Chromatographic Methods in Clinical Chemistry and Toxicology

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Contents

Preface

Among the vast array of methods available for biochemical analysis, chromatography occupies a venerable station. Few analytical methods have had such a vital impact on the development of clinical chemistry and toxicology. The roots of chromatography can be traced to the work of the Russian botanist Mikhail Semenovich Tswett, who separated plant pigments on a calcium carbonate column in 1903. Applications of this new technique to biochemical analysis were readily apparent. Chemical analysis of biological specimens is difficult because they contain a complex mixture of components, many of which react similarly with reagents selected to detect a specific compound. Analytical chemistry is not especially complicated when dealing with pure solutions, but the presence of many potentially interfering substances, as in biological matrices, creates a challenge. Chromatography answered that challenge by offering a method to separate components of a mixture, providing a pure specimen that could be measured by any of a number of analytical methods.

In this book, we have assembled a cross-section of contemporary applications of chromatographic methods used in clinical chemistry and toxicology. Chapter 1 focuses on quality assurance and quality control, emphasizing the importance of validating analytical methods that are used for clinical and forensic purposes. Merves and Goldberger cite the work of F. William Sunderman, to whom all clinical chemists and toxicologists owe a great debt of gratitude for his pioneering efforts to establish performance standards for clinical laboratories, including the first proficiency testing program ever devised. Sunderman was a fierce advocate of quality in laboratory medicine, and his dedication to that goal was pivotal in establishing national standards for laboratory practice in the USA, which were first codified in the Clinical Laboratory Improvement Act of 1967.

Just as any technology adapts to the applications for which it is suited, chromatography has evolved to meet the special needs of biochemical analysis. Packed column gas chromatography has been replaced by capillary column methods, which emerged in the late 1970s when fiber-optic technology provided a way to manufacture glass columns with micron-sized bores and lengths of 60 m and longer. Capillary gas chromatography revolutionized the analysis of drugs of abuse, with the ability to resolve congeneric compounds. Similarly, liquid chromatography has given way to the superior resolving ability of high-pressure (or -performance, depending on your etymological preference) liquid chromatography (HPLC). High-resolution derivatives of electrophoresis, a technique closely related to chromatography, have made the transition from research tools into commercial applications. Chromatography continues to evolve, and the versatility of this analytical method seems uniquely suited to clinical chemistry and toxicology applications.

Donald Catlin established the first Olympic drug testing laboratory for the 1982 Summer Games in Los Angeles, and he continues to operate the premier sports testing laboratory in the world. Catlin and co-workers Chang, Starcevic and Hatton, describe the application of liquid chromatography coupled with mass spectrometry for the detection of anabolic steroids in urine. Detection of exogenous steroids is challenging because structurally, the compounds are so closely related, and metabolic products of performance enhancing steroids that appear in the urine are often the same as naturally occurring products of steroid metabolism. Out of Catlins laboratory have come many innovations in the application of analytical chemistry to sports medicine, and as a consequence he has the distinction of making contributions both to clinical toxicology and to the forensic applications that ensure fairness in competitive sports.

Amitava Dasgupta applied HPLC to the detection and measurement of popular nutritional supplements. This is an important area of clinical investigation because the effects of these compounds on clinical laboratory tests are largely unknown. Dasgupta has explored this uncharted territory, providing an insight into the measurable effects dietary supplements have on laboratory tests. Le Bricon and colleagues used HPLC to measure L-dopa and L-tyrosine, as markers of malignant melanoma. Early detection of cancer is essential to the success of treatment, and the appearance of specific markers for this aggressive malignancy provides an important new tool in cancer therapy. Zhou and Johnston applied capillary zone electrophoresis, combined with liquid chromatography and mass spectrometry, to measure proteins in plasma. Proteomic analysis is a promising innovation in clinical biochemistry, and the work of Zhou and Johnston will make an important contribution to that field of study. Helander used HPLC to detect abnormally glycosolated transferrin, which is associated with alcohol abuse. Chan and Ho applied HPLC to the measurement of catecholamines, which are specific markers of neuroendocrine tumors.

This volume also contains several applications of toxicological interest. Larry Broussard describes the analysis of alcohols and inhalants by headspace gas chromatography. Wollersen and Musshoff developed liquid chromatographic methods for measuring organophosphorus pesticides, and Ropero-Miller used HPLC to detect and identify neurotoxins that are used as biochemical weapons. Marinetti developed an elegant GC-MS method for measuring γ-hydroxybutyrate and analogs, a regrettably popular group of abused drugs.

Analysis of heavy metals in biological specimens traditionally involves electrochemical or atomic absorption/emission techniques, but a few innovative methods involving chromatography have been pursued. Caruso and co-workers used a combination of liquid chromatography and an inductively coupled plasma detector to measure arsenic, mercury, selenium and platinum in a variety of biological matrices. Of particular interest is their ability to distinguish between the different compounds in which the metal occurs. Chromatography is ideally suited to their approach, in contrast to flame absorption and emission methods, which do not discriminate between various forms of the metal. Aggarwal, Fitzgerald and Herold pioneered a different approach to chromatographic measurement of heavy metals by generating volatile chelates that are separated by gas chromatography and detected by mass spectrometry.

The twelve applications described in this book illustrate the versatility of chromatographic methods and the range of applications to clinical chemistry and toxicology available with these powerful analytical techniques. Our intention was to provide an overview of useful methods, while emphasizing the contributions that chromatography continues to make in clinical laboratory medicine. We are indebted to our contributors for sharing their exemplary work in this field.

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Quality Assurance, Quality Control and Method Validation in Chromatographic Applications

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1.1 INTRODUCTION

Clinical chemistry and toxicology are also known as clinical biochemistry or clinical pathology, and they utilize a variety of analytical procedures to analyze body fluids to aid in medical prevention, diagnosis and treatment. Analytes commonly targeted include electrolytes, creatinine, albumin and other proteins, iron and prescription (therapeutic) and illicit drugs.

The focus of this chapter is on quality assurance and quality control issues facing clinical laboratories, with emphasis on chromatographic analysis. Chromatography is a versatile analytical technique with diverse applications including gas chromatography (GC), liquid chromatography (LC), GC or LC coupled with mass spectrometry (MS) and other qualitative and quantitative bioanalytical methods.

1.2 HISTORY

In his book, *Four Centuries of Clinical Chemistry*, Rosenfeld (1999) traces the evolution of laboratory medicine from medieval times to the present. Chemical testing in biological matrices began several centuries ago, with a very crude technique for detecting pregnancy. Egyptian women would urinate on wheat and other seeds, and then observe the seeds' growth patterns to reveal pregnancy, and it was supposed, the sex of the child. Subsequently,

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Egyptians, Persians, East Indians, Chinese and other cultures observed changes in urine in order to assess disease states. Slowly, they developed a better understanding of normal and abnormal biological processes. The sixteenth and seventeenth centuries marked a major turning point in diagnostic medicine: it became clear that observation alone was not sufficient to make many diagnoses, paving the way for laboratory investigation to become an integral part of medical practice. As a result, research and clinical laboratory facilities were established, and their contributions led to fundamental technological advances in clinical laboratory medicine.

The early nineteenth century was an active time for clinical chemistry, when the role of biochemical processes in physiology began to emerge. Scientists and physicians started to realize the importance of biochemical imbalances in many diseases. However, interest in laboratory medicine languished in the middle of the nineteenth century, mostly due to the limited advancements in technology that could be applied to biochemical analyses. In its early stages, the laboratory did not provide much help to the clinician; therefore, few resources were invested in this new field. Eventually, technology would fuel a re-emergence of laboratory medicine and establish its essential role in medical practice.

The term 'clinical chemical laboratory' was first used by Johann Joseph Scherer in the mid-1800s. Scherer was appointed director of the first independent hospital laboratory, in Würzburg, Germany. Although the term did not become part of the general German vocabulary for another 100 years (German-speaking areas used the term 'pathological chemistry' before 'clinical chemistry' became popular), Scherer played a key role in the development of many assays important during his career.

Medicine has advanced exponentially with diagnostic testing, and has, in turn, prompted further development in clinical chemistry and toxicology. In the late 1800s, medical technologists were trained to perform laboratory tests, eventually leading to the creation of private medical laboratory services. Eventually, it became clear that for medical laboratories to succeed, they needed to adopt specifications that would ensure the reliability of the qualitative and quantitative results they report. Quality control and assurance benefit the laboratory, because they increase productivity (Westgard and Barry, 1986) and maximize the cost-to-benefit ratio of medical laboratory testing (Kallner *et al*., 1999).

The impact of analytical imprecision and bias on medical decisions is mostly unknown. A large margin of error may be acceptable in some circumstances, whereas other clinical scenarios demand more accurate and precise laboratory measurements. Often, clinicians interpret laboratory results within the larger context of the patient's history and physical examination, but the influence of imprecision in laboratory data on a physician's assessment of a patient is not well documented (Kaplan, 1999). Klee *et al.* (1999) evaluated the effect of analytical bias on medical decisions involving three common laboratory tests: serum cholesterol testing for risk assessment of cardiac disease, serum thyroid-stimulating hormone measurement for the detection of hypothyroidism and serum prostate-specific antigen testing for prostate cancer risk assessment. In each case, the authors concluded that tighter control of analytical variability resulted in a greater certainty in patient classification.

Ensuring quality results in clinical chemistry and toxicology requires optimization of every aspect of the laboratory operation, including maintenance, waste disposal, analytical procedures, management, quality specifications, safety, cost management and overall strategies (Kallner *et al.*, 1999). Kaplan (1999) suggested five tools that would be helpful to consider when writing guidelines for clinical laboratories: the effect of performance on specific clinical decisions, effect of performance on broad-based clinical decisions, survey of

clinical opinions (i.e. when is an increase in error unacceptable), recommendations from professionals and societies and data from literature and proficiency testing.

Among clinical laboratories, there is a need for standardization to ensure that a result generated by one laboratory is comparable to the same measurement in another laboratory. As an example, therapeutic drug monitoring requires consistency of results over time, even when the measurements are being made in different laboratories (Groth, 1999). Several organizations are involved in establishing standards for clinical laboratory practice, including the National Academy of Clinical Biochemistry (NACB), the National Institute for Standards and Technology (NIST), the American Society of Clinical Pathologists (ASCP), the Clinical and Laboratory Standards Institute (CLSI), the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) and the International Organization for Standardization (ISO). These organizations are discussed in detail in a later section.

There has been significant progress in quality management over the last 50 years. For example, Levey and Jennings introduced statistical approaches to quality assurance in the 1950s. In the 1960s, Tonks, Barnett and Cotlove (among others) developed methods for controlling variability and established standards of quality (Westgard, 1999). In 1999, an international team of specialists with support from the International Union of Pure and Applied Chemistry (IUPAC) Clinical Chemistry Section, the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) and the World Health Organization (WHO) met for a 'consensus conference' to address laboratory quality goals (Kallner *et al.*, 1999). Other efforts towards improving laboratory quality are national, such as the set of guidelines for bioanalytical method validation in industry in the USA that was published by the Food and Drug Administration with help from the Biopharmaceutics Coordinating Committee in the Center for Drug Evaluation and Research in cooperation with the Center for Veterinary Medicine (FDA, 2001). There are also various guidelines written for different state and local jurisdictions.

Many challenges remain in establishing universal guidelines for laboratory quality. It should be noted that universal guidelines applicable to all analytical testing within clinical chemistry and toxicology cannot be set. Testing procedures have different goals that require unique specifications. For example, a semi-quantitative analytical protocol that involves a threshold concentration may only require the use of positive and negative controls, whereas quantitative assays have stricter specifications for linearity, accuracy and precision that require the use of multiple calibrators and controls.

1.3 DEFINITION OF QUALITY ASSURANCE AND QUALITY CONTROL

Quality assurance and quality control procedures are vital aspects of clinical chemistry and toxicology (Garfield *et al.* 2000; Tilstone, 2000; Ratliff, 2003). The terms quality assurance and quality control sometimes are used interchangeably, but the two terms have different meanings and should be used in different contexts. Quality assurance (QA) encompasses quality control, but also applies to virtually all aspects of laboratory operation. QA requires that all laboratory procedures are documented. Systematic internal and external review of these practices and documentation are essential to achieving optimal efficiency and accuracy (Rosenfeld, 1999). Key aspects of the QA process are laboratory accreditation, proficiency testing and staff education. Depending on the jurisdiction, there are many different types of

guidelines under the umbrella of QA. For example, the laboratories or technologists may be required to be certified by the state or other agencies. In addition, continuing staff education is essential for maintaining and updating credentials, and proficiency testing may be required in order to maintain certification.

Quality control (QC) originated from the industrial goal of producing each batch of product with consistently high quality, detecting and correcting process errors that result in defective products. Therefore, QC is an essential component of a laboratory QA program, because it provides a mechanism for monitoring the quality of laboratory results. To ensure that a set of analytical results meets the desired specifications, control specimens are included in each step of the procedure. These controls should include both normal and abnormal concentrations of analyte (Goldberger *et al.*, 1997).

1.4 PROFESSIONAL ORGANIZATIONS

As mentioned in Section 1.2, various professional organizations are involved in establishing standards for clinical laboratory practices and key organizations include the NACB, NIST, ASCP, CLSI, IFCC and ISO.

The National Academy of Clinical Biochemistry (NACB) is the Academy of the American Association for Clinical Chemistry (AACC). Its mission is 'to elevate the science and practice of clinical laboratory medicine by promoting research, education and professional development in clinical biochemistry and by serving as the leading scientific advisory group to AACC and other organizations' (http://www.nacb.org). It has six objectives which promote research, education, professional development, scientific advisory roles, recognition and infrastructure. Since the mid-1990s, the NACB has published several consensus-based laboratory medicine practice guidelines (LMPGs).

The National Institute for Standards and Technology (NIST) was established in 1901 as the National Bureau of Standards. The mission of NIST is 'to promote US innovation and industrial competitiveness by advancing measurement science, standards and technology in ways that enhance economic security and improve our quality of life' (http://www.nist.gov). The principal programs include NIST Laboratories, the Baldrige National Quality Program, the Manufacturing Extension Partnership and the Advanced Technology Program. As part of its commitment to the advancement of technology, NIST produces reference materials for bioanalysis. These standard reference materials are essential for validating the accuracy of analytical methods.

The American Society of Clinical Pathologists (ASCP) was founded in 1922 and its mission is to 'provide excellence in education, certification, and advocacy on behalf of patients, pathologists, and laboratory professionals' (http://www.ascp.org). The ASCP has developed many educational programs to advance the role of the laboratory in medical diagnosis (Rosenfeld, 1999).

In 1968, the National Committee for Clinical Laboratory Standards was established, and recently changed its name to the Clinical and Laboratory Standards Institute (CLSI) (http://www.nccls.org). The CLSI is a nonprofit organization that sponsors educational programs in order to promote national and international standards for clinical laboratories. This is a noteworthy task, especially since following these specifications is not mandatory and no enforcement agency exists to ensure each laboratory follows the CLSI guidelines. As part of its educational programs, the CLSI publishes consensus documents on laboratory

procedures, reference methods and evaluation protocols. A major goal of CSLI is to encourage high quality in the laboratory in order to maximize patient care benefits (Rosenfeld, 1999).

The International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) was established in the mid-1950s with goals to 'advance knowledge and promote the interests of biochemistry in its clinical (medical) aspects' (www.ifcc.org). IFCC was originally formed under IUPAC, but became independent in the late 1960s. However, it has maintained a close affiliation with IUPAC, and also WHO. Aside from its role in publishing guidelines and maintaining educational opportunities for its members, the IFCC plays a key role in developing national societies within countries without such resources.

The International Organization for Standardization (ISO) is a worldwide organization representing national standard societies from approximately 149 countries. ISO began in 1947 after a delegation of 25 countries met with the desire to 'facilitate the international coordination and unification of industrial standards' (http://www.iso.org). The ISO mission is to promote global standardization for exchange of services among nations. ISO publishes International Standards, which encourage intellectual, scientific, technological and economic exchange. For example, ISO 9000 is a set of four documents (9001–9004) that provide standards based on an international consensus for various aspects of quality assurance. In addition, ISO organizes Technical Advisory Groups to study quality control and assurance issues (Burtis and Ashwood, 2001).

1.5 INTERNAL QUALITY ASSURANCE AND CONTROL

Internal quality assurance and control procedures are designed to guarantee the reliability of the results generated by the laboratory; QA and QC ensure that the reported results contain the appropriate accuracy and precision through stringent monitoring of the analytical process, documentation of procedures and method validation.

1.5.1 Standard operating procedure manual

The standard operating procedure (SOP) manual contains the procedures validated by the laboratory; it is a complete set of instructions for pre-analytical, analytical and post-analytical methodology and also procedures for quality assurance/control, chain-of-custody and security. Each step in the handling of the specimen should be evaluated, optimized where possible and documented in the SOP. Important steps in the analytical process include collection, transport and accessioning of the specimen, sample preparation, isolation and detection of the analytes, production of the report and disposal of the specimen. This chapter focuses on the quality assurance and control issues for analytical method development and validation as well as statistical representation of the data.

1.5.2 Method development

Analytical methods are developed to meet specific needs. For example, pharmacodynamic and pharmacokinetic assessment of a new drug will require the ability to measure it in

biological matrices. These methods should be developed under the 'Good Laboratory Practice' (GLP) standards issued by the US Food and Drug Administration (FDA), and provide accurate and precise results. Typically, constraints are placed on the new methods, such as pre-established administrative reporting limits (e.g. cut-off concentrations) or existing QA/QC requirements (Goldberger *et al.*, 1997). Therefore, the intent and analytical goals must be established for the new protocol before the analytical technique is optimized on the bench. The analytical goals (also known as analytical quality specifications, performance standards or performance goals) are required not only for method development and validation, but also to help justify the purchase of new instrumentation and assist manufacturers in the design, construction and marketing of new equipment and reagents. In addition, the analytical goals provide specifications for relevant external quality assurance programs (Fraser *et al.*, 1999).

1.5.3 Method validation

Once a new analytical method has been developed, it must be optimized and standardized to meet the purposes for which it was developed. Validation studies confirm that a new assay has met its required performance specifications, and must be done before the analytical procedure can become a routine laboratory protocol. There are multiple approaches to validating analytical methods. The most common is to compare results from the new method with results obtained using an established, or reference, assay. Alternatively, standard reference materials (e.g. standards certified by NIST) can be analyzed by the new method to demonstrate its accuracy. When available, authentic specimens should be tested and compared against a previously validated protocol.

