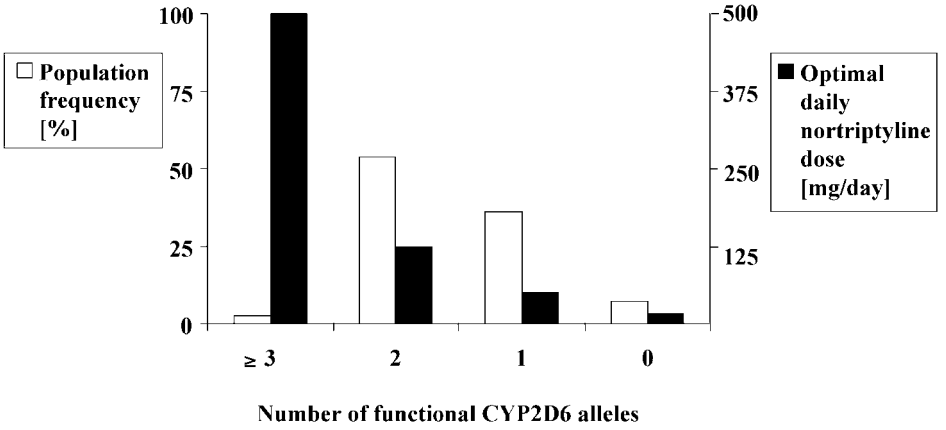

Fig. 13.3. Prevalence of CYP2D6 genotypes consisting of 0, 1, 2, and ≥3 functional alleles in Caucasians as determined by Sachse et al. [76] and correlation with the optimal daily dose of nortriptyline (according to Dalen et al. [77]).

[76,83,84]. The PM alleles CYP2D6*3, *4 and *6 are caused by single nucleotide mutations that lead to frame shifts or a splice defect, respectively, with disruption of the transcript [85–87]. In the case of CYP2D6*5, the entire gene is deleted as a consequence of homologous unequal cross-over events [88,89]. Additionally, chimeric genes due to hybridization with a nearby pseudogene have also been reported [90,91].

About 10–15% of Caucasians constitute a subgroup of so-called 'intermediate metabolizers' which demontrate a moderately impaired metabolic status between PM and EM. This subgroup is still not predicted satisfactorily although recent findings have identified the underlying genetic causes for 60% of all IMs [92,93].

Furthermore, inheritance of three or more functional alleles by gene duplication or gene amplification identifies the clinically important ultrarapid metabolizer (UM) phenotype with almost 100% specificity but can only explain 15–25% of all respective subjects [92,94]. The relevance of two SNPs recently detected within the 5′-UTR of the CYP2D6 gene for the establishment of the UM phenotype necessitates further investigation [94,95].

The clinical effects of the CYP2D6 metabolic phenotype and genotype might have far-reaching implications for rational drug treatment in clinical practice, especially in the treatment of neuro-psychiatric disorders. Treatment failure because of CYP2D6 ultrarapid metabolism can be effectively antagonized by concomitant administration of known inhibitors of CYP2D6 enzyme, such as paroxetine, to normalize the metabolic status [96]. However, remission of depressive disorder takes time and extension of treatment because of genetically determined inefficacy of antidepressants might prolong critical phases of suicidal risk [97]. Therefore, it might be of large therapeutic benefit to predict clinical non-response in suicidal depressive patients.

Additionally, the PM genotype has redundantly been associated with an increased risk of antipsychotic-induced extrapyramidal syndromes (EPS) when CYP2D6-metabolised neuroleptics are taken [98,99]. Occurrence or absence of adverse drug

events, especially severe extrapyramidal syndromes, has been reported to be the major determinant of medication compliance in schizophrenic patients [100].

A prospective study of 172 unselected psychotic in-patients who were treated with haloperidol also revealed a higher incidence of pseudoparkinsonic extrapyramidal syndromes in PM and a number-needed-to-treat of 20 for a significant benefit of pre-therapeutic CYP2D6 genotyping [101]. Another prospective clinical trial of 270 cancer patients who underwent chemotherapy showed that 50 patients would have to be genotyped for ultrarapid metabolism in order to predict insufficient antiemetic efficacy of 5-hydroxytryptamine type 3 receptor antagonists, tropisetron or ondansetron, and to prevent patients from severe emesis [102].

Another item of increasing importance is the economic aspect. A prospective one-year clinical study of 100 psychiatric in-patients revealed that CYP2D6 genetic variation tended to alter the duration of hospital stay and costs. Patients having a PM or an UM genotype demonstrated a tendency toward greater numbers of ADEs from medications and longer hospitalization, respectively [103].