Each analytical method will have a different purpose and therefore different analytical performance requirements. Some of the performance parameters are accuracy and precision, recovery, limits of detection (sensitivity) and quantitation, range of linearity, specificity, stability, carryover potential and ruggedness. Other methadological factors are selection of a reference standard, internal standard, derivatizing agent, ions for selected-ion monitoring in mass spectrometry, instrumentation and chromatographic performance (Goldberger *et al.*, 1997; Gerhards *et al.*, 1999; Wu *et al.*, 1999; Garfield *et al.*, 2000; Burtis and Ashwood, 2001; FDA, 2001; Jiménez *et al.*, 2002). These parameters can be assessed within the same batch (intra-assay, within-run) or between batches (inter-assay, between-run or day-to-day) using reference standards and quality control samples. Most standards and quality control materials have been verified and are available commercially, often in the form as lyophilized or liquid samples.

Accuracy and precision are the most important characteristics of an analytical method; they give the best indication of random and systematic error associated with the analytical measurement. Systematic error refers to the deviation of an analytical result from the 'true' value, and therefore affects the accuracy of a method. One the other hand, random errors influence the precision of a method (Kallner *et al.*, 1999). Ideally, accuracy and precision should be assessed at multiple concentrations within the linear range of the assay (low, medium and high concentration).

After a method has been validated, it can be used for routine laboratory analyses. There may be additional analytical considerations that are important in routine assays. Analysis of the specimens should be done in a timely manner, especially when the analyte is unstable.

Generally, specimens can be run singly unless duplicate measurements are required in order to improve precision and reliability. Quality controls should be included with each batch of specimens and the QC results monitored to detect trends in analytical performance that can potentially affect the accuracy of results.

1.5.4 Accuracy

Accuracy defines how closely the measured concentration agrees with the true (fortified) value. For most analytical applications, laboratories should achieve a level of accuracy within 5–10% of certified concentrations in reference materials. Accuracy is often expressed as a percentage difference from the true value:

$$\text{Accuracy}(\%) = [(\text{mean} - \text{true value})/\text{true value}] \times 100$$

Accuracy can also be expressed as the percentage of the mean to the true value:

$$\text{Accuracy}(\%) = (\text{mean/true value}) \times 100$$

Ideally, accuracy should be determined at multiple concentrations of the analyte. Since repeated measurements by any analytical method will produce a Gaussian distribution of results, Student's *t*-test provides a statistical way to assess the agreement of measured values with the target or 'true' value.

1.5.5 Precision

Precision describes the variability in a set of measurements, i.e. how closely repeated measurements on a single sample agree. Precision can be calculated for samples within an individual run (intra-assay precision) or across multiple runs (inter-assay precision). Precision is customarily expressed as the percentage coefficient of variation (%CV, or simply CV), which is the standard deviation expressed as a percentage of the mean:

$$\text{CV} = (\text{standard deviation/mean}) \times 100$$

Typically, laboratories should achieve a level of precision of $<10\%$; however, in some instances, the CV may be $>10\%$, especially at or near the lower limit of quantitation, when background noise is a larger portion of the total signal measured. The CV should be determined at multiple concentrations of the analyte.

1.5.6 Recovery

When a protocol requires some manipulation of the specimen prior to analysis, such as an extraction step, there may be opportunities for the analyte concentration to be diminished due to pre-analytical loss. Therefore, it may be important to calculate the recovery of the analyte. Analyte recovery can be calculated by comparing the detector response from the

analyte after it has been extracted to the detector response from an unextracted sample. The unextracted sample represents 100% recovery because it contains the 'true' concentration of the analyte. It is not necessary to achieve 100% recovery, but the recovery should be reproducible and evaluated at three separate concentrations (FDA, 2001).

1.5.7 Lower limits of detection (sensitivity) and quantitation

The lower limit of detection (LLOD) and the lower limit of quantitation (LLOQ) are helpful parameters when validating an analytical method. The LLOD is the lowest concentration of the analyte that can be detected. Customarily, the LLOD corresponds to the concentration of analyte producing a signal at least three times greater than the analytical background noise. Establishing an LLOD requires knowing the signal-to-noise ratio, acceptable chromatographic separation, predicted retention time and, for mass spectrometric (MS) applications, acceptable *m/z* ion ratios.

The LLOQ is the lowest concentration of the analyte that is detected with a specified accuracy and precision. In practice, the LLOQ often corresponds to a signal 5–10 times greater than the analytical background noise. As an example, the LLOQ might correspond to the concentration meeting all of the LLOD criteria, be quantified within 10% of the target concentration and produce a CV of $<10\%$. These parameters can be optimized by manipulating several factors, including the specimen volume, the detector threshold (gain), the type and condition of the chromatographic column, preconcentration of the analyte, the amount and type of internal standard, the extraction efficiency and the analytical method (Goldberger *et al.*, 1997).

1.5.8 Range of linearity

Most, though not all, analytical methods produce results that vary linearly with analyte concentration, so the term calibration 'curve' is something of a misnomer. The range of linearity of an analytical method is determined by measuring standard solutions at multiple concentrations (calibrators), sometimes using an internal or external standard for quantitation. Calibrators should be prepared in a matrix similar to authentic specimens, to compensate for potential matrix effects. The practical linear range, which encompasses the expected concentrations in authentic specimens, should be assessed, i.e. challenged at its upper and lower limits, each time the assay is run. An acceptable linear range depends on various criteria, such as chromatographic resolution, correct retention time, appropriate *m/z* ion ratios and a correlation coefficient (r) or coefficient of determination (R^2) >0.99 (Goldberger *et al.*, 1997).

There are various ways to derive a calibration curve. Multi-point calibration curves, for example, include a minimum of three different concentrations of the analyte. For semi-quantitative assays, a single-point calibration is common. The single point is usually the threshold concentration used to determine whether a specimen is positive or negative for the analyte of interest. Depending on the validation process and performance characteristics of the assay, a single-point calibration curve may also be used in quantitative applications over a limited range of linearity. A historical (pre-established) multi-point calibration curve may also be used, but only if the stability of the analytical method over time has been well established (Goldberger *et al.*, 1997).

1.5.9 Specificity

Specificity, or selectivity, is defined as the capability of the methodology to identify the analyte of interest in the presence of matrix components and potentially interfering substances (FDA, 2001; Goldberger *et al.*, 1997). Analytical interferences include endogenous matrix components, decomposition products, metabolites and other structurally related compounds. FDA guidelines (FDA, 2001) suggest that six blank specimens from different sources be analyzed for matrix effects. Fewer samples may be required when using methodology with MS detection.

In contrast, there are instances in which decreased specificity is beneficial, such as in assays designed to detect an entire class of compounds. For example, antibody cross-reactivity is essential in immunoassays used to screen for multiple drugs or drug classes. Screening methods ordinarily are paired with confirmatory testing methods that use a different analytical methodology with increased specificity.

1.5.10 Stability

Some analytes are not stable under certain transport, storage and analytical conditions. Therefore, it is essential to evaluate the stability of an analyte under a variety of conditions. Specimens may be collected with preservatives (e.g. sodium fluoride) and stored refrigerated or frozen. During analysis, the addition of an internal standard and/or the use of quality control samples can be helpful in evaluating the stability of an analyte during the entire analytical procedure.

For assays requiring specimens to be frozen, the stability of the analyte in a freeze–thaw cycle should be validated. FDA guidelines (FDA, 2001) suggest that analytes should be tested in triplicate over three freeze–thaw cycles at two different concentrations.

1.5.11 Carryover

Carryover is defined as the 'contamination of a sample by a sample analyzed immediately prior to it' (Goldberger *et al.*, 1997). Therefore, the validation process should assess carryover potential in highly concentrated specimens and the acceptable limit of carryover into otherwise negative samples. Suggested ways to minimize carryover include the use of solvent washes after each injection, the use of solvent blanks between cases, dilution of specimens prior to extraction, dilution of extracts prior to injection, occasional reassessment of the minimum carryover limit and injection of specimen extracts in ascending concentration order (determined by initial screening). Carryover is most often due to contamination by the sampling syringe exposed to the previous specimen. Therefore, when carryover is suspected, the specimen in question should be re-extracted, since re-injection from the already contaminated vial will produce the same carryover error.

1.5.12 Ruggedness

Ruggedness refers to the ability of an analytical method to withstand minor procedural changes without affecting the overall performance of the assay. The ruggedness of an assay

can be very important, particularly when multiple technicians or multiple laboratories perform the same procedure. Therefore, it is useful to determine the critical steps in the assay, such as pH, composition of solvent mixtures, derivatizing chemicals, temperature and incubation times (Goldberger *et al.*, 1997). In addition, specimen volume may be critical, so any adjustments to the volume of specimen should be validated. If specimen dilutions are required, the preferred diluent is blank (analyte-free) matrix (FDA, 2001).

1.5.13 Selection of a reference standard

Selection of a reference standard for most analytical methods is a straightforward task, involving acquisition of the relevant analyte in a state of known composition and purity. However, some analytes are not available in pure form, and structurally similar analogs (e.g. free base) may be used instead. Standard materials should be of a known purity, and the source, lot number, expiration date and certification of identity should be recorded (FDA, 2001).

1.5.14 Selection of an internal standard and standard addition

Analytes may be quantitated using an internal standard or by the method of standard addition. An internal standard is a structural analog (e.g. deuterium-labeled compound), a known amount of which is added to specimens prior to any preparatory steps such as extraction or hydrolysis. Ideally, the internal standard should be as chemically similar to the analyte of interest as possible, so that throughout the analytical procedure (e.g. extraction, chromatographic separation and ionization processes) and under the specified conditions (e.g. derivatization), its recovery will be similar to the analyte's. The concentration of analyte can then be determined from the ratio of the analyte response to the internal standard response.

The addition of an internal standard is advantageous because detection of the internal standard ensures that the extraction step successfully recovered analyte (if present). Any losses associated with preparatory steps are not critical, because this procedure relies on the ratio of concentrations, not absolute concentration.

Standard addition can be an alternative method for the quantitation of analytes. The general concept is to add known concentrations of the analyte to multiple aliquots of the unknown specimen. By extrapolation, the original concentration of the (unfortified) specimen can be determined. This technique is particularly helpful when a calibration curve cannot be generated using standards in a similar matrix.

1.5.15 Selection of derivatization agent

In the context of chromatographic applications, derivatization is the process of chemically altering a compound with the intent to improve the sensitivity with which it can be detected. Often, derivatization involves reacting polar groups (-NH, -OH and -SH) with perfluoryl or silyl derivatization reagents to increase the volatility of the compound. Some derivatives enhance the detector response, improving sensitivity. In other circumstances, it may be

beneficial to increase the mass of a compound to distinguish its ions from background noise or similar structures (Goldberger *et al.*, 1997). There are some of disadvantages to derivatization, such as requiring an extra step (often manual) in the analytical method and the absence of many derivatized products in commercial mass spectral libraries. Therefore, laboratories may need to produce their own reference libraries when using unusual derivatives for MS applications.

1.5.16 Selection of ions for selected-ion monitoring or full-scan analysis

Assays that utilize MS require the selection of ions that uniquely represent the analyte and internal standard without any potential interferences (Goldberger *et al.*, 1997). It is helpful to identify ions for both quantitative and qualitative purposes. Ratios between the ions can be helpful to confirm the compound of interest. The base peak (ion with the highest abundance in the mass spectrum) is often preferred as the quantitative ion because it allows for the greatest sensitivity.

1.5.17 Chromatographic performance

Methods involving chromatographic separations prior to detection require optimization of the chromatographic conditions for best performance. A Gaussian peak shape (no tailing or fronting), resolution (baseline separation from nearby peaks) and reproducible retention time are necessary for identification of the analytes of interest (Goldberger *et al.*, 1997).

1.5.18 Statistical evaluation of quality control

Proper interpretation of quality control data requires presentation in a format that makes systematic bias readily apparent. Based on the work of Shewhart, Levey and Jennings, Westgard and co-workers proposed in 1981, an algorithm for monitoring quality control in clinical laboratories (Westgard *et al.*, 1981). 'Westgard's Rules' provide guidance for determining when control values are acceptable or, alternatively, when control measurements reflect changes in the performance of the analytical method (Westgard and Barry, 1986; Burtis and Ashwood, 2001).

In a Levey–Jennings control chart, the *x*-axis represents time or number of analytical runs and the *y*-axis represents the control results (Figure 1.1a). Control values are plotted on the Levey–Jennings chart, and should fall within a statistically predicted range, based on a Gaussian distribution. The acceptable range of values is typically indicated by lines on the *y*-axis representing the upper and lower limits for control results (commonly ± 2 standard deviations or defined percentages from the mean control value). Westgard described a multi-rule procedure using the Levey–Jennings chart to detect deviations from Gaussian behavior that reflect analytical bias (Burtis and Ashwood, 2001).

Another way to present control data is the cumulative sum control chart. In this method, the *x*-axis is either time or number of control observations and the *y*-axis is the cumulative sum (cusum), or the difference between the measured control value and the predicted mean

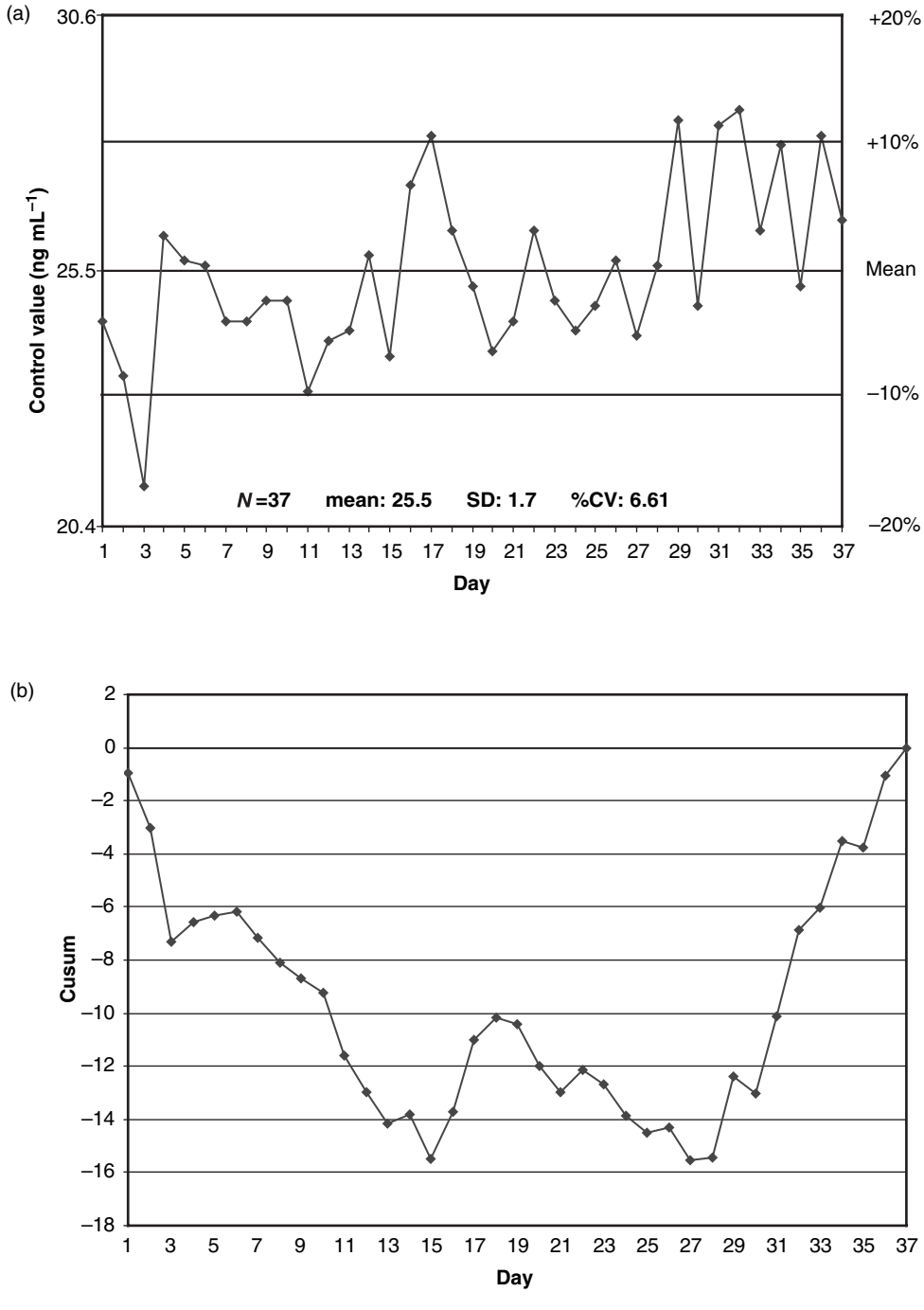

Figure 1.1 Examples of (a) Levey–Jennings and (b) cumulative sum (cusum) control charts using inter-assay quality control data from an alprazolam GC–MS assay. Since the cusum chart persents the cumulative sum of deviations from the mean, it is more sensitive to small biases that develop over time, whereas Levey–Jennings charts are most useful for detecting changes in the precision of the assay

concentration of the control material (Figure 1.1b). The cusum is calculated by adding this difference to the differences from previous control observations. Random errors should produce cusum values that cross the zero line (mean value) multiple times. However, if there are other sources of error, then the cusum will deviate from the mean value in one or the other direction. Other quality control charts, such as mean, standard deviation and range charts, are used in industry, but are not common in clinical chemistry and toxicology (Westgard and Barry, 1986).

1.6 EXTERNAL QUALITY ASSURANCE

External quality assurance (EQA) is fundamental to the standardization of clinical laboratory methods because it provides a means to compare results generated in one laboratory with those of peer laboratories subscribing to the same EQA program. EQA programs are especially beneficial since internal QA and QC procedures are limited in their ability to detect bias in analytical methods. Internal QA/QC can only detect errors that result in a deviation from the original method validation: inherent errors in the method may go unnoticed. Therefore, it is helpful to compare the results produced by a new method with those from other laboratories (Burtis and Ashwood, 2001). Monitoring the performance of laboratory procedures in a consistent manner keeps the laboratory accountable, and can reveal systematic errors that would otherwise be undetected. A prominent component of EQA is proficiency testing.