The CYP2D6 genotype has been associated to the pharmacokinetics of additional drugs with a narrow therapeutic index and a considerable spectrum of potential adverse events, such as metoprolol, propafenone, mexiletine, or dihydrocodeine [104,105]. A good correlation has been reported between CYP2D6 genotype and pharmacokinetics for propafenone but the clinical relevance remains unclear since the metabolites are also active [106]. In case of propafenone the antiarrhythmic efficacy was shown to depend on the genotype [107]. However, no association with proarrhythmic or therapy-limiting gastrointestinal side effects have been detected. A high correlation between CYP2D6 metabolism status and mexiletine pharmacokinetics has been reported and strong association with drug response is assumed but clinical data are lacking [108]. The metabolism of metoprolol is significantly affected by the CYP2D6 genotype but the relevance for clinical routine is also vague [104]. Serious proarrhythmic events in connection with antiarrhytmic drugs due to causative genetic variants have rather been related to rare mutations in cardiac ion channels [109].

As an interesting approach, preliminary quantitative dose recommendations adjusted to CYP2D6 genotypes based on the retrospective evaluation of pharmacokinetic and clinical data of various small-scale studies have recently been published [110,111]. However, the validity and benefit of these proposed "guidelines" for genotype-based dose adjustment will have to be thoroughly verified in prospective clinical studies which are strongly needed.

Screening before commencement of application of CYP2D6 substances is currently impeded by the unresolved questions concerning the association of allelic variants with pharmacodynamics and the therapeutic benefit in comparison to the costs of genotyping. Well-designed prospective clinical studies of much larger patient numbers should be conducted to clarify these important points. To date, despite the well-recognized importance of CYP2D6 genotype for the metabolism of respective drugs, CYP2D6 genotyping is mainly restricted to clinical studies in the drug development process of the pharmaceutical industry. One can speculate that the fast development of new cost- and time-sparing genotyping techniques will redeem the present objections and might allow for rapid pre-therapeutic genotyping in the near future.

13.7 CYTOCHROME P450 2C9 (CYP2C9)

Cytochrome P450 2C9 is an important drug metabolizing enzyme and accounts for ca.18% of cytochrome P450 protein content in the human microsomes [112]. It takes part in the metabolism of numerous drugs such as nonsteroidal antiinflammatory drugs, losartan, tolbutamide, warfarin, phenytoin or carbamazepine [113]. In the gene encoding for CYP2C9 protein, genetic polymorphism was found which gives rise to significant differences in interindividual variation in drug disposition. In addition to the most common allele CYP2C9*1 (wild type), a 430 C > T single-nucleotide polymorphism in exon 3 and 1075 A > C single-nucleotide polymorphism in exon 7 are the most common polymorphisms resulting in the amino acids exchange i.e. Arg144Cys (CYP2C9*2 allele) and Ile359Leu (CYP2C9*3 allele), respectively [114]. Individuals expressing the defect alleles (poor metabolizers) are more sensitive to adverse events upon administration of drugs metabolized by CYP2C9. In the Caucasian population, wild type is found in about two-thirds of individuals, whereas one-third individuals express heterozygous genotype CYP2C9*1/*2 or CYP2C9*1/*3. Prevalence of subjects with two low-activity alleles i.e. CYP2C9*2/*2, CYP2C9*2/*3 and CYP2C9*3/*3 accounted for less than 2.5% in these populations. In contrast, genetic CYP2C9 polymorphism is unusual in African-American and Asian populations [115]. Individuals possessing at least one defect allele CYP2C9*2 or CYP2C9*3 exhibit decreased biotransformation of drugs metabolized by CYP2C9, although CYP2C9*3 allele seems to be of primary importance for decreased enzymatic activity [116]. Because many substrates of CYP2C9 are drugs with narrow therapeutic range (e.g. warfarin or phenytoin), questions about the potential clinical utility of genotyping or phenotyping screening for CYP2C9 polymorphism prior to pharmacotherapy appear to be justified. There is no standard CYP2C9 phenotyping assay. The most important substrates which may be used for phenotyping include tolbutamide, phenytoin, flurbiprofen, losartan and warfarin. The largest database is available for tolbutamide (oral antidiabetic agent) supporting its use as a selective in vivo CYP2C9 probe [117]. Numerous plasma and urine tolbutamide metrics have been proposed for phenotyping. Tolbutamide AUC or tolbutamide plasma concentration 24h after dosing appear as the most promising parameters derived from plasma concentrations [118]. The urinary metabolic ratio (hydroxytolbutamide + carboxytolbutamide/tolbutamide) determined in the 6 to 12 hour urine collection period was up to now considered as a gold standard for CYP2C9 phenotyping regarding its reliability and non-invasiveness. Recent results indicate, however, that other urine metrics are preferable such as 0- to 12-hour urinary amount of 4′-hydroxytolbutamide and carboxytolbutamide which better correlated with 4′-OH-tolbutamide formation clearance [115]. A possible hypoglycemia in poor metabolizers of CYP2C9 upon administration of standard phenotyping tolbutamide dose (500 mg) was reported to limit tolbutamide use as phenotypic probe [117]. This problem could be, however, minimized by coadministration of oral glucose during phenotyping studies [115] or by intake of low 125 mg tolbutamide doses in connection with a highly sensitive LC-MS/MS assay [118].