Proficiency testing in clinical laboratories began in the late 1940s as a response to lack of reproducibility among laboratories (Rosenfeld, 1999). In 1947, Belk and Sunderman published the results of a study in which they surveyed clinical laboratories in Pennsylvania for accuracy among common chemical testing procedures in hospital laboratories (Sunderman, 1992). Thereafter, other states conducted similar studies, and eventually monthly samples were mailed to various laboratories worldwide and the statistical summaries were returned to these laboratories, comparing their results with other laboratories performing the same analyses. The Sunderman Proficiency Testing Service diligently continued this program for 36 years until 1985, when the American Society of Clinical Pathologists took over the service. Since then, many other organizations, such as College of American Pathologists, the American Association of Bioanalysts and American Association for Clinical Chemistry have provided various forms of proficiency testing. Combined, these external testing procedures have helped to standardize testing among laboratories and enhance the overall quality of laboratory practices.

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2

Liquid Chromatographic-Mass Spectrometric Measurement of Anabolic Steroids

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2.1 INTRODUCTION

The growing availability of liquid chromatography–tandem mass spectrometry (LC-MS-MS) instruments to life science researchers has sparked an exponential flurry of efforts to discover new possibilities for the detection and quantitation of anabolic steroids in a wide variety of samples. Of all the directions and applications that have been explored to date, the area of greatest potential impact on science focuses on natural androgens in human samples, as opposed to synthetic anabolic steroids or animal samples.

The quantitation of natural androgens has been attempted in a wide variety of human samples, from urine and serum to prostate biopsy samples and testicular fluid. Analytical studies have also been conducted on standards and various *in vitro* media. Because the incidence of one particular androgen-dependent disease, prostate cancer, is on the rise, many studies revolve around its diagnosis and monitoring. In the wake of research connected to prostate cancer and the related benign prostatic hyperplasia, we can hope to gain insights into more basic aspects of human physiology and pathology. After all, androgens are sex steroids associated with a number of physiological developments and responses such as the development of reproductive systems, sexual differentiation and maintenance of secondary sexual characteristics. The balance of male and female hormones can elicit biological responses with a profound impact on health and behavior.

Testosterone (T), the male hormone *par excellence*, is primarily produced in the testis. It is metabolized to the more potent dihydrotestosterone (DHT). Physiological T concentrations reflect the balance between biosynthesis and metabolism. Therefore, T is a prime

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target of quantitative assays because T measurements make it possible to determine its pharmacokinetics, production rate and clearance. In turn, these can be used in clinical research studies to study androgen metabolism after physiological or pharmacological interventions or to fine tune replacement doses.

T is most commonly measured in serum, and it is one of the hormones most commonly measured in serum. T circulates in plasma non-specifically bound to albumin, specifically bound to sex hormone binding globulin and unbound (free). Clinicians use serum T measurements to diagnose and monitor various disorders such as hypogonadism (androgen deficiency), testicular dysfunction, hirsutism, virilization, alopecia, prostate disease, adrenal hyperplasia and aging, in addition to anomalies caused by exposure to chemicals or hormone disruptors.

Most of these measurements are still routinely performed with immunoassays because of their simplicity, rapidity and relatively low cost, and despite occasional concerns about their reliability and validity. Serum total T has also been quantitated by gas chromatography–mass spectrometry (GC-MS), liquid chromatography–mass spectrometry (LC-MS) and LC-MS-MS. MS-based methods are often used in research studies or to confirm immunoassay results. The advantage of chromatography coupled with MS is the high specificity not available with immunoassays because they are susceptible to cross-reactions. An immunoassay might measure a host of structurally related compounds in addition to the target analyte.

Recent clearance studies use stable isotope dilution methods. MS methods make it possible to measure deuterated and endogenous species simultaneously and specifically. LC-MS methods offer advantages over GC-MS methods, such as streamlined sample preparation (no derivatization necessary), high recovery and sensitivity and superior specificity.

This chapter will focus primarily on natural anabolic steroids in human samples because of the potential impact of improved analysis and quantitation by LC-MS-MS on our understanding of reproduction and disease. However, LC-MS methods for synthetic steroids or animal samples have also been published, therefore they will be reviewed first, but only briefly.

2.2 LC-MS ANALYSIS OF SYNTHETIC STEROIDS OR ANIMAL SAMPLES

In veterinary medicine, boldenone, a synthetic anabolic steroid (Figure 2.1), is commercially available, hence it is a concern in the horseracing industry. Pu *et al*. (2004) used ion-trap LC-MS analysis to detect boldenone conjugates (sulfate and glucuronide) and their 17-epimers in horse urine after intramuscular administration of boldenone undecylenate. Soon afterwards, Ho *et al*. (2004) reported the occurrence of endogenous boldenone sulfate in the urine of uncastrated male horses, and quantitated it by quadrupole time-of-flight (Q-TOF) LC-MS-MS.

Also known as endogenous in male horses and prohibited as a doping agent is 19-nortestosterone (nandrolone; Figure 2.1). Kim *et al*. (2000a) validated an LC-MS-MS method for the detection and quantitation of three different commercially available esters, but 19-nortestosterone esters are rapidly hydrolyzed in horse plasma, which limits the usefulness of this method in racehorse doping control.

Another synthetic anabolic steroid available as a veterinary product is stanozolol (Figure 2.1), whose metabolites were investigated by McKinney *et al.* (2004) by ion-trap LC-MS in horse urine after intramuscular injection.

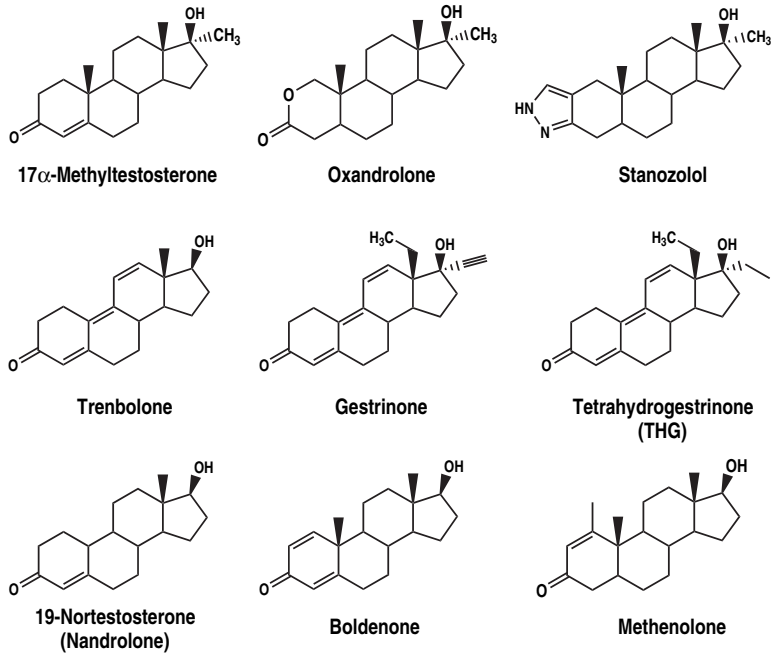

Figure 2.1 Examples of synthetic androgen analytes

Yu *et al*. (2005) developed an LC-MS-MS screen for deconjugated anabolic steroids in horse urine and characterized the method using horse urine samples spiked with 15 prohibited anabolic steroids. In an excretion study in two horses, methenolone acetate was administered by mouth, methenolone (Figure 2.1) was detected in urine and the 17-epimethenolone metabolite was detected for a longer time.

Racehorse anti-doping work is not the only area that requires screening for prohibited anabolic steroids in animals. The drugs act as growth promoters in cattle and this use is prohibited by European regulations, hence analytical methods have been developed to detect residues in meat for enforcement purposes. Joos and Van Ryckeghem (1999) described a procedure for the LC-MS-MS analysis of 36 anabolic steroids found in animal kidney fat matrices. The method's limits of detection are low enough to meet regulations. In Japan, where maximum residue limits were established in 1995, Horie and Nakazawa (2000) developed a method for the determination of trenbolone (Figure 2.1) and zeranol (an anabolic agent but not a steroid) in bovine muscle and liver tissues by LC-MS with selected-ion monitoring (SIM).

Draisci *et al*. (2000) used LC-MS-MS to quantitate T (Figure 2.2), 19-nortestosterone and their 17-epimer metabolites in bovine serum and urine, and subsequently stanozolol and its major metabolite (Draisci *et al*., 2001) and boldenone (Draisci *et al*., 2003) in bovine urine. Van de Wiele *et al*. (2000) also worked on stanozolol in cattle urine and feces, with or without derivatization before LC-MS-MS analysis. For trenbolone, Buiarelli *et al*. (2003) developed and characterized an LC-MS-MS method for bovine urine and serum. Van Poucke and co-workers extended the list of LC-MS-MS target analytes to four anabolic steroids (Van Poucke and Van Peteghem, 2002), then 21 anabolic steroid residues in bovine urine (Van Poucke *et al*., 2005).

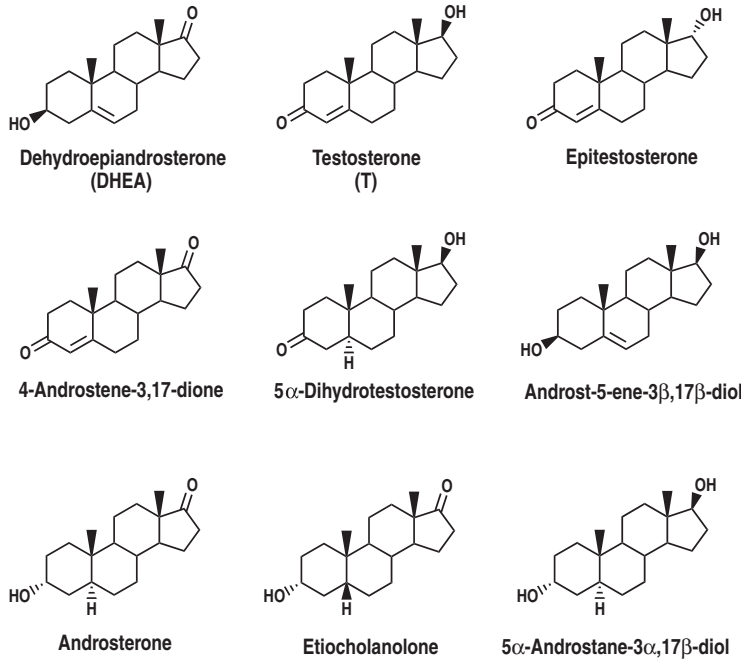

Figure 2.2 Examples of natural androgen analytes in human samples. Glucuronide or sulfate conjugates of these steroids are formed with the C-3 or C-17 hydroxyl group

Poelmans *et al*. (2002) reviewed analytical approaches to the detection of stanozolol and its metabolites.

Nielen *et al*. (2001) focused on a related facet of the illegal use of growth promoters in cattle, namely the detection of anabolic steroids in illegal cocktails. They presented a Q-TOF LC-MS-MS method with the aim of measuring accurate mass and calculating elemental composition for identification purposes.

Turning to the abuse of anabolic steroids by human competitors, Shackleton *et al*. (1997) conducted LC-MS analyses of pharmaceutical T esters in human plasma because of the potential applications to doping control, especially if sensitivity could be improved.

Leinonen *et al*. (2002) compared LC-MS-MS with electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) for unconjugated (free) anabolic steroids in human urinary extracts. The selected analytes were synthetic steroids or their metabolites, known to be misused in sports and known to be excreted in urine unconjugated, namely oxandrolone (Figure 2.1), the 3′-hydroxy metabolite of stanozolol and the 6β-hydroxy metabolite of 4-chlorodehydromethyltestosterone.

Kim *et al*. (2000b) used GC-MS, LC-MS and LC-MS-MS to study the metabolites of gestrinone (Figure 2.1), a contraceptive and potential sports doping agent, in human urine.

Anti-doping scientists had suspected for years that underground chemists supply performance-enhancing substances to athletes compelled to cheat, including ‘designer’ drugs made and used only to beat the test. This was proven when the anabolic steroid

tetrahydrogestrinone (THG; Figure 2.1), a new chemical entity, was identified by Catlin *et al*. (2004) in a used syringe anonymously turned in to the US Anti-Doping Agency. LC-MS and LC-MS-MS played key roles in the identification and urine sample screening procedures. Thevis *et al*. (2005) reasoned that chemical modifications of steroids usually alter their molecular weights, used to detect and identify them in doping control samples, but might not alter their nuclei or some of their characteristic fragments or product ions. When suspicious product ions have been detected, precursor ion scan experiments can help identify unknown steroids.

Another reality that anti-doping scientists must face is that some athletes accused of using banned anabolic steroids offer explanations such as the inadvertent consumption of various alleged sources of the substances. To assess the theory that uncastrated boar meat consumption can result in an adverse finding in a doping control urine test, Le Bizec *et al*. (2000) tested volunteers and detected 19-norandrosterone and 19-noretiocholanolone for about 24 h after intake. The same team identified and quantitated the boar meat 19-norsteroids responsible for this finding (De Wasch *et al.*, 2001).

To help provide anti-doping scientists with reference standards for LC-MS work, Kuuranne *et al*. (2002) carried out the enzyme-assisted syntheses of the glucuronides of methyltestosterone and nandrolone (Figure 2.1), and developed a new LC-MS method to control the synthetic product purity.

Watching the world become awash in steroid sex hormones, abused in animals and humans, Lopez de Alda *et al*. (2003) felt the need to review LC-MS and LC-MS-MS methods for the determination of these and other analytes in the aquatic environment.

In the only publication on the LC-MS analysis of synthetic steroids or animal samples that is unrelated to any steroid misuse, Magnusson and Sandstrom (2004) reported on a quantitative analysis of eight T metabolites in rat intestine mucosa.

2.3 LC-MS ANALYSIS OF NATURAL ANDROGENS IN HUMAN SAMPLES

Table 2.1 summarizes publications describing the measurement of natural anabolic steroids as standards or in human samples. Listed are analytes, samples, sample preparation, LC conditions, MS-MS conditions, whether the assay is quantitative or qualitative and references.

In anticipation of potential studies involving endogenous steroids, including neurosteroids, or exogenous steroids, or metabolites, Ma and Kim (1997) reported results obtained by LC-MS analysis by APCI or ESI of 29 steroid standards. Twelve of those had an androstane nucleus. They were either 3-one-4-enes or unconjugated ketones or they had only hydroxyl groups. The use of different LC mobile phases for each ionization mode helped to optimize the sensitivity.

Under the heading of natural androgens in human samples falls one kind of drug abuse – sports doping or the use of athletic performance- enhancing substances – which is prohibited by major sports organizations. If athletes take pharmaceutical T, the level of T in their urine will increase, but the level of epitestosterone, a natural isomer with no known function, will not, therefore the ratio of the two, of T/E, will increase. In the 1980s, the International Olympic Committee defined a T/E ratio of >6 as an adverse finding that anti-doping laboratories ought to report, and in 2005 the cut-off was lowered to 4. To help study and

Table 2.1 Summary of LC-MS analyses relevant to natural androgens in human samples

Analyte	Sample	Sample preparation[a]	LC injection volume, stationary phase	MS-MS	Quantitative	Comments	Ref.
29 steroids (12 androstane-type), 3-one,4-enes, unconjugated ketones, hydroxyls only	Standards	None	C_{18}	LC-MS APCI and ESI full-scan and SIM	NA		Ma and Kim (1997)
T glucuronide, T sulfate, epitestosterone glucuronide, epitestosterone sulfate	Standards	None	C_{18}	LC-MS and LC-MS-MS triple quadrupole ESI	NA	Explored potential to detect T misuse in sports doping	Bowers and Sanaullah (1996)
8 glucuronide standards: T, epitestosterone, nandrolone, androsterone, 5α-estran-3α-ol-17-one, 5β-estran-3α-ol-17-one, 17α-methyl-5α-androstane-3α,17β-diol, 17α-methyl-5β-androstane-3α, 17β-diol	Standards	None	No LC	Triple quadrupole ESI and APCI negative and positive ion mode	NA	Potential application to sports doping control analysis	Kuuranne *et al.* (2000)
T, dihydrotestosterone, 4-androstene-3,17-dione	100 μL cell culture medium	No IS, no sample preparation except automated: column-switching C_4-alkyldiol-silica restricted access SPE column	100 μL, C_{18}	Triple quadrupole ESI positive ion mode	Y	Comparison with RIA	Chang *et al.* (2003)
T, hydrocortisone, SR 27417	1 mL spiked human plasma	IS d_3-T C_8 disc		Ion-trap APCI positive ion mode	Y		Tiller *et al.* (1997)

Dehydroepieandrosterone and sulfate, androstenedione, T	Standards and 760 μL spiked serum	IS deuterated analogs, precipitation by adding 1140 μL IS solution, analyze supernatant	1700 μL, C_{18}	Triple quadrupole APPI positive ion mode	Y	Comparison with 7 RIAs	Guo *et al.* (2004)
Androstenediol 3-sulfate, dehydroepieandrosterone sulfate	100 μL human serum	Deuterated analogs, protein precipitation, SPE, 30 μL acetonitrile–ammonium bicarbonate reconstitution	C_{18}	LC-MS ion trap ESI negative ion mode	Y	Comparison of healthy subjects with prostate cancer or benign prostate hyperplasia patients	Mitamura *et al.* (2003)
Dehydroepieandrosterone sulfate	5 μL human plasma	IS deuterated analog, add aqueous ethanol, supernatant through C_{18}, reconstitute in 100 μL liquid phase	Capillary LC	Triple quadrupole ESI negative ion mode	Y		Liu *et al.* (2003)
T	2 mL human serum	IS d_3-T C_8 disc		Triple quadrupole APCI positive ion mode	Y	Comparison with 6 IAs	Wang *et al.* (2004a)
T, d_3-T	2 mL human serum	IS 19-nortestosterone, sodium acetate buffer, 5-mL ether extractions 100 μL ethanol reconstitution	15 μL, C_{18}	Triple quadrupole APCI positive ion mode	Y	Assay applied to 38 samples from normal volunteers Influence of ethnicity and age in normal men	Starcevic *et al.* (2003) Wang *et al.* (2004b)
T	50 μL human serum	IS d_2-T, 100 μL zinc sulfate–methanol precipitation	40 μL, C_{18}	Triple quadrupole APCI positive ion mode	Y	Sample preparation and GC-MS vs LC-MS-MS comparison, T measurement in women	Cawood *et al.* (2005)

(*continued*)

Table 2.1 (*Continued*)

Analyte	Sample	Sample preparation[a]	LC injection volume, stationary phase	MS-MS	Quantitative	Comments	Ref.
T glucuronide, dihydrotestosterone glucuronide	50 μL human urine	IS d_3-T, filtration	C_{18}	Ion-trap ESI negative ion mode	Y	Comparison of benign prostate hyperplasia and normal men	Choi *et al.* (2003)
T glucuronide, T sulfate, epitestosterone glucuronide, epitestosterone sulfate	2 mL human urine	IS d_3-T glucuronide, d_3-epitestosterone sulfate, SPE, aqueous methanol elution, 20 μL water reconstitution	5 μL	Triple quadrupole ESI positive ion mode	Y	To detect T misuse in sports doping?	Bean and Henion (1997)
T glucuronide, T sulfate, epitestosterone glucuronide, epitestosterone sulfate	Standards and 3 mL human urine	IS deuterated analogs, SPE C_{18} cartridge, aqueous methanol elution, 100 μL mobile phase reconstitution	10 μL, C_{18}	Triple quadrupole ESI positive ion mode	Y	To detect T misuse in sports doping?	Borts and Bowers (2000)
T, epitestosterone, dehydroepiandrosterone, androsterone, etiocholanolone, their glucuronides and their sulfates	2 mL human urine	IS d_3-epitestosterone glucuronide, d_3-T sulfate, d_3-T, SPE, methanol elution, 50 μL aqueous methanol reconstitution	5 μL	Triple quadrupole ESI positive and negative ion mode	Ratios	To profile urinary steroids for doping control	Buiarelli *et al.* (2004)

Dehydroepiandrosterone metabolites: androst-5-ene-3β,17β-diol, androst-4-ene-3,17-dione, T, 5α-dihydrotestosterone, androsterone, 7α-hydroxydehydro-epiandrosterone	Human prostate homogenate incubation mixture	IS d_4-DHEA, hexane–ethyl acetate extraction, C_{18}, aqueous acetonitrile	C_{18}	LC-MS ion-trap APCI full-scan mode	No	Comparison with GC-MS results	Mitamura *et al.* (2002)
Dihydrotestosterone	Human serum and prostate biopsy tissue	Alkaline extraction, SPE, *N*-methylpyridinium polar derivatization		LC-MS ESI positive ion mode	Y	Cancer patients before and after androgen deprivation therapy	Nishiyama *et al.* (2004)
T, dihydrotestosterone	10 mg human prostate tissue	IS d_3-T, aqueous methanol, Oasis HLB cartridge, *N*-methylpyridinium polar derivatization	C_{18}	Triple quadrupole ESI positive ion mode	Y	Comparison of sujects with benign prostate hyperplasia and prostate cancer	Higashi *et al.* (2005)
T, dihydrotestosterone, estradiol, 5α-androstane-3α,17β-diol	20 μL human testicular fluid	IS dimethylbenzoyl-phenylurea, one-step ether extraction	C_{18}	Triple quadrupole ESI positive ion mode	Y	Comparison with RIA	Zhao *et al.* (2004)

[a]IS, internal standard.

understand such cases, and to help resolve basic questions about steroid excretion, Bowers and Sanaullah (1996) worked out a direct method for the quantitation of four analyte standards, namely the conjugates (glucuronides and sulfates) of T and its naturally occurring but inactive epimer, epitestosterone (Figure 2.2), by LC-MS and LC-MS-MS. The following year, Bean and Henion (1997) reported the quantitation of the same four analytes in human urine. Using [16,16,17-$^{2}H_3$]testosterone glucuronide and [16,16, 17-$^{2}H_3$]epitestosterone sulfate as internal standards, they extracted 2 mL of urine by solid-phase extraction and monitored *m/z* 465–289, 465–271 and 465–253 for T glucuronide and epitestosterone glucuronide, and *m/z* 468–292 and 372–273 for the trideuterated internal standard analogs. The positive ion mode was substantially more sensitive than the negative ion mode for T glucuronide, and almost as sensitive for epitestosterone sulfate. Switching mode would have been impractical with the instrument used by the authors because the analytes eluted too close to each other. Calibration curves were prepared in urine for T glucuronide and epitestosterone glucuronide from 50 to 1000 nmol L^{-1} and for T sulfate and epitestosterone sulfate from 10 to 200 nmol L^{-1}. LODs for all four target analytes and for the two internal standards were in the low nanomolar range. Subsequently, Borts and Bowers (2000) tried to overcome ion suppression by using deuterated analogs of all four analytes. They extracted 3 mL of urine and extended the calibration curves to cover 0–1500 nmol L^{-1}. Comparisons with a GC-MS method and with published population data showed agreement. These methods targeting the urinary glucuronides and sulfates of T and epitestosterone were published between 1996 and 2000, but their potential applications in human sports doping analysis, to control the abuse of T, have not become a reality. Perhaps this is in part because the method fails to close a loophole in the detection of T abuse. Athletes determined to cheat might take both T and epitestosterone, to benefit from T while keeping T/E below the cut-off. Quantitating urinary conjugates might not have helped to distinguish such users from innocent athletes, because like GC-MS, LC-MS-MS is unable to distinguish natural from pharmaceutical T. By the late 1990s, this loophole was closed by the introduction of isotope ratio mass spectrometry, which is able to detect a measurable difference in carbon-13 between natural and pharmaceutical T.

Buiarelli *et al*. (2004) extended the above analytical approach to many more related steroids when they published a method for the direct analysis of 15 urinary anabolic steroids in a single run, namely T, epitestosterone, dehydroepiandrosterone (DHEA), androsterone, etiocholanolone, their sulfates and their glucuronides (Figure 2.2). They extracted 2 mL of human urine by solid-phase extraction with methanol elution and reconstituted the residue in aqueous methanol in the presence of deuterated internal standards (d_3-epitestosterone glucuronide, [16,16,17-$^{2}H_3$]testosterone sulfate and [16,16,17-$^{2}H_3$]testosterone), then monitored, for example, *m/z* 289–97 and 109 for T and epitestosterone, *m/z* 367–97 for their sulfates, and *m/z* 463–113 and 287 for their glucuronides. The method does not achieve quantitation, but it allows the estimation of ratios, which makes it possible to monitor the urinary steroid profile, which is useful for monitoring the abuse of anabolic steroids.

In a study also designed to pave the way for LC-MS-MS of steroid glucuronides, Kuuranne *et al*. (2000) compared ESI and APCI, in positive and negative ion modes, MS-MS of eight steroids synthesized by an enzyme-assisted approach. They determined the best ionization method, ESI in the positive ion mode, and the MS data allowed them to distinguish isomers such as T glucuronide and epitestosterone glucuronide or 5α- and 5β-nandrolone glucuronide.

Turning to cell culture media, Chang *et al*. (2003) worked out a specific and sensitive method for the quantitation of anabolic hormone residues without any sample preparation except for two automated steps. The tandem MS transitions used were *m/z* 289.2 to 97.1 to monitor 4-androstene-3,17-dione (Figure 2.2) and T and *m/z* 291.3 to 255.3 for DHT. Calibration curves were prepared in the range 0.05–100 ng mL^{-1} for T (~0.17–347 nmol L^{-1}) and 4-androstene-3,17-dione (~0.17–350 nmol L^{-1}) and 1.0–100 ng mL^{-1} (~3.4–345 nmol L^{-1}) for DHT. Recoveries ranged from 86.6 to 91% depending on the analyte and concentration. Matrix effects were assessed by varying the proportions of cell culture medium and loading buffer used to prepare the steroid standards. The method was validated. Comparison with radioimmunoassay (RIA) indicated that LC-MS-MS provided better accuracy, specificity and sensitivity and a wider dynamic range while sparing workers the handling of and exposure to radiochemicals. The method has been applied in a study of the effects of chemical exposure on male sex hormone homeostasis in cultured human cell lines. In that the method targets free steroids, if it were applied to urine samples it would require a deconjugation step.

Plasma and serum are biological samples commonly collected from subjects in clinical studies for basic research. Tiller *et al*. (1997) spiked plasma extracts with T, hydrocortisone, a corticosteroid (not an anabolic steroid), and SR 27417, a platelet-activating factor, to prepare a calibration curve with nine concentrations for analysis by LC–ion trap MS. For T, the best ions for quantitation were deemed to be *m/z* 97, 109 and 253. The authors found the full-scan mode helpful in identifying the best ions for quantitation and matrix contaminants.

Guo *et al*. (2004) took advantage of the improved sensitivity achieved by using APPI to develop a method for profiling nine steroid hormones, including three androgens, and applied it to a spiked plasma pool. The androgen analytes were dehydroepiandrosterone (DHEA; Figure 2.2), DHEA sulfate, androstenedione and T. The other steroids were estrogen, progestins and corticoids. Sample preparation consisted of protein precipitation by adding, to 760 µL of plasma, a solution containing all deuterated analog internal standards. The transitions monitored were *m/z* 271 to 213, 287 to 97 and 289 to 97 for DHEA and DHEA sulfate, androstenedione and T, respectively. The report includes a comparison with seven RIAs and assessments of precision, recovery and accuracy.

Liu *et al*. (2003) focused their method paper on the instrumental set-up for on-line capillary LC coupled with MS-MS via a low flow-rate interface, hoping that miniaturization would improve sensitivity for many biomedical applications. The system design and performance were tested on DHEA sulfate and pregnenolone sulfate quantitation from 5 µL of plasma from one male volunteer. Sample preparation was simple – addition of aqueous methanol and of the deuterated analog internal standards, protein precipitation, supernatant filtration through a C_{18} bed, evaporation and reconstitution in 100 µL of mobile phase, of which 20 µL were injected for LC-MS-MS analysis. The DHEA sulfate concentration found was in agreement with literature values based on GC-MS and LC-MS studies.

Working on actual serum samples, Wang *et al*. (2004a) measured morning serum T concentrations in samples from 62 eugonadal and 60 hypogonadal men (25 baseline samples, 35 after transdermal T replacement therapy) in order to compare a reference method by LC-MS-MS with six commonly used immunoassays, four or them automated and two manual. In this study, the analyte was T, therefore it was possible to use d_3-T as internal standard for LC-MS-MS analysis. The method was validated using protocols specified by the

US Food and Drug Administration and considered the reference method in accordance with the literature and in view of the widely accepted superior specificity. The calibration curve for T was linear up to 2000 ng dL^{-1} (~69 nmol L^{-1}) and the limit of quantitation (LOQ) was 20 ng dL^{-1} (~0.69 nmol L^{-1}). Recovery ranged from 71.4 to 77.0% depending on the concentration.

Because the serum T values were spread over a wide range (<50–1500 ng dL^{-1} or ~2–52 nmol L^{-1}) and the number of samples was large (>100), the method comparison should not be affected by individual variability and it should hold true in studies by other workers.

The immunoassays were biased towards either lower or higher values, or they overestimated or underestimated serum T levels. The results confirmed previously published opinions that for the immunoassays, adult male reference ranges need to be established in each individual laboratory, as opposed to being provided by the automated instrument manufacturer. At low concentrations, the immunoassays were biased and suffered from a lack of precision and accuracy. Therefore, it seemed that they cannot be used to measure T levels accurately in serum from females or pre-pubertal males, unless those level are abnormally high.

The same team developed an LC-MS-MS method to quantitate T and trideuterated T ([16,16,17-$^2H_3$]testosterone or d_3-T) in human serum, for clinical studies involving administration of d_3-T (Starcevic *et al.*, 2003). To a serum aliquot (2 mL), 19-nortestosterone internal standard, sodium acetate buffer (pH 5.5) and diethyl ether (5 mL) were added, the mixture was shaken and centrifuged and the ether recovered and evaporated to dryness. The residue was reconstituted in methanol (100 μL) and injected (15 μL) into an LC–triple quadrupole MS system operated in the positive ion mode. After chromatography on a C_{18} column with gradient elution, T was monitored with the transition *m/z* 289 to 97, the calibration curve was linear in the range 0.5–20 ng mL^{-1} (~1.7–69 nmol L^{-1}), the LOQ was 0.5 ng mL^{-1} (~1.7 nmol L^{-1}) and the recovery was 91.5%. d_3-T was monitored with the transition *m/z* 292 to 97, the calibration curve was linear in the range 0.05–2 ng mL^{-1} (~0.17–7 nmol L^{-1}), the LOQ was 0.05 ng mL^{-1} (~0.17 nmol L^{-1}) and the recovery was 96.4%. Accuracy and precision were determined. For the 19-nortestosterone internal standard, the transition was *m/z* 275 to 109. The serum T concentrations in 38 healthy subjects ranged from 2.5 to 14.0 ng mL^{-1} (~8.7–48.6 nmol L^{-1}) in baseline samples and samples collected during an infusion of d_3-T. Good sensitivity was achieved for d_3-T. The ideal internal standard would have been trideuterated T, but it was the analyte, hence 19-nortestosterone was used, and the authors hoped that ion suppression or instrument instability would affect it to the same extent as T and d_3-T. The transitions to monitor were selected so as to avoid interfering peaks. Immunoassays cannot be used to quantitate T and d_3-T separately because they cannot distinguish between the two, therefore LC-MS-MS provides a unique advantage.

Wang *et al*. (2004b) then used the Starcevic *et al*. method to determine T metabolic clearance and production rates in normal men by stable isotope dilution and LC-MS-MS, to assess the influence of ethnicity and age. Subjects underwent a constant infusion of d_3-T, serum d_3-T concentrations were measured by LC-MS-MS and serum total T (= T + d_3-T) was measured by RIA. There were no ethnic differences. Values were lower in older than younger men. This is the first report of the use of LC-MS-MS to quantitate labeled and unlabeled analogs of an anabolic androgenic steroid in a clearance study. The amount of

d_3-T infused was small and the LC-MS-MS method has sufficient sensitivity to allow the measurement of production rate and metabolic clearance rate with a small enough d_3-T dose to avoid perturbing the endogenous production of T.

Cawood *et al*. (2005) improved Wang *et al*.'s method by reducing the plasma or serum sample volume 40-fold from 2 mL to 50 μL. The estimated T functional LOQ of 0.3 nmol L^{-1} is not far from the d_3-T LOQ of 0.05 ng mL^{-1} (~0.17 nmol L^{-1}) in the Starcevic *et al*. method, which targeted low d_3-T levels but had no need to measure particularly low T levels (T LOQ 0.5 ng mL,$^{-1}$ ~1.7 nmol L^{-1}). In Cawood *et al*.'s study, to prepare the samples, d_2-T internal standard and precipitating reagent containing zinc sulfate and methanol were added (100 μL) and the supernatant (40 μL) was analyzed. After chromatography on a C_{18} column with isocratic elution, T was monitored with the transition from *m/z* 289.1 to 96.7. The calibration curve was linear from 0.25 to 100 nmol L^{-1}. The method is simple, quick, robust, accurate and validated. Not only does it make it possible to measure low T levels (<8.0 nmol L^{-1} serum, low enough for most clinical purposes) in women, but also it is suitable for routine high-throughput use.

In view of the major role played by DHT in the development of benign prostatic hyperplasia, Choi *et al*. (2003) set out to quantitate simultaneously T glucuronide and the glucuronide of its active metabolite, DHT, in human urine, in hope of assessing the activity of 5α-reductase, the enzyme that converts T to DHT. Their method used [16,16,17-$^2H_3$]testosterone-17β-glucuronide as internal standard. A calibration curve was prepared using urine stripped of steroids, then spiked with 0.2–400 μg L^{-1} (~0.4–860 nmol L^{-1}) for T glucuronide and 3–200 μg L^{-1} (~6–430 nmol L^{-1}) for DHT glucuronide. Recoveries were 94 and 97% for T glucuronide and DHT glucuronide, respectively. Limits of quantitation were 1 μg L^{-1} (~2 nmol L^{-1}) for T glucuronide and 3 μg L^{-1} (~6 nmol L^{-1}) for DHT glucuronide. The assay was applied to 24-h urine samples from 27 patients, 19 with benign prostatic hyperplasia and eight healthy. For T glucuronide, there was no significant difference between patients and controls. In contrast, the levels of DHT glucuronide were significantly lower in the patients. This seems contrary to published results where patients with benign prostatic hyperplasia had higher plasma and tissue concentrations of DHT, unless the lower urinary concentration indicates lower glucuronidation and higher free DHT in plasma and tissue. The ability to measure urinary T glucuronide and DHT glucuronide may help in assessing a patient's androgen status in any condition where abnormal 5α-reductase activity is suspected.

Mitamura *et al*. (2003) quantitated androstenediol 3-sulfate and DHEA sulfate in human serum, in the presence of the deuterated analogs as internal standards, by LC–ion-trap MS in the negative ion mode. Calibration curves were linear in the range 10–400 ng mL(~26–1020 nmol L^{-1}) for the diol sulfate and 0.05–8 μg mL^{-1} (~0.1–21 nmol L^{-1}) for DHEA sulfate. The assay was applied to samples from 14 healthy men, 19 prostate cancer patients and seven patients with benign prostatic hyperplasia. The concentration ranges overlapped but were lower in the cancer patients. This showed that the method was practical. It needs to be applied to larger populations despite the challenge of finding healthy controls among older men.

In an earlier study, Mitamura *et al*. (2002) used LC-MS in the full-scan mode and GC-MS to identify six dehydroepiandrosterone metabolites formed by incubation with a human prostate homogenate of androst-5-ene-3β,17β-diol (major metabolite), androst-4-ene-3,17-dione, T, 5α-DHT (identified by GC-MS but not LC-MS due to insufficient sensitivity), androsterone (Figure 2.2) and 7α-hydroxydehydroepiandrosterone.