Warfarin, a racemic mixture of the enantiomers, S- and R-warfarin, is the most widely prescribed anticoagulant agent. S-warfarin, which is 3 to 5 times more potent than

R-warfarin, is mainly eliminated through 6- or 7-hydroxylation via CYP2C9. Thus, the activity of CYP2C9 regulates the steady-state plasma concentration of S-warfarin, being consequently of significant importance for anticoagulant response [119]. On the other hand, genetic CYP2C19 polymorphism seems not to be relevant for safe therapy with warfarin, although CYP2C19 takes part in biotransformation of R-warfarin [120]. The appropriate therapy is based on evaluating of international normalized ratio (INR) and requires constant assessment of the possible risks of over- and underanticoagulation resulting in increased risk of hemorrhage or lack of efficacy, respectively. Increased risk of bleedings upon a therapy with warfarin was observed in poor metabolizers of CYP2C9 and a subsequent dosage adjustment was required [121]. Patients with variant alleles of CYP2C9 requires lower maintenance doses of warfarin, have a longer induction period and are at higher risk for bleeding upon therapy. Thus, it is suggested that CYP2C9 genotyping may identify a population of patients at an increased risk of bleeding complications due to anticoagulant therapy [122,123].

Phenytoin, a hydantoin anticonvulasant, is another drug with a narrow therapeutic index and individual dose requirements. 4′-hydroxylation of phenytoin accounts for about 80% of its elimination and is predominantly mediated via CYP2C9 [119]. Insufficient biotransformation of the phenytoin can result in symptoms of drug intoxication. It was reported that polymorphism of CYP2C9 as well as of CYP2C19 contributes to variability in phenytoin pharmacokinetics. However, in patients with variant alleles of CYP2C19 the maximal elimination rate was decreased up to 14%, whereas the Ile359Leu mutation of CYP2C9 (CYP2C9*3) was associated with a 40% decrease in the parameter as compared with the wild type [124]. Individualized therapy with antiepileptic drugs based on pharmacogenetic tests could contribute to optimal safety and efficacy therapeutic profiles in the future.

Losartan is a potent angiotensin II type 1 (AT_1) receptor antagonist used in the treatment of hypertension and congestive heart failure. It is metabolized by CYP2C9 and CYP3A4 to its active metabolite, E-3174 [125]. CYP2C9 polymorphism was shown to result in interindividual differences in oxidation and activation of the drug [126]. For irbesartan, another angiotensin II type 1 receptor antagonist, an influence of CYP2C9 polymorphism on the effects of antihypertensive treatment was also observed [127]. Patients heterozygous for CYP2C9*2 demonstrated stronger reduction of diastolic and systolic blood pressure compared to patients homozygous for CYP2C9*1 (wild type). Therefore the authors suggested that CYP2C9 genotype might play a role in prediction of therapeutic response. Although irbesartan plasma concentration was not measured in this study, it is suggested that the different therapeutic response between CYP2C9 genetic variants could be explained with a slower elimination of irbesartan and thus greater blood concentrations of the drug in CYP2C9*2 carriers.

The importance of CYP2C9 for oxidative biotransformation of numerous non-steroidal antiinflammatory drugs (e.g. 4′-hydroxylation of R- and S-flurbiprofen or 2- and 3-hydroxylations of R- and S-ibuprofen) was supported by many clinical trials and in vitro studies [119]. The potential clinical importance of CYP2C9 polymorphism during therapy with NSAID was subject to evaluation in some clinical trails. For instance, hydroxylation of S-ibuprofen was significantly decreased in heterozygous and homozygous carriers of CYP2C9*3 allele, whereas CYP2C9*2 variant exerted no

significant effect [128]. In contrary, for orally administered diclofenac, which is 4′-hydroxylated by CYP2C9, no evidence of impaired metabolism in carriers of CYP2C9 genetic variants was determined [129]. Celecoxib, a highly selective inhibitor of cyclooxygenase (COX)-2 also metabolized predominantly by CYP2C9, was shown to undergo markedly slower biotransformation in carriers of CYP2C9*3 variant allele than in wild type individuals [130]. Medical impact of CYP2C9 polymorphism in patients receiving analgesic drugs need to be evaluated in further clinical trials.

For tolbutamide (see above) the following dose adjustments for CYP2C9 poor metabolizers were suggested: half of the standard dose for CYP2C9*1/*3 and CYP2C9*2/*3 carriers and 20% of the standard dose for CYP2C9*3/*3 carriers [128]. These gentoype-based dose recommendations should be, however, verified in clinical trials with clinical endpoints.

Especially, for drugs with narrow therapeutic index (e.g. warfarin and phenytoin), it appears to be preferable to proceed with the prospective evaluation of genotype to facilitate optimal efficacy of therapy and limit adverse drug reactions in patients.