Nishiyama *et al.* (2004) set out to quantitate DHT in human serum and prostate biopsy tissue using LC-MS with charged derivatization to the *N*-methylpyridinium derivative. Prostate tissue DHT was dissolved in alkaline solution, extracted by SPE and derivatized before LC-MS analysis [ESI, positive ion mode, selected reaction monitoring (SRM)]. Pretreatment values in patients with ($N = 69$) or without ($N = 34$) prostate cancer were, for serum DHT, 423.9 pg mL^{-1} ($\sim$1.46 nmol L^{-1}) with SD 243.2 pg mL^{-1} ($\sim$0.84 nmol L^{-1}) or 462.5 pg mL^{-1} ($\sim$1.60 nmol L^{-1}) with SD 274.6 pg mL^{-1} ($\sim$0.95 nmol L^{-1}), respectively, and for prostate DHT the values were 5.61 ng g^{-1} ($\sim$0.0193 nmol g^{-1}) with SD 1.96 ng g^{-1} ($\sim$0.0067 nmol g^{-1}) or 5.19 ng g^{-1} ($\sim$0.0179 nmol g^{-1}) with SD 2.50 ng g^{-1} ($\sim$0.0086 nmol g^{-1}), respectively. In 30 cancer patients who received androgen deprivation therapy with castration and flutamide, serum DHT fell from 503.4 pg mL^{-1} ($\sim$1.74 nmol L^{-1}) with SD 315.9 pg mL^{-1} ($\sim$1.09 nmol L^{-1}) to 38 pg mL^{-1} ($\sim$0.13 nmol L^{-1}) with SD 31.2 pg mL^{-1} ($\sim$0.11 nmol L^{-1}), and prostate DHT from 5.44 ng g^{-1} ($\sim$0.0188 nmol g^{-1}) with SD 2.84 ng g^{-1} ($\sim$0.0098 nmol g^{-1}) to 1.35 ng g^{-1} ($\sim$0.0047 nmol g^{-1}) with SD 1.32 ng g^{-1} ($\sim$0.0046 nmol g^{-1}). These results showed that not all of the prostate DHT was gone after treatment. This was the first report on changes in prostate DHT upon androgen deprivation therapy.

Higashi *et al*. (2005) quantitated DHT and T in prostate tissue using LC-MS-MS with the same charged derivatization. Although the samples in this study were obtained after prostatectomy, the 10-mg sample size is consistent with the amount of tissue expected from a needle biopsy. After adding the internal standard, 19,19,19-[$^2H_3$]T, the tissue was homogenized, including in the extraction solvent, aqueous methanol, in which it was heated. After purification through an Oasis HLB cartridge and derivatization, LC-MS-MS analysis with ESI in the positive ion mode was applied. Recoveries were quantitative. Limits of detection were 1.0 ng g^{-1} ($\sim$0.003 nmol g^{-1}) of tissue for both analytes. The method was used to quantitate T and DHT in the prostates of patients with benign prostatic hyperplasia ($N = 7$) and prostate cancer ($N = 3$). The range for DHT was 5.18 ± 1.32 ng g^{-1} ($\sim 0.0179 \pm \sim 0.0046$ nmol g^{-1}) in the first seven patients' tissues. That it was not detected in the three cancer patients is consistent with the expected effects of neoadjuvant hormone therapy. T was below the LOQ of 1.0 ng mL^{-1} ($\sim$3 nmol L^{-1}) in all samples, an observation consistent with the literature and with the knowledge that T is rapidly converted to DHT in the prostate. DHT is indeed the more important analyte of the two since its elevated levels are believed to be a major factor in the pathogenesis of prostate cancer.

Zhao *et al*. (2004) determined the steroid composition of human testicular fluid using LC-MS-MS. After a one-step diethyl ether extraction, they monitored T (*m/z* 289.0–108.8), DHT (*m/z* 291.0–255.0) and 5α-androstane-3α,17β-diol (*m/z* 275.2–257.1) (Figure 2.2), and also estradiol, which is an estrogen, not an anabolic steroid. Calibration curves were linear in the range 0.1–50 ng mL^{-1} ($\sim$0.3–170 nmol L^{-1}) for T, 0.02–1 ng mL^{-1} ($\sim$0.07–3 nmol L^{-1}) for DHT and 2–10 ng mL^{-1} ($\sim$7–34 nmol L^{-1}) for 5α-androstane-3α,17β-diol. Androgen recoveries ranged from 60 to 86% depending on the compound. In samples obtained by percutaneous testicular aspiration from 10 male volunteers, T concentrations were 572 ± 102 ng mL^{-1} ($\sim 1990 \pm 350$ nmol L^{-1}), a range similar to that previously measured by RIA by the same authors.

For the other two androgens, the concentrations were below the limit of detection by RIA but measurable by LC-MS-MS and therefore reported for the first time. This method should be of help in studying the relationship between intratesticular T and spermatogenesis in men.

2.4 CONCLUSION

The earliest clinical studies concerned with androgens measured them mostly by RIA, therefore they suffered from potential cross-reactions. Chromatographic methods coupled with mass spectrometry, namely GC-MS and LC-MS, brought much needed specificity, and the ability to distinguish deuterated, non-radioactive androgens from unlabeled analogs. As a result, stable isotope dilution studies made it possible to give to volunteers small enough doses of labeled T to avoid perturbing endogenous production and yet assess production and clearance rates. The advent of LC-MS-MS brought such higher throughputs than GC-MS and even superior specificity that it is now considered the gold standard. LC-MS-MS measurement of anabolic steroids is a sharper research tool that opens new windows into the understanding of issues of global importance such as human reproduction, contraception, prostate cancer and aging.

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3

High-performance Liquid Chromatography in the Analysis of Active Ingredients in Herbal Nutritional Supplements

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3.1 INTRODUCTION

Herbal dietary supplements (herbal remedies) are readily available in the USA from herbal stores without prescriptions. Chinese medicines are an important component of herbal remedies available today. Ayurvedic medicines are widely used in India and some preparations are available in the USA. Unlike Western medicines, herbal supplements are crude plant extracts and both active ingredients and undesired components are present side by side. Moreover, the amounts of active ingredients may vary widely among different batches of the same products or between different manufacturers.

Herbal remedies can be toxic and have side-effects, or may show significant drug–drug interactions with Western medicines. Ginseng, St John's wort, ma huang, kava, ginkgo biloba, Danshen, chan su, feverfew, garlic, ginger, saw palmetto, comfrey, pokeweed, hawthorne, dong quai and cat's claw are used by the general population in the USA. Common herbal remedies and their intended uses are summarized in Table 3.1. Gulla *et al.*[1] published a survey of 369 patient–escort pairs and reported that 174 patients used herbs. Most common was ginseng (20%), followed by echinacea (19%), ginkgo biloba (15%) and St John's wort (14%).[1] Klepser and Klepser reported their opinions regarding safe and unsafe herbal products. Many herbs that have been classified as unsafe include comfrey, life root, borage, calamus, chaparral, licorice and ma huang. Relatively safe herbs are feverfew,

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Table 3.1 Intended use of some herbal medicines

Herbal medicine	Intended use
Ginseng	Tonic capable of invigorating users physically and, mentally and also used for stress relief
Danshen	Stimulation of heart
Chan Su (toxic)	Heart tonic
St John's wort	Treatment of mood disorders, particularly depression
Ginkgo biloba	Promoted mainly to sharpen mental focus in otherwise healthy adults and also in people with dementia
	Improvement of blood flow in brain and peripheral circulation
Valerian	Treatment of insomnia
Echinacea	Immune stimulant that helps increase resistance to cold, influenza and other infections, wound healing
Feverfew	Relief from migraine headache and arthritis
Garlic	Lowering cholesterol and blood pressure; preventing heart attacks and stroke
Aloe	Healing wounds, burns, skin ulcers. Also used as a laxative
Senna	Laxative
Kava[a]	Promoted for relief of anxiety and stress; sedative
Pokeweed	Antiviral and antineoplastic. Eating uncooked berry or root may cause serious poisoning
Comfrey[a]	Repairing of bone
Chaparral[a]	General cleansing
Ephedra[a]	Herbal weight loss products

[a]These herbal products may cause serious toxicity and there are also some reported cases of death due to use of such herbal supplements.

garlic, ginkgo, Asian ginseng, saw palmetto, St John's wort and valerian.[2] Common adverse reactions due to use of herbal remedies are summarized in Table 3.2.

The US Food and Drug Administration (FDA) mandates that only medicines have to be proven to be safe before release to market. Herbal products are classified as 'dietary supplements' and are marketed pursuant to the Dietary Supplement Health and Education Act of 1994. Herbal products are regulated differently in other countries. In the UK, for

Table 3.2 Toxicity of commonly used herbs

Toxicity	Herbal product
Allergic reaction	Aloe, chamomile, echinacea, garlic, cat's claw
Cardiovascular	Ephedra, danshen, chan su[a], oleander
Carcinogenic	Aloe, chaparral, comfrey, senna
Dermatological	Garlic, kava, St John's wort
Hepatic	Chaparral, comfrey[a], valerian
Hematological	Feverfew, garlic, ginkgo
Neurological	Ephedra, ginkgo, kava, St John's wort, valerian
Renal	Cat's claw, chaparral, ephedra, licorice

[a]Banned by the FDA.

example, any product not granted a license as a medical product by the Medicines Control Agency is treated as a food and cannot carry any health claim or medical advice on the label. Similarly, herbal products are sold as dietary supplement in The Netherlands. In Germany, documents called the German Commission E monographs are prepared by an interdisciplinary committee using historical information, chemical, pharmacological, clinical and toxicological studies, case reports, epidemiological data and manufacturer's unpublished data. If a herb has an approved monograph, it can be marketed.

Active components of herbal supplements can be measured using various analytical techniques, including high-performance liquid chromatography (HPLC) with photodiode-array detection, HPLC combined with mass spectrometry (MS), gas chromatography (GC), thin-layer chromatography (TLC) and immunoassays. Understanding concentrations of active ingredients maybe useful in evaluating herbal remedy-induced toxicity or the magnitude of interactions between herbal supplements and Western drugs.

3.2 St JOHN'S WORT

St John's wort is a very popular herbal antidepressant. The most widely available St John's wort preparation in the USA comes from the dried alcoholic extract of hypercian, a perennial aromatic shrub with bright yellow flowers that bloom from June to September. The flowers are believed to be most abundant and brightest around 24 June, the day traditionally believed to be the birthday of John the Baptist. Therefore, the name St John's wort became popular. The German Commission E monograph indicates that St John's wort can be used in supportive treatment of anxiety and depression. Many chemicals have been isolated from St John's wort, including hyperforin, adhyperforin, hypericin, pseudohypericin, protohypericin, protopseudoquercetin, isoquercitrin, rutin, amentoflavone, flavonoids and xanthones. However, hypericin, hyperforin and 1,3,5,7-tetrahydroxyxanthone are unique to St John's wort.[3]

3.2.1 Drug interactions with St John's wort

Interactions between St John's wort and digoxin are clinically significant. Johne *et al.* reported that 10 days' use of St John's wort could result in a 33% decrease in trough serum digoxin concentrations and a 26% increase in peak concentrations.[4] Durr *et al.* also confirmed the lower digoxin concentrations in healthy volunteers who concurrently took St John's wort.[5] Because St John's wort increases the metabolism of drugs by inducing liver enzymes, co-ingestion of St John's wort with warfarin, cyclosporin, oral contraceptives, protease inhibitors and other drugs has led to reported interactions and reduced therapeutic efficacy. Barone *et al.* reported two cases where renal transplant recipients started self-medication with St John's wort. Both patients experienced sub-therapeutic concentrations of cyclosporin and one patient developed acute graft rejection due to low cyclosporin level. In both patients, termination of the use of St John's wort returned their cyclosporin concentrations to therapeutic levels.[6] Another report describes a kidney transplant patient with a steady-state cyclosporin trough level between 100 and 130 ng mL^{-1} who suddenly had a sub-therapeutic cyclosporin level of 70 ng mL^{-1}, despite an increase in daily dose. The patient was using a tea containing St John's wort. After stopping St John's wort, his

Table 3.3 Common Drug–Herb Interactions

Herbal product	Interacting drug	Comments
Ginseng	Warfarin	Ginseng may decrease effectiveness of warfarin
	Phenelzine	Toxic symptoms: headache, insomnia, irritability
St John's wort	Paxil	Lethargy, incoherent, nausea
	Digoxin	Decreased AUC, peak and trough concentration of digoxin, may reduce effectiveness of digoxin
	Cyclosporin/FK 506	Lower cyclosporin/FK 506 concentrations due to increased clearance may cause transplant rejection
	Theophylline	Lower concentration thus decreases the efficacy of theophylline
	Indinavir	Lower concentration may cause treatment failure in patients with HIV
	Oral contraceptives	Lower concentration/failed birth control
Ginkgo biloba	Aspirin	Bleeding because ginkgo can inhibit clotting factors
	Warfarin	Hemorrhage
	Thiazide	Hypertension
Kava	Alprazolam	Additive effects with CNS depressants, alcohol
Garlic	Warfarin	Increases effectiveness of warfarin, bleeding
Ginger	Warfarin	Increases effectiveness of warfarin, bleeding
Feverfew	Warfarin	Increases effectiveness of warfarin, bleeding
Dong quai	Warfarin	Dong quai contains coumarin Dong quai increases INR[a] for warfarin/bleeding
Danshen	Warfarin	Increase effectiveness of warfarin due to reduced elimination
Borage oil	Phenobarbital	May lower seizure threshold
Evening primrose oil	Phenobarbital	May lower seizure threshold
Licorice	Spirolactone	May offset the effect of spirolactone

[a]International normalized ratio for prothrombin time.

cyclosporin level increased to 170 ng mL^{-1} after 5 days.[7] A significant reduction in the area under the plasma concentration curve (AUC) for tacrolimus was also observed in 10 stable renal transplant patients receiving St John's wort. Interestingly, no interaction was observed with mycophenolic acid.[8] St John's wort also reduced the AUC for the HIV-1 protease inhibitor indinavir by a mean of 57% and decreased the extrapolated trough by 81%. A reduction in indinavir exposure of this magnitude could lead to treatment failure.[9] A reduced plasma level of methadone was observed in the presence of St John's wort, resulting in reappearance of withdrawal symptoms.[10] Major interactions between St John's wort and Western medicines, and common drug–herb interactions, are summarized in Table 3.3.

3.2.2 Measurement of active ingredients of St John's wort using HPLC

Commercially available St John's wort is not prepared following rigorous pharmaceutical standards and wide variations in concentrations of active ingredients have been reported. One report involving the determination of hypericin and hyperforin content among eight

brands of commercially available St John's wort preparations indicated that the hypericin content varied from 0.03 to 0.29% and the hyperforin content from 0.01 to 1.89%. One product (Nature's balance) had the lowest concentration of hypericin (0.03%). Although some products had hypericin and hyperforin contents comparable to the reported concentrations, other products did not match the claim of hypericin concentration on the package insert.[11] Li and Fitzloff described a rapid reversed-phase HPLC method for the determination of the major constituents of St John's wort, rutin, hyperoside, isoquercitrin, quercitrin, quercetin, pseudohypericin, hypericin and hyperforin. The specimens were extracted into methanol by two sonication steps (30 min each) at low temperature. The extraction efficiency for the major active ingredients of St John's wort was around 99%. HPLC analysis was carried out using a reversed-phase C_{18} column and a mobile phase gradient of water–acetonitrile–methanol–trifluoroacetic acid. The run time was 60 min and the analytes were measured with a photodiode-array detector.[12] Ruckert *et al.* described an HPLC method with electrochemical detection for the determination of hyperforin in St John's wort preparations. They used an isocratic mobile phase consisting of 10% ammonium acetate buffer (0.5 M, pH 3.7)–methanol–acetonitrile (10:40:50 v/v/v) and a flow-rate of 0.8 mL min^{-1}. Hyperforin was detected ampherometrically with a glassy carbon electrode at a potential of +1.1 V versus a silver/silver chloride/3 M potassium chloride reference electrode. The limit of detection was 0.05 ng of hyperforin on-column.[13] Mauri and Pietta used HPLC coupled simultaneously with a diode-array detector and an electrospray mass spectrometer for the analysis of hypercium extract. Hypericin, psudohypericin, hyperforin and adhyperforin were separated and identified based on their UV and mass spectra.[14]

Several studies indicate that hypericin and hyperforin are unstable and may degrade on exposure to light, heat or air.[15] Ang *et al.* studied the effects of pH and light exposure on the active ingredients of St John's wort, including pseudohypericin, hypericin, hyperforin and adhyperforin, in aqueous buffer solution and non-alcoholic, non-carbonated fruit-flavored beverages. The components were separated using a Luna C_8 column (250 × 2 mm i.d., particle size 5 μm) and a mobile phase delivered at a rate of 0.2 mL min^{-1} consisting of a 30-min linear gradient from 50 to 80% acetonitrile with a constant 3 mM ammonium formate, which was maintained for an additional 30 min. The authors used mass spectrometric detection in the negative electrospray mode and found that components of St John's wort were unstable under acidic aqueous conditions. Major degradation products of hyperforin in acidic aqueous solutions were identified as furohyperforin, furohyperforin hydroperoxide and furohyperforin isomer a.[16] Active ingredients of St John's wort are more stable when stored in the dark; under exposure to light, hypericin, hyperforin and adhyperforin all decomposed rapidly. Liu *et al.* reported the influence of light and solution pH on the stability of phloroglucinols (hyperforin and adhyperforin) and naphthodianthrones (hypericin, pseudohypericin, protohypericin and protopseudohypericin) extracted with methanol from St John's wort powder. They used liquid chromatography combined with mass spectrometry for analysis and found that when exposed to light, both hyperforin and adhyperforin in the extract rapidly degraded regardless of pH and complete degradation was observed within 12 h. In contrast, when protected from light, minimal degradation was observed even after 36 h. Under light and at neutral pH, phloroglucinols and naphthodianthrones showed different stability behaviors. The marked increase in oxidation when hyperforin is exposed to light even at neutral pH may indicate a susceptibility to light-induced free radical reactions where one of the end products is hyperforin hydroperoxide. However, hypericin (closed-ring structure) is not susceptible to light-induced formation of free radicals.[17]

3.2.3 Analysis of St John's wort extract with other analytical techniques

Although HPLC, in addition to HPLC combined with mass spectrometry (MS), is the most common method for the analysis of active components of St John's wort, Seger *et al.* used both HPLC-MS and GC-MS for the analysis of a supercritical fluid extract of St John's wort. Supercritical fluid extraction of plant material with carbon dioxide yields extracts enriched with lipophilic components. In addition to the dominating phloroglucinols hyperforin ($36.5 \pm 1.1\%$) and adhyperforin ($4.6 \pm 0.1\%$), the extracts mainly contained alkanes (predominately nonacosane), fatty acids and wax esters. The non-polar components tended to accumulate in a waxy phase resting at the top of the hyperforin-enriched phase. Highly polar compounds (naphthodianthrones) were not found. For the GC-MS analysis, the authors used electron ionization MS analysis (scan range: 40–640 amu). Ten oxygenated hyperforin derivatives were identified.[18]

Bilia *et al.* reported the efficiency of two-dimensional homonuclear ^{1}H–^{1}H correlated spectroscopy and two-dimensional reverse heteronuclear shift correlation spectroscopy in evaluating the composition of phloroglucinols, flavonols and naphthodianthrones in a dried extract of St John's wort. They successfully assigned carbon resonances for these three classes of compounds and also identified shikimic and chlorogenic acids, sucrose, lipid, polyphenols and traces of solvent (methanol) during the extraction process. This rapid technique is an alternative to HPLC, TLC or capillary GC for the analysis of St John's wort preparations.[19] Another report utilized near-infrared reflectance spectroscopy (NIRS) for the determination of two major constituents of St John's wort, including hyperforin.[20] HPLC was used as the reference method.

3.2.4 Measurement of hypericin and hyperforin in human plasma using HPLC

Several reports indicate that the magnitude of interactions between a Western drug and St John's wort depends on the concentrations of the active ingredients of St John's wort in plasma. Mai *et al.* reported that a St John's wort product containing a low amount of hyperforin did not affect the cyclosporin pharmacokinetics in renal transplant patients, but the group of patients who received St John's wort containing higher amounts of hyperforin showed significantly lower concentrations of cyclosporin and needed a 65% increase in their cyclosporin dose in order to maintain therapeutic levels.[21] Hyperforin induces cytochrome P450 mixed function oxidase, the liver enzyme responsible for the metabolism of many drugs.[22] Mannel also concluded that hyperforin is probably responsible for the induction of liver enzyme (CYP3A4) via activation of nuclear/pregnane and the xenobiotic receptor.[23]

Hyperforin concentration in human plasma can be measured using HPLC with UV detection at 287 nm. A Luna C_{18} column (150×4.6 mm i.d., particle size 3 μm, Phenomenex) was used and the mobile phase was prepared by adding a methanol–acetonitrile organic phase (3:2 v/v) to water so that the final composition of the organic phase was 92% and the aqueous phase 8%. Finally, 2 mL of formic acid and 2 mL of triethylamine were added to 1000 mL of mobile phase and the pH of the final mobile phase was 3.2. Hyperforin-containing or spiked plasma was mixed with acetonitrile and finally hyperforin was extracted using a solid-phase extraction column. Benzo[*k*]fluoranthene was used as the internal standard. The

limit of detection was 4 ng mL^{-1} and the limit of quantitation was 10 ng mL^{-1}.[24] Bauer *et al.* described HPLC combined with UV detection for the determination of hyperforin and HPLC combined with fluorimetric detection for the determination of hypericin and psudohypericin in human plasma. They used liquid–liquid extraction. The limit of quantitation was 10 ng mL^{-1} for hyperforin and 0.25 ng mL^{-1} for both hypericin and pseudohypericin.[25]

HPLC-MS can also be utilized for analysis of active components of St John's wort in human plasma. Pirker *et al.* used liquid–liquid extraction and HPLC-MS-MS for the simultaneous determination of hypericin and hyperforin in human plasma and serum. For sample preparation, 1 mL of plasma containing hypericin and hyperforin was mixed with 0.4 mL of DMSO and 0.15 mL of acetonitrile, followed by mixing for 30 s and extraction into 1 mL of ethyl acetate–hexane (70:30 v/v). After removing the ethyl acetate–hexane layer, the residue was extracted again and both organic phases were combined and concentrated under nitrogen for further analysis. The recovery of hyperforin (89.9–100.1%) was much higher than that of hypericin (32.2–35.6%). The assay was linear for hypericin concentrations between 8.4 and 28.7 ng mL^{-1} and for hyperforin concentrations from 21.6 to 242.6 ng mL^{-1}.[26] Riedel *et al.* also described an HPLC method combined with MS-MS for the determination of hypericin and hyperforin concentrations in human plasma. They used liquid–liquid extraction with ethyl acetate and hexane and used a reversed-phase (RP-18) column for analysis. The limit of quantitaion was 0.05 ng mL^{-1} for hypericin and 0.035 ng mL^{-1} for hyperforin. The hypericin assay was linear between 0.05 and 10 ng mL^{-1} and the hyperforin assay between 0.035 and 100 ng mL^{-1}.[27]

3.3 HERBAL SUPPLEMENTS WITH DIGOXIN-LIKE IMMUNOREACTIVITY

Several herbal supplements, including several Chinese medicines, have digoxin-like immunoreactivity because some components have structural similarity to digoxin. Digoxin immunoassays available commercially may use either a monoclonal antibody specific to digoxin or polyclonal antibodies. In general, assays that employ polyclonal antibodies against digoxin, such as the fluorescence polarization immunoassay (rabbit polyclonal antibody) and microparticle enzyme immunoassay (both marketed by Abbott Laboratories), are subject to more interference by Chinese medicines than other digoxin immunoassays based on monoclonal antibodies. Several Chinese medicines such as chan su, lu-shen-wan, danshen and Asian and Siberian ginseng interfere with various digoxin immunoassays.[28–35]

The Chinese medicine chan su is prepared from the dried white secretion of the auricular and skin glands of Chinese toads (*Bufo melanostictus* Schneider and *Bufo bufo gargarzinas* Gantor). Chan su is also a major component of traditional Chinese medicines lu-shen-wan and kyushin.[36] These medicines are used for the treatment of tonsillitis, sore throat, furuncle, palpitations, etc., because of their anesthetic and antibiotic action. Chan su, given in small doses, also stimulates myocardial contraction, has an anti-inflammatory effect and is analgesic. The cardiotonic effect of chan su is due to its major bufadienolides such as bufalin, cinobufagin and resibufogenin.[37] At high dosages, chan su causes cardiac arrhythmia, breathlessness, convulsion and coma. Death of a Chinese woman after ingestion of Chinese herbal tea containing chan su has been reported.[28] Structural similarity between bufadienolides and digoxin accounts for the digoxin-like immunoreactivity of chan su. Fushimi and Amino reported a serum digoxin concentration of 0.51 nmol L^{-1} (0.4 ng mL^{-1}) in a healthy

volunteer after ingestion of kyushin tablets containing chan su as the major component.[29] Panesar reported an apparent digoxin concentration of 1124 pmol L^{-1} (0.88 ng mL^{-1}) in healthy volunteers who ingested lu-shen-wan pills.[30]

Ingestion of chan su and related drugs prepared from toad venom cause digoxin-like immunoreactivity in serum. Moreover, it caused positive interference (falsely elevated serum digoxin levels) in serum digoxin measurement by fluorescence polarization immunoassay (FPIA) (Abbott Laboratories) and negative interference (falsely lowered serum digoxin values) by the microparticle enzyme immunoassay (MEIA) (Abbott Laboratories). Other digoxin immunoassays, such as EMIT 2000, Synchron LX system (Beckman), Tina-quant (Roche Diagnostics) and turbidimetric (Bayer Diagnostics), are also affected but the magnitudes of interference are less significant than in the FPIA assay. The chemiluminescent assay (CLIA) marketed by the Bayer Diagnostics is free from such interferences. The interfering components in chan su are very strongly bound to serum proteins and are absent in the protein-free ultrafiltrates. On the other hand, digoxin is only 25% bound to serum protein and is present in the ultrafiltrate. Therefore, monitoring free digoxin concentration eliminated this interference of chan su in serum digoxin measurement. Another way to eliminate this interference is to use a specific CLIA (Bayer Diagnostics).[31]

Danshen is a Chinese medicine prepared from the root of the Chinese medicinal plant *Salvia miltiorrhiza*. This herb has been used in China for many centuries for treating various cardiovascular diseases, including angina pectoris. Danshen caused modest interference with polyclonal-based digoxin immunoassays such as MEIA and FPIA. A digoxin CLIA (Bayer), EMIT 2000 digoxin assay and Roche and Beckmann digoxin assays are also free from interference from Danshen.[32]

McRae reported a case where ingestion of Siberian ginseng was associated with an elevated digoxin level in a 74-year-old man. In this patient, the serum digoxin level had been maintained between 0.9 and 2.2 ng mL^{-1} over a period of 10 years. After ingestion of Siberian ginseng, his serum digoxin level increased to 5.2 ng mL^{-1}, although the patient did not experience any sign of digoxin toxicity.[33] Another report indicated that Siberian ginseng produces only modest interference in the FPIA and MEIA digoxin assays. Asian ginseng also showed modest positive (FPIA) and modest negative (MEIA) interference. Again, the EMIT 2000, Bayer (both turbidimetric and CLIA), Roche (Tina-quant) and Beckman (Synchron LX system) digoxin assays were free from interference from both Asian and Siberian ginseng.[34,35]

3.3.1 Use of HPLC for the determination of chan su, danshen and ginsengs

Major bufadienolide components of chan su and related Chinese medicines can be analyzed by TLC and HPLC. Hong *et al.* described an HPLC protocol for the determination of bufalin, cinobufagin and resibufogenin, the major active ingredients of chan su and liu-shen-wan. They extracted bufadienolides from Chinese medicines using chloroform and ultrasonication and then applied HPLC with an RP-18 column and methanol–water (74:26 v/v) as mobile phase. They reported differences in composition of bufadienolides in 11 different pills that they analyzed and concluded that the variability of active constituents in products available to the public in Hong Kong may represent a hazard to public health.[38] Another report described the use of TLC using silica gel for the analysis of chan su. The TLC plates were developed using chloroform–methanol–water (75:20:5 v/v/v). The authors also developed an HPLC technique

using a linear water–methanol gradient and an RP-18 column. The compounds eluted were detected at 220 and 300 nm.[39] Wang *et al.* described an HPLC method for the simultaneous determination of four bufadienolides in human liver. They used solid-phase extraction, a reversed-phase column and photodiode-array detection. The detection limits of the method were 0.4 ng for cinobufotalin and bufalin and 0.5 ng for cinobufagin and resibufogenin.[40]

An extract of Radix Salvia miltiorrhiza is used in the Chinese herbal product danshen and related medicines used as heart tonics. Shi *et al.* successfully separated components of danshen (tanshinones including cryptotanshinone, tanshinone I and tanshinone IIA) using a C_{18} column (150 × 4.6 mm i.d., particle size 5 μm). The mobile phase was methanol–tetrahydrofuran–water–glacial acetic acid (20:35:44:1 v/v/v/v), employing isocratic elution at a flow-rate of 1.0 mL min^{-1}. The analytes were detected by measuring their UV absorption at 254 nm. The method was successfully applied to the analysis of five kinds of Chinese herbal medicines containing danshen.[41] Hu *et al.* used LC-MS for the quantitative determination of dihydrotanshinone I, cryptotanshinone, tanshinone I and tanshinone IIA. These components were separated using a reversed-phase C_{18} column and quantification was based on the $[M + H]^+$ fragments produced under collision activation conditions and the selected reaction monitoring mode.[42] Zhang *et al.* studied the metabolism of phenolic acids from Salvia miltiorrhiza roots in rats using HPLC-UV and HPLC-MS and identified danshenu, caffeic acid, ferulic acid, isoferulic acid and methylated ferulic acid as metabolites.[43]

The analysis of the active components of ginseng using HPLC combined with UV or MS detection has been extensively studied. Harkey *et al.* analyzed 25 commercial ginseng products available in the USA for the presence of marker compounds using HPLC-MS-MS. They concluded that although all products were labeled correctly and marker compounds were found in all preparations, there were wide variations in the concentrations of marker products, suggesting poor quality control and standardization in manufacturing such products.[44] Bonfill *et al.* described a reversed-phase HPLC assay for the simultaneous quantitative determination of several ginsenosides, Rb(1), Rb(2), Rc, Rg(1), Re and Rf, in ginseng products. Chromatographic separation can be achieved in less than 20 min using a diol column and UV detection at 203 nm.[45] Another method employed HPLC combined with negative ion electrospray MS for determination of three ginsenosides [Rb(1), Rc, and Re] in six different samples of ginseng, including a liquid extract, capsules, tea bags and instant tea. The authors found at least one ginsenoside in four of the six products studied.[46] Zhu *et al.* reported a comparative study on the triterpene saponins of 47 samples of ginseng using HPLC. They selected 11 ginsenosides as markers and found 10-fold variations in ginsenoside concentrations between different products.[47]

Sun *et al.* studied ginsenoside concentrations in rat plasma using LC-MS. After solid-phase extraction and HPLC separation, the chloride adduct anions of Rg(1), Rh(1) and aglycone protopanaxatriol (PPT) were analyzed in the selected-ion monitoring mode. The detection limit was 20 pg for Rg(1), 100 pg for Rh(1) and 10 pg for PPT. Chromatographic separation was achieved in less than 8 min.[48]

3.4 HERBAL REMEDIES AND ABNORMAL LIVER FUNCTION TESTS

Consumption of kava has been associated with increased concentrations of γ-glutamyl-transferase (GGT), suggesting potential hepatotoxicity. Escher and Desmeules described a

case in which severe hepatitis was associated with kava use. A 50-year-old man took three or four kava capsules daily for 2 months and liver function tests showed 60–70-fold increases in AST and ALT. Serology was negative for hepatitis A, B and C, CMV and HIV. The patient eventually received a liver transplant.[49] Humberston *et al.* also reported a case of acute hepatitis induced by kava-kava.[50] Other cases of hepatotoxicity due to the use of kava have been documented.[51] In January 2003, kava extracts were banned in the entire European Union and Canada. The FDA strongly cautioned against using kava. Eleven cases of serious hepatic failure and four deaths have been reported in association with kava use. There are also 23 reports indirectly linking kava-kava with hepatotoxicity.[52]

Chaparral can be found in health food stores as capsules and tablets and is used as an antioxidant and anti-cancer herbal product. Leaves, stems and bark in bulk are also available for brewing tea. However, this product can cause severe hepatotoxicity. Several reports of chaparral-associated hepatitis have been reported. A 45-year-old woman who took 160 mg of chaparral per day for 10 weeks presented with jaundice, anorexia, fatigue, nausea and vomiting. Liver enzymes and other liver function tests showed abnormally high values (ALT 1611 U L^{-1}, AST 957 U L^{-1}, alkaline phosphatase 265 U L^{-1}, GGT 993 U L^{-1} and bilirubin 11.6 mg dL^{-1}). Hepatitis, CMV and EBV were ruled out. A liver biopsy showed acute inflammation with neutrophil and lymphoplasmocytic infiltration, hepatic disarray and necrosis. The diagnosis was drug-induced cholestatic hepatitis, which in this case was due to the use of chaparral.[53] Gordon *et al.* reported a case where a 60-year-old woman took chaparral for 10 months and developed severe hepatitis for which no other cause was found. On admission her bilirubin was 12.4 mg dL^{-1}, ALT 341 U L^{-1}, AST 1191 U L^{-1} and alkaline phosphatase 186 U L^{-1}. Her prothrombin time was 15.9 s, but all tests for viral causes were negative. Eventually she received a liver transplant.[54]

Comfrey is a perennial herb used for the prevention of kidney stones; nourishing and repairing bone and muscle and for the treatment of injuries such as burns and bruises. In Australia, comfrey is classified as a poison and its sales have been restricted in several regions. Many different commercial forms of comfrey are marketed, including oral and external products. Commercial comfrey is usually derived from the leaves or roots of *Symphytum officinale* (common comfrey). However, some products are also derived from Russian comfrey. Russian comfrey contains a very toxic pyrrolizidine alkaloid, echimidine, which is not found in common comfrey. However, common comfrey contains other hepatotoxic alkaloids, namely 7-acetylintermedine, 7-acetyllycopsamine and symphytine. The metabolites of these alkaloids are very toxic to the liver.[55] Ridker *et al.* documented hepatic venocclusive disease associated with consumption of comfrey root.[56] Long-term studies in animals have also confirmed the carcinogenicity of comfrey in animal models.[57]

Germander has been used as a remedy for weight loss and general tonic. Germander tea made from the aerial parts of the plant has been used for many centuries. Twenty-six cases of germander-induced liver toxicity have been reported in Europe. A 55-year-old woman taking 1600 mg per day of germander became jaundiced after 6 months. Her bilirubin was 13.9 mg dL^{-1}, AST 1180 U L^{-1}, ALT 1500 U L^{-1} and ALP 164 U L^{-1}. Serological tests for hepatitis viruses were negative. A liver biopsy suggested drug-induced hepatitis. Germander therapy was discontinued and the hepatitis resolved in 2 months.[58] Bosisio *et al.* described an HPLC method for detection of teucrin A, the active component of germander, in beverages.[59]

3.4.1 Use of GC-MS and HPLC for the measurement of active components

Duffield *et al.* used GC combined with chemical ionization MS for the identification of human urinary metabolites of kava lactones following ingestion of kava prepared by the traditional method of aqueous extraction from the plant *Piper methysticum*. All seven major and several minor kava lactones were identified in human urine. Metabolic transformations include reduction of the 3,4-double bond and/or demethylation. Ring-opening products of kava lactones were also detected in human urine.[60] Gaub *et al.* used HPLC combined with coordination ionspray MS, where charged complexes are formed through addition of central complexing ions such as sodium, silver and cobalt, for the analysis of kava extracts.[61]

Phenolic components of chaparral, including lignans and flavonoids, can be analyzed using HPLC combined either with UV or MS detection.[62] Mroczek *et al.* utilized cation-exchange solid-phase extraction and ion-pair HPLC for the simultaneous determination of *N*-oxides and free base pyrrolizidine alkaloids of comfrey. The recoveries were 80% for retrorsine *N*-oxide, 90% for retrorsine and 100% for senkirkine as assessed by both TLC and HPLC.[63] Schaneberg *et al.* developed a reversed-phase HPLC method with evaporative light scattering detection for the simultaneous determination of hepatotoxic pyrrolizidine alkaloids including riddelline, riddelline *N*-oxide, senecionine, senecionine *N*-oxide, seneciphylline, retrorsine, integerrimine and lasisocarpine, and also heliotrine.[64]

Teucrin A is the major ingredient of germander responsible for its toxicity and it is also considered a marker for germander use. Teucrin A accounts for approximately 70% of the *neo*-clerodane diterpenoids found in the extract of germander (*Teucrium chamaedrys*). Avula *et al.* reported an HPLC method for the simultaneous analysis of nine *neo*-clerodane diterpenoids from germander. They used a reversed-phase Phenomenex Luna C_{18} (2) column (150×4.6 mm i.d., particle size 5 μm) and an acetonitrile–water gradient at a flow-rate of 1 mL min^{-1} for the separation. The limit of detection was 0.24–0.9 μg mL^{-1} using photodiode-array detection.[65]

3.5 GINKGO BILOBA

Ginkgo biloba is prepared from dried leaves of the ginkgo tree (Ginkgo biloba) by organic extraction (acetone–water). After the solvent has been removed, the extract is dried and standardized to contain a constant faction of flavonoids (usually 24%) and terpenes (usually 6%). Most commercial dosage forms contains 40 mg of this extract. Ginkgo biloba is sold in the USA as a dietary supplement intended to improve blood flow in brain and peripheral circulation. It is used mainly to sharpen mental focus and to improve diabetes-related circulatory disorders. The German Commission E approved the use of ginkgo for memory deficit, disturbances in concentration, depression, dizziness, vertigo and headache. Ginkgo leaf contains kaempferol-3-rhamnoglucoside, ginkgetin, isoginketin and bilobetin. Several flavone glycosides have also been isolated from ginkgo (ginkgolide A and B). Other substances isolated from ginkgo include shikimic acid, D-glucarica acid and anacardic acid. Several chemicals found in ginkgo extracts, especially ginkgolide B, are potent antagonists to coagulation factors, and also have antioxidant effects.