13.8 CYTOCHROME P450 2C19 (CYP2C19)

CYP2C19 is responsible for the metabolism of the range of commonly prescribed drugs such as diazepam, omeparazole and other proton pump inhibitors, propranolol or imipramine, to name a few. In the past two decades, CYP2C19 genetic polymorphism has become the subject of detailed analysis which significantly contributed to understanding of interindividual and interethnic differences in pharmacokinetic of drugs metabolized by this enzyme. Initially, the polymorphism was described as genetically determined deficiency in hydroxylation of the antiepileptic drug mephenytoin in a family study [131]. In the following studies, (S)-mephenytoin hydroxylase could be identified as CYP2C19. Using urinary recovery of 4-hydroxymephenytoin or the urinary S:R enantiomeric ratio of mephenytoin excreted in urine during 8 h after its administration, the majority of the population could be precisely divided into extensive and poor metabolizers for CYP2C19. Mephenytoin seems to be the preferred probe substrate for CYP2C19, but omeparazol or proguanil are also used for CYP2C19 phenotyping. However, some discordance between CYP2C19 genotyping and phenotyping results was determined in studies comparing results of both procedures in patients. Old age, liver or renal diseases and advanced metastatic cancer seem to be confounding factors which may result in poor metabolizer phenotype in genetic extensive metabolizers [132,133].

So far, seven genetic variant alleles of the CYP2C19 that are responsible for the majority of the poor metabolizer phenotypes have been identified. The most common non-functional alleles are CYP2C19*2 and CYP2C19*3. They are caused by single base exchanges in exon 5 (CYP2C19*2 or m1) and 4 (CYP2C19*3 or m2) resulting in splicing defect in exon 5 with premature termination of protein synthesis or a premature stop codon with a truncated protein, respectively [134]. For the two most frequent variant alleles, simple genetic tests are available allowing for a relatively quick and reliable identification of defect alleles. The poor metabolizer phenotype is inherited as

an autosomal recessive trait and it results from owing of two nonfunctional alleles. The interethnic variation in the frequency of the poor metabolizer phenotype for mephenytoin oxidation is significant: poor metabolizers represent about 2.8% of Caucasian populations, 14.3% of Chinese, 14.0% of Koreans and 21.3% of Japanese [135]. The activity of CYP2C19 in different populations is in accordance with the amount of CYP2C19 protein in liver microsomes e.g. in Caucasians 1.4% compared with 0.8% of total CYP450 amount in Japanese [136]. Natural consequence of the varying interethnic distribution of CYP2C19 polymorphism is varying drug disposition among different populations. The prime example could be interindividual variation in biotransformation of proton pump inhibitors, especially of omeprazole 5-hydroxylation, which is predominantly mediated by CYP2C19. In a systematic review on pharmacogenetic studies of proton pump inhibitors Chong and Ensom evaluated the effects of CYP2C19 polymorphism on the clinical outcomes of therapy with the drugs i.e. eradication rates of Helicobacter pylori [137]. The results of the most studies supported the hypothesis that clinical outcomes in Helicobacter pylori eradication are dependent on CYP2C19 polymorphism with better efficacy in poor metabolizer. Only in few studies, notably performed with rabeprazole, a similar cure rate irrespective of CYP2C19 genotype was demonstrated. It is not surprising as rabeprazole undergoes mainly non-enzymatic metabolism and both CYP3A4 and CYP2C19 contribute to the fraction of metabolism mediated enzymatically [138]. In a prospective study evaluating efficacy of Helicobacter pylori dual therapy (amoxicillin as the only antibiotic in addition to omeprazole), CYP2C19 genotype was associated with efficacy of bacteria eradication showing cure rates 28.6%, 60%, and 100% in the rapid-, intermediate-, and poor-metabolizer groups, respectively [139]. Thus, in poor metabolizers for CYP2C19 the dual therapy seems to be sufficient. As most of the pharmcogenetic studies were performed in Japanese men, the clinical relevance of CYP2C19 genetic polymorphism in other ethnic groups and women remains to be evaluated.

Metabolism of diazepam, a commonly prescribed benzodiazepine with anxiolytic, sedative and muscle relaxant activities is also affected by CYP2C19 polymorphism. In a study evaluating the effects of the CYP2C19 gene on diazepam metabolism in Chinese subjects, a significant difference in the mean clearance of diazepam with regard to the CYP2C19 genotype could be determined [140]. Homozygous extensive metabolizers showed the highest clearance followed by heterozygous extensive metabolizers and poor metabolizers for CYP2C19 suggesting that CYP2C19 polymorphism results in impaired metabolism of diazepam in a gene-dosage effect manner. For this reason, poor metabolizers for CYP2C19 could be at higher risk for adverse drug reactions upon therapy with diazepam. CYP2C19 was demonstrated to play a key role in metabolizing of antimalaric drug proguanil to its active metabolite i.e. cycloguanil. The urinary metabolic ratio of proguanil over cycloguanil shows a pronounced interindividual variability correlating with CYP2C19 activity and for this reason is also used as phenotypic metric for this enzyme. However, if the genetic CYP2C19 polymorphism could be relevant for failure in malaria treatment or prophylaxis due to decreased concentrations of active metabolite is questionable. A study by Kaneko et al. in 95 subjects infected with Plasmodium falciparum or vivax revealed a similarly high antimalarial efficacy of the proguanil therapy in extensive and poor metabolizers. It was

suggested that the parent compound proguanil has as well significant intrinsic efficacy against Plasmodium so the therapy could be effective independent of proguanil biotransformation to cycloguanil [141].