A common reported adverse effect of ginkgo is bleeding. Spontaneous intracerebral hemorrhage occurred in a 72-year-old woman who had been taking 50 mg of ginkgo three times per day for 6 months.[66] Fessenden *et al.* reported a ginkgo-associated case of postoperative bleeding after laparoscopic cholecystectomy.[67] Concurrent use of ginkgo and non-steroidal anti-inflammatory drugs (NSAIDs) or anticoagulants should be avoided because ginkgolide B is a potent inhibitor of platelet activating factors. Hauser *et al.* reported a case of bleeding complications after liver transplant in a 59-year-old patient who was using of ginkgo biloba. Seven days after a second liver transplant, subpherenic hematoma appeared in this patient. Three weeks later, an episode of vitreous hemorrhage was documented. No further bleeding occurred after the patient stopped taking ginkgo biloba.[68]

3.5.1 Analysis of components of ginkgo biloba by HPLC

Ginkgolic acids in ginkgo biloba extract can be analyzed by HPLC after liquid–liquid extraction of an aqueous commercially available extract of ginkgo biloba with ethyl acetate or aliphatic hydrocarbons such as hexane. Analysis can be carried out using HPLC combined with UV detection with a photodiode array (200–550 nm) or by HPLC-MS.[69] Tang *et al.* combined reversed-phase HPLC (C_{18} column) and a mobile phase composed of methanol and water (33:67 v/v) for the analysis of ginkgolides and bilobalide in ginkgo extract. Samples were extracted with ethyl acetate and purified by passage through an aluminum oxide column. They used evaporative light scattering detection of the compounds eluting from the column.[70] On-line dialysis is an alternative to the conventional extraction technique for isolating compounds of interest from a complex matrix. Chiu *et al.* developed a method for measuring ginkgolide A and B and bilobalide from ginkgo biloba extract using a self-assembled microdialysis device coupled to an HPLC instrument. They dialysis efficiencies for ginkgolide A and B and bilobalide were between 97.8 and 100.7%. They used a Zorbax SB-C_{18} column (150 × 4.6 mm i.d., particle size 5 μm) and the detection wavelength was set at 219 nm. The mobile phase was methanol–acetonitrile–0.01 M phosphate buffet (pH 5.0) (30:5:65 v/v/v).[71] HPLC combined with photodiode-array detection can also be used for the quantitative determination of five selected flavonol compounds (rutin, quercitrin, quercetin, kaempferol and isorhamnetin) which can be used as markers for quality control of ginkgo biloba extracts. Separation of these compounds was achieved using a Phenomenex Luna C_{18} (2) column (250 × 2.0 mm i.d., particle size 5 μm). The temperature of the column was maintained at 45 °C and the mobile phase was acetonitrile–formic acid (0.3%) with a one-step linear gradient and a flow-rate of 0.4 ml min^{-1}. The limits of detections were 2.76, 0.77, 1.11, 1.55 and 1.03 μg ml^{-1} for rutin, quercitrin, quercetin, kaempferol and isorhamnetin, respectively.[72] Dubber and Kanfer applied HPLC-MS-MS for the accurate determination of two flavonolglycosides, rytin and quercitrin, together with quercetin, kaempferol and isorhamnetin in several ginkgo biloba oral formulations. A one-step gradient of acetonitrile–formic acid (0.3%) at a flow-rate of 0.5 mL min^{-1} was used and the column temperature was maintained at 45 °C. Baseline separations of these compounds were achieved using a run time of 20 min.[73]

Wang *et al.* studied the disposition of quercetin and kaempferol in 10 adult volunteers following oral administration of ginkgo biloba extract. Quercetin and kaempferol were determined in human urine using reversed-phase HPLC. Quercetin and kaempferol were excreted from urine mainly as glucuronides.[74] An HPLC protocol using the ion-pairing

technique has been reported for the rapid analysis of 4-*O*-methylpyridoxine in human serum. This compound is present in ginkgo seeds and, when consumed in large quantities, can cause vomiting and convulsions. The authors used fluorescence detection (excitation wavelength 290 nm, emission wavelength 400 nm) and achieved a detection limit of 5 pg. The analysis time was 30 min.[75] This method can be applied to the detection of 4-*O*-methylpyridoxine in human serum.

3.6 ECHINACEA

Echinacea, a genus of flowering plants including nine species that grow in the USA, is a member of the daisy family. Three species are found in common herbal preparations: *Echinacea angustifolia*, *Echinacea pallida* and *Enchinacea purpurea*. Native-American Indians considered the plant a blood purifier. In the USA, echinacea is used in oral dosage as an immune stimulant that helps increase resistance to cold and influenza. Fresh herb, freeze-dried herb and the alcoholic extract of the herb are all commercially available. The ability of oral echinacea preparations to prolong the time of onset of an upper respiratory infection, was compared with placebo in 302 volunteers from an industrial plant and several military institutions. Although subjects felt better after taking echinacea, there was no statistically significant difference with regard to the time of onset of upper respiratory tract infection.[76] Another study also examined the efficacy of echinacea in preventing colds and upper respiratory tract infection. The authors found no difference between the placebo group and the group that took echinacea.[77]

The Australian Adverse Drug Reaction Advisory Committee received 11 reports of adverse reactions associated with echinacea use between July 1996 and September 1997. There were three reports of hepatitis, three of asthma, one of rash, myalgia, and nausea, one of utricaria and one of anaphylaxis. There are other published reports of echinacea associated with contact dermatitis and anaphylaxis.[78]

3.6.1 Analysis of active components of echinacea by HPLC

A simple TLC method was reported for the identification of a marker compound in echinacea that demonstrated a blue fluorescence at an excitation wavelength of 366 nm after staining with a spray reagent containing ethanol, trifluoroacetic acid and zinc.[79] Chicoric acid is the main phenolic compound present in *Echinacea purpurea* roots and another component is caftaric acid. Echinacoside and cynarin are found in *Echinacea padilla* and *Echinacea angustifolia*. Perry *et al.* reported an HPLC protocol for analysis of these components.[80] Molgaard *et al.* reported a reversed-phase HPLC protocol for the analysis of caffeic acid derivatives in echinacea; naringenin was used as an internal standard.[81] Pellati *et al.* used an 80% methanol in water solution and magnetic stirring for extraction of caffeic acid derivatives (caftaric acid, chlorogenic acid, caffeic acid, cynarin, echinacoside and cichoric acid) from echinacea roots. The extracts were analyzed on a HPLC column with gradient elution and photodiode-array detection.[82] Luo *et al.* used HPLC coupled with UV photodiode-array detection and electrospray ionization MS for the simultaneous analysis of caffeic acid derivatives and alkamides in the roots and extracts of *Echinacea puppurea*.[83]

3.7 VALERIAN

Valerian is a perennial herb that grows in North America, Europe and western Asia. The crude valerian root, or rhizome, is dried and used as is or as an extract. Valerian is available as a capsule, oral solution or tea, and is used as a sleeping pill. The recommended dose is one or two tablets before bedtime, depending on the amount of valerenic acid in the preparation. The German Commission E has approved valerian as a sleep-promoting and calmative agent. The chemical components of valerian are valeranone, valerenic acid, isoeugenyl isovalarerate, valepotriate, isovaltrate and didrovaltrate. At least 37 valepotriates and seven alkaloids have been isolated from valerian. It is believed that valepotriates are responsible for the sedative activity. Valerenic acid can exert pentobarbital-like central nervous system depressant activities. However, valerian's mechanism of action has not been fully elucidated.

Leathwood *et al.* conducted a double-blind crossover study with 128 volunteers and concluded that, compared with placebo, valerian significantly improved subjective sleep quality in habitually poor or irregular sleepers.[84] However, placebo effects were marked in some studies, and in some cases beneficial effects of valerian were not seen until after 2–4 weeks of therapy. The adverse effects of valerian include gastrointestinal upset, allergies, restless sleep, headache and mydriasis. Valerian overdoses have the major effect of central nervous system depression.[85]

3.7.1 Analysis of components of valerian by HPLC

Shohet *et al.* analyzed 31 commercial valerian preparations available in Australia, including tea, tablets, capsules and liquids, by HPLC for valepotriates, valerenic acid and valerenic acid derivatives. The concentrations of valerenic acid and its derivatives ranged from 0.01 to 6.32 $\mu g\ g^{-1}$ of the product; powdered capsules on average contained the highest concentrations of valerenic acid and liquid preparations had the lowest concentrations.[86] Torrado studied the *in vitro* release of valerenic and hydroxyvalerenic acids from valerian tablets. The valerenica acid and hydroxyvalerenic acid concentrations were measured by HPLC using a C_{18} Kromasil column (200×4.6 mm i.d., particle size 5 μm) and a mobile phase of methanol–aqueous 0.5% (v/v) orthophosphoric acid (75:25 v/v). The flow-rate was 1 $mL\ min^{-1}$. The uncoated tablets had the fastest release profile whereas the coated tablets showed very different release patterns, depending on the type of formulation.[87]

3.8 FEVERFEW

Feverfew (*Tanacetum parthenium*) is a short perennial shrub that grows along fields and roadsides. In the 1970s, the use of feverfew as an alternative to traditional medicines for relief from arthritis and migraine headache gained popularity. Feverfew is available as the fresh leaf, dried powdered leaf, in capsules and tablets, as a fluid extract and in oral drops. During a migraine episode, serotonin is released from platelets, so serotonin antagonists (for example, methysergide) are used to treat migraine symptoms. An *in vitro* study using a bovine platelet bioassay has shown that parthenolide, and also other sesquiterpene lactones found in feverfew, inhibit serotonin release by platelets.[88] Feverfew may inhibit serotonin release from platelets in the same manner as methysergide, an ergot alkaloid.[89] Feverfew

may also irreversibly inhibit prostaglandin synthesis by interfering with cyclooxygenase and phospholipase A_2. Parthenolide and epoxyartemorin, found in feverfew, have been shown to inhibit irreversibly thromboxane B_2 and leukotrine B_4.[90] Johnson evaluated the efficacy of feverfew in 17 patients with at least a 2-year history of migraines. The patients took 50 mg of feverfew or placebo. Patients in the placebo group had migraines more often than patients receiving feverfew.[91] Murphy *et al.* conducted a randomized double-blind study of feverfew for the prophylaxis of migraine in 60 patients who had had migraines for at least 2 years. All migraine-related medicines were stopped before the study. The patients received one feverfew capsule (70–114 mg) or placebo four times daily for 4 months. The number of migraine attacks was significantly lowered by feverfew.[92]

Adverse effects associated with feverfew use include dizziness, heartburn, indigestion, bloating and ulceration of oral mucosa. Some 18% of 300 feverfew users reported adverse effects, with mouth ulceration reported by 11.3% of subjects. Discontinuation of feverfew may produce muscle and joint stiffness, rebound of migraine symptoms and anxiety and disrupt sleeping patterns. Feverfew should be avoided in pregnancy because it is purportedly associated with spontaneous abortion in cattle and uterine contraction in term human pregnancy. Because of the potential of feverfew to inhibit cyclooxygenase, it interacts with anticoagulants and increases the antiplatelet effect of aspirin.

3.8.1 Analysis of parthenolide by HPLC

Zhou *et al.* described a sensitive method for the quantification of parthenolide in feverfew using HPLC. The compound was extracted into acetonitrile–water (90:10 v/v) and separated on a Comosil C_{18} HPLC column (150 × 4.6 mm i.d., particle size 5 μm). The mobile phase was acetonitrile–water (55:45 v/v) at a flow-rate of 1.5 mL min^{-1}, and parthenolide was detected by its UV absorption at 210 nm. The analysis time was only 6 min and the detection limit was 0.10 ng of parthenolide on-column. The spiked recovery of parthenolide was 99.3%.[93] Nelson *et al.* studied variations in the parthenolide content of feverfew products available commercially using a similar HPLC method, and observed wide variations in parthenolide content in a single dose (0.02–3.0 mg) of feverfew among the various preparations.[94] Curry *et al.* measured plasma concentrations of parthenolide by solid-phase extraction and mass spectrometric detection. Although the limit of detection by this method was 0.5 ng mL^{-1}, the authors did not observe any detectable concentration of parthenolide even after a daily oral dose containing 4 mg of parthenolide.[95]

3.9 GARLIC

Garlic has been promoted as a dietary constituent that lowers cholesterol and blood pressure, thereby reducing the risk of heart attacks and stroke. Garlic contains various sulfur-containing compounds derived from allicin. Allicin is formed from alliin by the action of allinase. Allinase is released when garlic is chopped. Allicin then produces diallyl sulfide, diallyl disulfide, diallyl trisulfide and other related sulfur compounds. Cooking destroys allinase. Allicin produces the characteristic odor of garlic. There have been several studies evaluating the medical efficacy of garlic. Silagy and Neil performed a meta-analysis of eight prospective randomized studies. All of the studies used dried garlic powder

(600–900 mg daily, equivalent to 1.8–2.7 g of fresh garlic). The overall mean systolic blood pressure reduction was 7.7 mmHg (range 50–17.2) with garlic, and the overall mean reduction in diastolic pressure was 5.0 mgHg (range 3.4–9.6). None of the studies assessed compliance and some studies did not mention the position (e.g. supine) when blood pressure was measured.[96] The reduction in blood pressure was variable and small, and more clinical trials are needed in order to determine whether garlic is an effective treatment for mild hypertension. Jabbari *et al.* studied the difference between swallowing and chewing garlic on levels of serum lipids, lipid peroxidation, creatinine and cyclosporine in 50 renal transplant recipients, and concluded that swallowed garlic had no effect on the lipid profile in serum but chewed garlic reduced lipids in serum, a lipid peroxidation marker (malondialdehyde), in addition to blood pressure.[97]

Hypersensitivity to garlic has been reported. Topically applied garlic can cause garlic burn and an allergic dermatitis. Garlic can increase the effectiveness of warfarin, causing bleeding. A person taking warfarin should avoid garlic. The use of garlic should be stopped 7–10 days before surgery because it can prolong bleeding time. Postoperative bleeding has been reported with garlic alone.[98,99]

3.9.1 Measurement of components of garlic by HPLC

Arnault *et al.* described an ion-pair chromatographic method for the simultaneous analysis of allin, deoxyallin, allicin and dipeptide precursors in garlic products. They developed a rapid HPLC protocol using a mobile phase containing heptanesulfonate as an ion-pairing reagent and photodiode-array UV detection, and also electrospray ionization ion-trap MS detection.[100] Rosen *et al.* determined allicin, *S*-allylcysteine and volatile metabolites of garlic in breath, plasma and simulated gastric fluid using headspace sampling and GC-MS the for analysis of volatiles from breath and HPLC-MS for the determination of *S*-allylcysteine in plasma. For the determination of concentrations of volatiles in breath, a short-path thermal desorption device was used to volatilize the analytes. The absorption trap was spiked with toluene-d_8 and naphthalene-d_8 as internal standards. The desoprtion time was 3 min and the desoprtion temperature was 150 °C. The GC column used was a DB-1 methylsilicone capillary column (0.25 μm film thickness, 60 m × 0.32 mm i.d.), and the mass spectrometer was operated in electron ionization mode (70 eV). The mass scanning range was 35–450 amu. The HPLC system for the analysis of allicin used a Supelco 25 cm × 4.6 mm i.d. C_{18} column with photodiode-array detection (195 nm) and an isocratic mobile phase composition of acetonitrile–water (30:70 v/v). The major volatile component found in breath was allyl methyl sulfide. After consumption of raw garlic, limonene and *p*-cymene were also detected in breath.[101]

3.10 EPHEDRA (MA HUANG) AND RELATED DRUGS

Ma huang (ephedra) is commonly found in herbal weight loss products that are often referred to herbal fen-phen. Some weight loss clinics and herbal outlets promote 'Herbal fen-phen' as an alternative to fenfluramine, the prescription drug that has been withdrawn from the market due to toxicity. Herbal fen-phen products sometimes contain St John's wort and are sold as 'herbal prozac'. Ephedra-containing products are also marketed as decongestants, bronchodilators and stimulants. Other promoted uses include bodybuilding and enhancement

of athletic performance. 'Herbal ecstasy' is also an ephedrine-containing product, which can induce a euphoric state. Ephedra is a small perennial shrub with thin steams. The plant rarely grows over 30 cm tall. Some of the better known species include *Ephedra sinica* and *Ephedra equisentina* (collectively called ma huang from China. The German Commission E report recommended against the use of ephedra in patients with high blood pressure, glaucoma or thyrotoxicosis.