Taken together, assessment of genetic CYP2C19 polymorphism could be of particular importance for Asians, as the frequency of poor metabolizers is several fold greater in this population than among Caucasians. Genotyping or phenotyping for CYP2C19 activity could be an important clinical tool contributing to better treatment efficacy. Although there are established phenotyping and genotyping methods for assessment of CYP2C19 activity, they remain a research tool being neither available to most practicing physicians nor routinely used for individualization of pharmacotherapy in patients.

13.9 CYTOCHROME P450 3A4 (CYP3A4)

The CYP3A subfamily, which consists of four CYP3A genes (CYP3A4, CYP3A5, CYP3A7 and CYP3A43), is one of the most important fraction of drug metabolizing enzymes [142,143]. It is responsible for the oxidative metabolism of about 50% of all prescribed drugs undergoing relevant metabolism such as HIV protease inhibitors, calcium channel blockers (e.g. nifedipine), hydroxymethylglutaryl coenzyme A-reductase inhibitors, antineoplastic drugs (e.g. cyclophosphamide, ifosfamide, docetaxel, paclitaxel, doxorubicin, etoposide, teniposide or vinca alkaloids) or immunosuppressants. In addition, CYP3A is responsible for the metabolism of endogenous steroids (e.g. testosterone) or activation of dietary mycotoxins (e.g. aflatoxin) which are potential carcinogenic factors. The main sites of CYP3A expression in humans are the liver, small intestine and kidney [144]. In the liver, CYP3A represents about 30% of total CYP450 proteins [145]. In the intestinal mucosa, CYP3A enzyme content accounts for about 70% of CYP proteins [146]. CYP3A4 is the best explored enzyme in the CYP3A subfamily. The CYP3A4 gene which contains 13 exons was assigned to chromosome 7 at q22.1 [147]. Whereas a significant percentage of the white population is not able to express functional CYP3A5 protein, CYP3A4 seems to be universally expressed in humans. Because most substrates for CYP3A5 are also biotransformed by CYP3A4, the lack of active CYP3A5 may not become manifest [109]. Pronounced interindividual differences in the CYP3A4 protein content in the liver are accompanied by variability in the metabolic activity exceeding 30-fold in some populations [148]. It was suggested that interindividual and interethnical variations in activity of CYP3A4 could affect the safety and efficacy profile of drugs which are substrates for this enzyme. One of the best-researched reasons for this variability are drug interactions. Intake of eythromycin (or other macrolide antibiotics), ketoconazole or grapefruit products could result in pronounced inhibition of CYP3A4 activity whereas upon chronic treatment with rifamipcin, carbamazepin or dexamethasone, an induction of CYP3A4 expression is observed. The recent identification of orphan nuclear receptor PXR (pregnane X receptor, known also as SXR) may elucidate the mechanism underlying the interactions. PXR/SXR was shown to bind to a response

element in the CYP3A4 promoter and is regulated by various xenobiotics. Numerous drugs such as synthetic steroids (e.g. dexamethasone, spironolactone or RU486), rifampicin and clotrimazol were demonstrated to activate the receptor [149]. PXR/SXR seems to play a significant role in regulating CYP3A4 and MDR1 transcription thus contributing to drug clearance in the liver and small intestine. In addition to environmental factors, a large genetic contribution to variability in CYP3A4 activity is postulated, i.e. at least 60% for orally administered substrates [150]. Several publications in the last years threw some light on the genetic basis of polymorphic expression of CYP3A subfamily. In a study by Eiselt et al., 18 variants within the protein-coding regions of CYP3A4 were identified in DNA samples of Middle- and Western Europe Caucasians, however, a very low frequency (below 1%) for the most of the minor alleles was assessed [143]. The authors suggested that the expected quantitative effect of CYP3A4 variant proteins could result in maximal two-fold decrease in drug clearance. In addition, in carriers of CYP3A4 polymorphic variants, altered individual sensitivity to several carcinogens was assumed. On the other hand, Lamba et al. found no correlation between any of the 28 determined CYP3A4 single nucleotide polymorphisms and low hepatic CYP3A4 protein expression or low CYP3A4 activity in vivo [151].

Because current data on genetic reasons for variation in CYP3A4 activity are inconclusive, phenotyping is the method of choice for this enzyme. There are several established probe substrates for CYP3A4 phenotyping such as: nifedipine, dapsone, cortisol, erythromycin and midazolam. The best validated and most widely accepted procedures are [^{14}C-N-methyl]erythromycin breath test and midazolam phenotyping [152]. Phenotyping for CYP3A4 activity with cortisol (urinary ratio 6-β-hydroxy-cortisol/cortisol) is worth mentioning as it is certainly the only method without adverse reactions since cortisol is an endogenous substrate [153]. Because of independent regulation of intestinal and hepatic CYP3A4, two different metrics have to be used for the two activities. It has been shown repeatedly that intravenous midazolam clearance closely reflects hepatic CYP3A4 activity while intestinal availability of orally administered midazolam shows a very close correlation to intestinal CYP3A4 activity (Fig. 13.4) [154].

As mentioned above, CYP3A4 is known to participate in the metabolism of numerous antineoplastic drugs. Possible effects of impaired CYP3A4 function for cytostatics disposition are complex since as a result of CYP3A4 drug biotransformation active as well as inactive metabolites are formed. For instance, lower enzymatic CYP3A4 activity is associated with an increased plasma concentration of the active cyclophosphamide metabolite i.e. 4-OH-cyclophosphamide, which is formed predominantly by CYP2B6. However, for ifosfamide, another oxazaphosphorine alkylating agent, inhibition of CYP3A4 contributes to the increased formation of its metabolite dechloroethylifosfamide deprived of antineoplastic activity [155]. During formation of this metabolite, chloroacetaldehyde is formed, which is thought to be neurotoxic. Thus, for most of these drugs, the impact of individual CYP3A4 activity on therapeutic outcome remains to be examined. However, data on docetaxel, a chemotherapeutic agent used in the treatment of various solid tumors and metabolized by CYP3A4, suggest that CYP3A4 phenotyping may be worthwhile. For this drug, a close relationship between the drug

clearance and the phenotypic measurement of CYP3A4 activity using the [^{14}C-N-methyl]erythromycin breath test or midazolam clearance was demonstrated [156,157]. The authors suggested that phenotyping for CYP3A4 activity before therapy onset could be helpful in individualizing the dose of docetaxel and thus it could improve the efficacy of the treatment and reduce the drug toxicity.

Another drug group affected by variable CYP3A4 activity are HIV protease inhibitors such as ritonavir, saquinavir and lopinavir. All these agents undergo extensive first-pass metabolism by hepatic cytochrome 3A4 and are known for pronounced interactions with other CYP3A4 substrates [158]. The HIV protease inhibitor ritonavir has recently been demonstrated to bind to PXR/SXR activating its target genes and thus regulating expression of CYP3A4 [159]. Knowledge of molecular basis of CYP3A4 activity regulation offers the possibility of developing in vitro assays predicting drug interactions in humans.

In the light of current knowledge, genetic polymorphism affecting CYP3A4 activity may not primarily be found in the CYP3A4 gene but rather in regulating elements. Thus, a role for genotyping may emerge also for this enzyme. Currently, CYP3A4

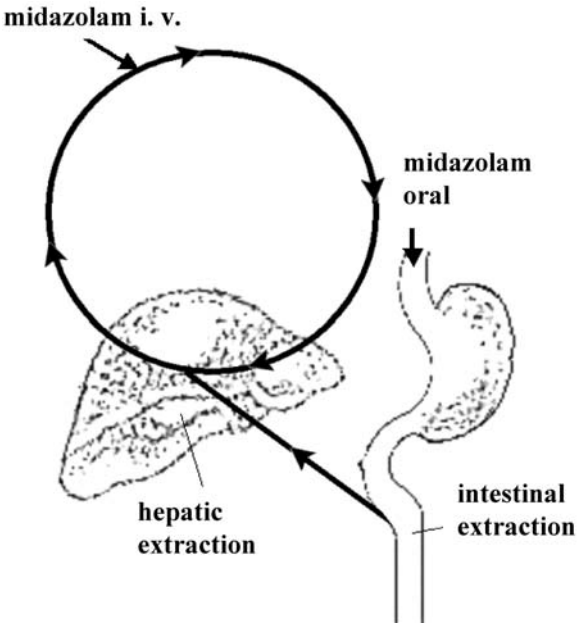

Fig. 13.4. Phenotyping for intestinal and hepatic CYP3A4 activity using midazolam. Midazolam administered intraenously (i.v.) is metabolized exclusively by hepatic CYP3A4, with midazolam clearance as the appropriate metric. Midazolam administered orally undergoes a two-step first pass metabolism by hepatic and intestinal CYP3A4; the intestinal extraction is a metric for intestinal CYP3A4 and can be calculated from midazolam pharmacokinetics following oral administration if the hepatic clearance is known.

phenotyping, first of all in patients treated with antineoplastic agents metabolized via CYP3A4, seems to allow tailored drug dosing and optimal drug exposure.

13.10 P-GLYCOPROTEIN (PGP)

P-glycoprotein (PGP) is an integral membrane transport protein that is encoded by the multidrug-resistance (MDR)-1 gene and functions as an energy-dependent efflux pump of metabolites and xenobiotic toxins from the intracellular space to the outside [160]. The protein belongs to the ATP-binding cassette superfamily of transporters [161] and despite of extensive research its physiological role still remains some kind of enigmatic. Its main function may be the protection of cells from toxic substances and to regulate intracellular homeostasis of various compounds of metabolic cycles. The far-reaching relevance of PGP may not become apparent unless the organism is exposed to unphysiological external conditions or unless there is a significant skew in metabolism. Mice completely lacking the MDR1 gene were viable, fertile and healthy from a clinical point of view. However, they demonstrated a reduced clearance of vinblastine and a transport defect for other drugs (ivermectin, cyclosporin A, dexamethasone, ondansetron, loperamide and digoxin) with increased concentrations in various tissues including the brain [162–167].

PGP is expressed in a variety of normal tissues, such as the intestine, kidney [168], the blood-brain barrier [169], liver [170], lymphocytes [171], and bone marrow [172]. It shows a broad substrate spectrum of various substance classes with different therapeutic indications as displayed in Table 13.1. Some substrates are also potent inhibitors of PGP-mediated transport (Table 13.2) and interesting enough, PGP may be functionally linked with the cytochrome P450 3A4 because of co-localization in the gut and a largely overlapping spectrum of substrates, inhibitors and inducers [173].

The outstanding relevance of PGP for the pharmacokinetics and therapeutic effectiveness of numerous drugs has extensively been studied, particularly in cancer therapy where the impact of a high PGP expression level on cytotoxicity of anticancer agents and resistance of tumor cells is a problem known for long [174,175]. PGP overexpression of tumor cells is partially conferred by MDR1 gene amplification [176] but the PGP expression is also modulated by other factors [177]. For instance, intestinal expression of PGP is significantly induced by rifampicin and several other drugs. Prazosin and progesterone also stimulate PGP-mediated drug transport [178].

Various functional polymorphisms have been identified within the coding sequence of the MDR1 gene. However, surprisingly the synonymous C3435T single nucleotide substitution (SNP) has been shown to be associated with the intestinal level of PGP expression and activity in human and to exert influence on the bioavailability of certain drugs. Individuals who were homozygous for the 3435C allele showed approximately 2-fold higher duodenal PGP expression levels than subjects with a TT genotype while heterozygotes demonstrated intermediate expression levels. What is more, the PGP C3435T polymorphism significantly affected digoxin pharmacokinetics after oral administration. There was an inverse correlation between genotype-based level of expression and both mean maximum plasma concentrations C_{max} and area under the

TABLE 13.1
CLINICALLY RELEVANT PGP SUBSTRATES
(HTTP://WWW.AIDSINFONYC.ORG/TAG/SCIENCE/PGP.HTML)

Indication	Substrate
Chemothepeutic agent	Doxorubicin
	Daunorubicin
	Vinblastine
	Vincristine
	Paclitaxel
	Etoposide
Immunosuppressant	Cyclosporin A
	FK506
Steroids	Aldosterone
	Hydrocortisone
	Cortisol
	Corticosterone
	Dexamethasone
HIV protease inhibitors	Amprenavir
	Indinavir
	Nelfinavir
	Ritonavir
	Saquinavir
Digitalis glycoside	Digoxin
Antiarrhythmic agent	Quinidine
Antibiotic	Erythromycin
Anti-tuberculous agent	Rifampin
Dye	Rhodamine 123

TABLE 13.2
CLINICALLY RELEVANT PGP INHIBITORS
(HTTP://WWW.AIDSINFONYC.ORG/TAG/SCIENCE/PGP.HTML)

Indication	Substrate
Immunosuppressant	Cyclosporin A
HIV protease inhibitors	Ritonavir
	Saquinavir
	Nelfinavir
Calcium channel blocker	Verapamil
Progesterone antagonist	Mefipristone
Antiestrogen	Tamoxifen
Antiarrhythmic agent	Quinidine
Antifungal agent	Ketoconazole
Sedative agent	Midazolam

References pp. 343–353

plasma concentration-time-curve AUC [177]. The authors hypothesized that an unidentified regulatory polymorphism in strong physical linkage with the C3435T polymorphism could be causative for the observed differences in PGP expression level. The relevance of the C3435T polymorphism for alteration of PGP function has also been reported by other groups. For example, CD56+ natural killer cells from healthy individuals with a CC genotype exhibited lower accumulation of rhodamine 123 as monitored by lower fluorescence signals in comparison with homozygous 3435T subjects [179].

PGP has been proposed to regulate the degree of drug uptake into the brain at the blood-brain barrier and high PGP levels may limit the efficacious accumulation of anticonvulsants [166,180]. This mechanism corroborates well with recent findings of a study where the C3435T polymorphism was shown to be strongly associated with drug resistance in epilepsy. Patients with the 3435CC genotype had a 75% probability to experience antiepileptic drug resistance, irrespective of the type of epilepsy or antiepileptic drug used [181]. Furthermore, there is evidence of influence of MDR1 genotypes on the availibility of the immunosuppressant drug cyclosporin in transplant recipients.

Recently, the C3435T polymorphism has been shown to be in strong linkage disequilibrium with a G2677T polymorphism leading to an amino acid substitution and altered PGP function. However, there are conflicting results of a clinical study on the influence of the MDR1 genotype on the fexofenadine pharmacokinetics since, in contrast to the findings by Hoffmeyer et al., the 2677T variant was associated with enhanced in vitro PGP activity and lower bioavailability in humans [182].

Recent studies suggest that the PGP genotype might have significant implication in HIV therapy. The MDR1 C3435T polymorphism was related to bioavailability and efficacy of the antiretroviral agents efavirenz and nelfinavir. Patients with the CC genotype showed higher median plasma concentrations than heterozygotes and 3435T homozygotes, respectively. Contrarily, patients with the TT genotype experienced a greater rise in CD4-cell count and a better recovery of naive CD4-cells than carriers of the 3435C allele. The authors interpreted the results as evidence of an influence of the C3435T polymorphism on availability of antiretroviral agents in restricted target-compartments and of a predictive value for immune recovery in HIV therapy [183]. However, prospective data on long-term outcome are lacking. The therapeutic relevance of the C3435T polymorphism in antiretroviral therapy challenges further investigation since intensified HIV therapy utilizes combinations of protease inhibitors. For example, the bioavailability of saquinavir is dramatically increased by coadministration of ritonavir [184]. Thus, the influence of the MDR1 genotype on pharmacokinetics might not be decisive for the clinical response. However, the C3435T polymorphism might exert influence on admittance of antiretroviral drugs to separated compartments and pharmacological sanctuary sites. Furthermore, considerable interethnic differences in allelic distribution must be taken into account. While the 3435CC genotype occurred only in about 25% of white Caucasian subjects the prevalences were found to value 83% and 61% in West African and African American populations, respectively [185,186]. These findings may have significant and so far underestimated implications on the therapy with a large variety of clinically important and frequently administered drugs.

13.11 PERSPECTIVE

Several decades ago, it has been recognized that individual activity of drug metabolizing enzymes may be related to differences in drug effects. Genotyping for drug metabolizing enzymes and for drug transporters has become increasingly reliable, simple and cheap, e.g. by light cycler and gene chip technologies (see chapter 12). Likewise, phenotyping made major progress, mainly by increased analytical sensitivity brought about by LC-MS/MS with the possibility to administer very low doses of marker drugs. Thus, the tools for assessment of individual activity of many highly variable human enzymes and/or transporters with outstanding relevance for pharmacokinetics are available. For instance, today genotyping identifies >99% of CYP2D6 poor metabolizers [92], individual elimination rates of tolbutamide may be derived from the contribution of individual CYP2C9 alleles [128], or 2/3 of the variation in docetaxel pharmacokinetics may be explained by individual CYP3A4 activity [156], as outlined in the sections for the individual enzymes. Furthermore, many pharmaceutical companies incorporated genotyping and phenotyping for drug metabolizing enzymes and drug transporters in the drug development process.

Few studies however have been conducted to relate enzyme and transporter activities to drug effects in patients. Most of such studies are retrospective and have been done in small populations only. The few examples for prospective investigations include the assessment of the relationship between CYP2D6 genotype to efficacy and tolerability of haloperidol [101], or to the antiemetic effects of 5-hydroxytryptamine type 3 receptor antagonists [102]. A quantitative proposal for dose adjustment as it has been made for antidepressants based on CYP2D6 and CYP2C19 genotype is a rare exception [111]. With the exception of TPMT (chapter 13.2), no appropriate clinical studies clearly assessing the therapeutic benefit of dose personalization based on enzyme or transporter activity are available to our knowledge. As a consequence, information on individual protein activity currently is not being applied to personalize drug therapy. Because of the lack of data, despite its potential such procedures to date cannot yet be recommended outside clinical studies.

What is needed are large prospective randomized studies comparing traditional dose adjustment based on clinical effects only with additional dose personalization based on enzyme and/ transporter activity. In these trials, both average efficacy/tolerability and the degree of interindividual variation in these aspects should be compared. From such studies, algorithms and software tools (expert systems) need to be derived to take enzyme and'/or transporter activity into account for dose calculation. Finally, the cost-effectiveness of the approach must be addressed. Only then, it will be possible to judge whether the promising approach of genotyping and/or phenotyping to predict pharmacokinetics and effects of its substrates is worthwhile to be considered in clinical medicine.

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Subject index