(−)-Ephedrine is the predominant alkaloid of ephedra plants. Other phenylalanine-derived alkaloids found in ephedra plants are (+)-pseudoephedrine, (−)-norephedrine, (+)-norpseudoephedrine, (+)-*N*-methylephedrine and phenylpropanolamines. Ephedrine is a potent central nervous system (CNS) stimulant. Because ephedra is both an α- and β-adrenergic agonist, ingestion of quantities over 50 mg lead to a rise in blood pressure, heart rate and cardiac output.

Haller and Benowitz evaluated 140 reports of ephedra-related toxicity and concluded that 31% of the cases were definitely related to ephedra toxicity and a further 31% were possibly related; 47% of reports of ephedra toxicity involved cardiovascular problems and 18% involved problems with the CNS. Hypertension was the single most frequent adverse reaction, followed by palpitation, tachycardia, stroke and seizure. Ten events resulted in death and 13 events caused permanent disability. The authors concluded that use of dietary supplements that contains ephedra may pose a health risk.[102]

3.10.1 Analysis of active components of ephedra-containing products

(+)-Pseudoephedrine and (−)-ephedrine in ephedra-containing herbs can be analyzed using ion-pair reversed-phase HPLC with sodium dodecyl sulfate after solid-phase extraction from herbal preparations. The mobile phase was water–acetonitrile–phosphoric acid (650:350:1 v/v/v) containing 0.5% sodium dodecyl sulfate, at a flow-rate of 1 mL min^{-1}. The column temperature was maintained at 50 °C and detection was achieved using a photodiode array at 210 nm. *N*-Benzyldiethylamine was used as the internal standard.[103] A simple GC-MS method for the determination of ephedrine alkaloids and 2,3,5,6-tetramethylpyrazine in ephedra has also been reported. The sample was extracted with diethyl ether and analyzed by GC without derivatization. A capillary column (30 m × 250 μm i.d.) coated with 5% phenylmethylsilicone was used, and the detection limits were 0.4, 0.7 and 0.02 ng for ephedrine, pseudoephedrine and 2,3,5,6-tetramethylpyrazine, respectively.[104] Cottiglia *et al.* isolated and characterized two novel phenolic glycosides, 4-hydroxy-3-(3-methyl-2-butenyl)phenyl β-D-glucopyranoside and *O*-coumaric acid β-D-allopyranoside, from *Ephedra nebrodensis* using NMR and MS.[105]

Ephedrine and pseudoephedrine have been measured in guinea pig plasma using HPLC with fluorescence detection, following precolumn derivatization with 5-dimethylamino-napthalene-1-sulfonyl chloride in acetonitrile. The mobile phase was 0.6% phosphate buffer (pH 6.5)–methanol (3:8 v/v).[106] Jacob *et al.* developed an LC–atmospheric pressure chemical ionization MS-MS method for the quantitaion of various alkaloids found in ephedra-containing dietary supplements and also in plasma and urine from subjects using these supplements. Using this method, the concentrations of ephedrine, pseudoephedrine, norephedrine, norpseudoephedrine, methylephedrine, methylpseudoephedrine and caffeine were determined in low nanogram quantities in plasma and urine. The analytical cycle time for this method was 12 min.[107]

3.11 CONCLUSIONS

Despite many reported toxic effects and drug interactions resulting from the use of alternative medicines, they remain popular and most users consider them safe. Labeling of herbal products may not accurately reflect their content, and adverse events or interactions attributed to a specific herb may be due to misidentification of the plant or contamination of the plant with pharmaceuticals or heavy metals. The addition of pharmaceuticals to Chinese herbal products is a serious problem. Of 2069 samples of traditional Chinese medicines collected from eight hospitals in Taiwan, 23.7% contained pharmaceuticals, including caffeine, acetaminophen, indomethecin, hydrochlorothiazide and prednisolone.[108,109] A fatal case of hepatic failure due to contamination of a herbal supplement with nitrosofenfluramine has been reported. Analysis of the herbal supplement also revealed the presence of fenfluramine.[110] Cole and Fetrow reported the presence of colchicine in gingko biloba and echinacea preparations. They also reported the case of a 23-year-old woman who had a serum digoxin concentration of 3.66 ng mL^{-1} after taking a herbal product that was found to contain digitalis lantana as an unlabeled constituent.[111] Heavy metal contamination is a major problem with Asian medicines. Ko reported that 24 of 254 Asian patent medicines collected from herbal stores in California contained significant quantities of lead, 36 contained arsenic and 35 contained mercury.[112] Contamination with lead and other heavy metals is common in Indian Ayurvedic medicines.[113] Analysis of active ingredients in serum and other biological fluids is an important step towards understanding the toxicity of herbal products, the magnitude of interactions between herbal supplements and pharmaceuticals and the detection of the presence of contaminants in these preparations.

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4

Measurement of Plasma L-DOPA and L-Tyrosine by High-Performance Liquid Chromatography as a Tumor Marker in Melanoma

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4.1 INTRODUCTION

Melanoma is the leading cause of death from skin diseases: one out of five patients diagnosed with melanoma will die from this cancer. The incidence of melanoma is increasing by 4% per year in the USA and is reaching up to 10 per 100 000 per annum in Europe (lifetime risk ~1 in 200). Melanoma is a malignancy of melanocytes, cells of neuroectodermic origin migrating essentially to the skin during embryogenesis.

The primary risk factor for melanoma is childhood sun exposure and prevention through education towards children and parents is essential. Individuals whose skin type and/or genetic background place them as high risk should also adopt sun protection behavior. Only ~10% of melanomas are probably related to a genetic context, which involves CDKN2 and/or CDK4 genes coding for cyclin-dependent kinase (CDK) inhibitors. The American Joint Committee on Cancer (AJCC) melanoma classification is based on the tumor–node–metastasis (TNM) system.[1] This defines four different stages and some sub-stages based, for example, on

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histopathological criteria of the primary tumor. Early surgical removal of the primary tumor is the best curative treatment of melanoma. Disease progression is associated with poor prognosis due to the high metastatic potential. In the treatment of metastasizing melanoma, chemotherapy is disappointing (15–20% response to dacarbazine), even with the introduction of adjuvant therapy with interleukin-2 and interferon-α.

Efforts of researchers are concentrated on early detection of tumors and recurrence, selection of high-risk patients for adjuvant therapy and monitoring of treatment efficiency. In the last 25 years, a wide range of molecules have been investigated as tumor markers in melanoma.[2] Unfortunately, most have a limited role in screening, early diagnosis and staging due to their low sensitivity. A non-specific enzyme, lactate deshydrogenase (LDH), has recently been introduced in the revised AJCC melanoma staging system[1] (stage IV, M1c: elevated serum levels at the time of staging). Available laboratory techniques include molecular biology [such as reverse transcriptase polymerase chain reaction (RT-PCR)] for tyrosinase and other melanoma-associated mRNAs such as MART-1, GalNAc-T, PAX-3 and MAGE-A3, immunoassays [such as enzyme-linked immunosorbent assays (ELISAs)] for melanoma antigens and high-performance liquid chromatography (HPLC) for melanin precursors.

The present review will focus on the analysis of L-DOPA and L-tyrosine by HPLC in plasma. Both are involved in the first step of melanogenesis controlled by a melanocyte-specific enzyme, tyrosinase. The plasma L-DOPA/L-tyrosine ratio has been evaluated as a tumor marker in melanoma during a 10-year collaboration between the Biochemistry Laboratory, the Dermatology Department of Saint-Louis Hospital (AP-HP) in Paris (France) and the Dermatology Department of the National Center of Oncology in Sofia (Bulgaria).

4.2 MELANOGENESIS

Melanocytes produce pigments of complex heterogeneous polyphenol-like structure, known collectively as melanins. In humans, melanins play a major role in photoprotection based on their radical scavenging abilities. They are responsible for the production of different colored patterns in hair and superficial epidermis.

4.2.1 Overview of the pathway

Two basic types of melanins exist in mammals: reddish brown phaeomelanins and brownish black eumelanins. Both are produced by enzymatically catalyzed and chemical reactions in specific subcellular melanogenic compartments, known as melanosomes.

H. S. Raper started the most important studies on melanogenesis in the period 1920–35. The classical Raper–Mason scheme postulated a linear pathway involving L-tyrosine, L-DOPA, L-DOPAquinone, L-DOPAchrome, dihydroxyindoles and indolequinones, leading to the end-products melanins. During the last decade, new technologies such as the molecular biology of the pigmented-related genes have substantially modified the traditional concept of melanogenesis.[3,4] For example, melanin synthesis in mammals is catalyzed by at least two other metalloenzymes, the tyrosinase-related proteins 1 and 2.

The amino acid L-tyrosine is the starting material of melanin biosynthesis. The first step of melanogenesis is centered on tyrosinase (EC 1.14.18.1), a copper-containing glycoprotein. Until recently, this enzyme was believed to catalyze the hydroxylation of L-tyrosine

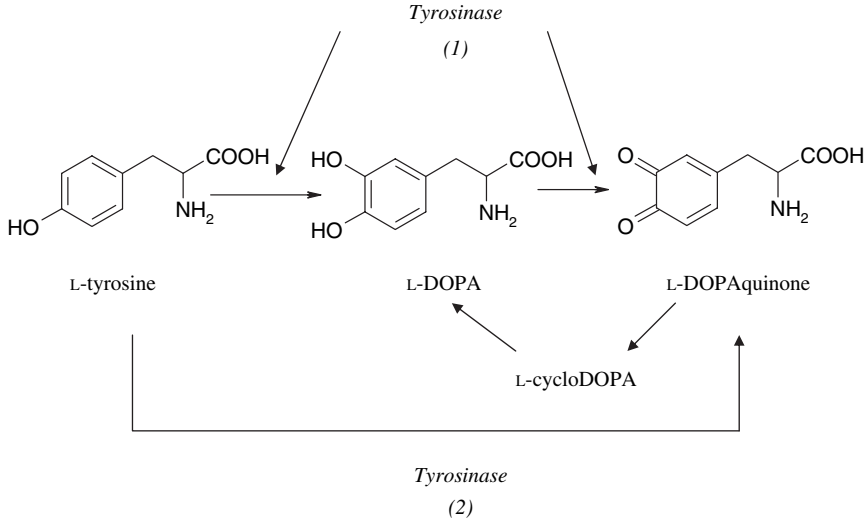

Figure 4.1 Early phases of the melanogenesis pathway. The first steps are critically regulated by the melanocyte-specific enzyme tyrosinase. L-DOPA is directly formed from L-tyrosine (1) and/or indirectly via L-DOPAquinone (2). Adapted from *Melanoma Research*, **9**, Letellier S, Garnier JP, Spy J, Stoitchkov K, Le Bricon T, Baccard M, Revol M, Kerneis Y, Bousquet B. Development of metastases in malignant melanoma is associated with an increase of plasma L-DOPA/L-tyrosine ratio, pages 389–394, 1999, with permission from Lippincott Williams & Wilkins

to 3,4-dihydroxyphenylalanine (L-DOPA) and the subsequent oxidation of L-DOPA to L-DOPAquinone (Figure 4.1). This classical role of tyrosinase has been contested, and L-DOPA could be indirectly formed from L-tyrosine via L-DOPAquinone. The catalytic activities of the human melanogenic enzymes, including tyrosinase, are still a matter of debate.

Eumelanogenesis and phaeomelanogenesis pathways diverge at the point where L-DOPAquinone, a reactive intermediate, undergoes either a reductive endocyclization yielding L-DOPAchrome, the precursor of the eumelanogenic pathway, or a reductive addition of thiols by cysteine to give the catechol 5-*S*-cysteinylDOPA (5-*S*-CD). The different 5-*S*-CD isomers are then oxidized to form phaeomelanins.

4.2.2 Potential tumor markers

Malignant melanocytes present defective melanosomes and tend to exhibit up-regulated melanogenesis. Melanogenuria (in the form of dark urine) is observed in some patients with widespread disease. End-product pigments, enzymes and melanin precursors or intermediates of the melanogenesis have therefore been measured in urine and blood from melanoma patients for more than 30 years.

Based on its key regulating role, the melanocyte-specific enzyme tyrosinase has been evaluated by the measurement of its activity (abandoned due to poor sensitivity), substrates and/or metabolites (L-tyrosine, L-DOPA) and, more recently, by the detection and now

quantification of specific mRNAs, mostly in blood (plasma or serum). Tyrosinase appears to be the most reliable mRNA target for RT-PCR: it is a specific marker of melanocytic differentiation, expressed both in primary and metastatic melanoma. Many researchers aiming at the detection of melanoma cells in blood, bone marrow, lymph nodes and sentinel nodes have pursued this promising line of investigation. Unexpectedly, the results are so far extremely variable and sometimes negative in advanced melanoma patients.

Other evaluated serological markers from the melanogenesis pathway include the phaeomelanin metabolite 5-*S*-CD (see Ref. 5 for an review of 5-*S*-CD in melanoma with 2648 samples taken from 218 patients) and to a lesser extent the eumelanin metabolite 6-hydroxy-5-methoxyindole-2-carboxylic acid. Since 5-*S*-CD is very sensitive to light and oxidation, analysis requires immediate centrifugation, freezing of the samples after blood collection and exclusion of light and air-oxygen.

4.3 L-DOPA ALONE

4.3.1 Urine analysis

Blois and Bonda[6] in 1976 first detected elevated L-DOPA levels in urine of patients with metastatic disease using ion-exchange column chromatography with colorimetric detection. L-DOPA excretion levels appeared to be proportional to tumor burden and to provide earlier evidence of distant metastases than imaging techniques. This relation with disease stage was confirmed by Faraj *et al.*[7] using an enzyme radioimmunoasassay; surgical intervention substantially decreased the urinary output of L-DOPA.

Incomplete 24-h urine collection, interfering substances, tedious sample preparation and low L-DOPA concentration in diluted samples have limited the routine use of urinary L-DOPA, even with the development HPLC coupled with electrochemical detection.[8,9] The reversed-phase HPLC method proposed by Ito *et al.*[10] used a simple alumina extraction, a procedure also applicable to serum (and even tissues). In addition, these sensitive and specific HPLC methods allowed the separation of both L-DOPA and 5-*S*-CD.[8–10]

4.3.2 Blood (plasma or serum) analysis

Using a radioenzymatic technique, Faraj *et al.*[11] measured plasma L-DOPA in 98 melanoma patients. For those without metastases, the mean L-DOPA plasma concentration ($1.01 \pm 0.12\ \mu g\ L^{-1}$, $n = 21$) was not different from that of normal controls ($n = 32$). However, it was increased ($p < 0.001$) in patients with metastases to regional lymph nodes ($2.08 \pm 0.46\ \mu g\ L^{-1}$, $n = 65$) and more distant metastases ($8.40 \pm 3.50\ \mu g\ L^{-1}$, $n = 12$). The development from regional to distant metastases in four patients was accompanied by a 4–6-fold increase in the concentration of plasma L-DOPA. This clinical study, for the first time, suggested that measurement of L-DOPA in plasma might be useful in melanoma patients, at least in those with metastatic disease. Measuring L-DOPA by the technique of Faraj *et al.*[11] was, however, cumbersome: it should be enzymatically converted to dopamine and further to 3-*O*-[^{3}H]methyldopamine, before being extracted and characterized by radiochromatography.

4.4 L-DOPA/L-TYROSINE RATIO

Since L-tyrosine is the main precursor of melanins and a substrate of tyrosinase, the L-DOPA/L-tyrosine ratio could more accurately reflect tyrosinase activity than L-DOPA alone. In 1997,[12] we developed two reversed-phase HPLC techniques, one with electrochemical detection to measure simultaneously L-DOPA, norepinephrine (NE), epinephrine (E), dopamine (DA), and DOPAC (3,4-dihydroxyphenyl acetic acid) (all compounds easily oxidizable between +0.15 and +0.50 V), and one with fluorimetric detection to measure L-tyrosine (and phenylalanine) on the same blood sample.

4.4.1 Technical aspects

These two HPLC techniques, slightly modified since the original publication, are fully described in Table 4.1; analytical performances[12] are summarized in Table 4.2. All procedures

Table 4.1 Reversed-phase HPLC determination of L-DOPA and L-tyrosine[a]

Samples	
Plasma[b] or serum	Li heparinate[b]
Centrifugation	2000 *g* (10 min, 4 °C)
Storage	−80 °C
HPLC system	
Column	C_{18} reversed-phase (250 × 4 mm i.d.); integrated precolumn (4 × 4 mm); 5 µm Purospher particles (Merck)
Pump/injector	515 model/WISP 717 (Waters)
L-DOPA procedure (1 mL plasma or serum)	
Internal standard	+ 100 µL 3,4-hydroxybenzamide (DHBA, 200 nmol L^{-1})
Isolation	Alumina adsorption[b], final desorbing volume: 800 µL. 0.2 mol L^{-1} $HClO_4$; 150 µL of eluate injected
Mobile phase	0.05 mol L^{-1} phosphate buffer with 0.02 mol L^{-1} EDTA, 1.125 mmol L^{-1} sodium octanesulfonic acid (7% methanol), pH 2.65
Elution, run time	Isocratic: 1 mL min^{-1} flow-rate, 30 min
Detection	Coulometric (5100 A detector, ESA), analytical cell (5011 ESA) in oxidative mode (optimal potential: +0.35 V).
L-Tyrosine procedure (1 mL plasma or serum)	
Internal standard	+ 50 µL *p*-hydroxyphenylacetic acid (PHPA) (2.5 mmol L^{-1})
Deproteinization	+ 0.5 mL 1 mol L^{-1} trichloracetic acid, followed by centrifugation at 2000 *g*, 4 °C (10 min); 10 µL injected
Mobile phase	0.08 mol L^{-1} acetate buffer, with 0.02 mol L^{-1} sodium EDTA, 1.8 mmol L^{-1} sodium lauryl sulfate (10% methanol), pH 3.8
Elution, run time	Isocratic: 1 ml min^{-1} flow-rate, 10 min
Detection	Fluorimetric: excitation at 275 nm, emission at 305 nm (RF 535 detector, Shimadzu)

[a] All chemicals from Sigma-Aldrich (Saint-Quentin Fallavier, France). Other suppliers: Merck (Darmstadt, Germany), Waters (St Quentin en Yvelines, France), ESA (Bedford, MA, USA), Shimadzu (Kyoto, Japan).

[b] See Ref. 12 for more details.

Table 4.2 Characteristics of the L-DOPA and L-tyrosine HPLC procedures[a]

L-DOPA procedure	
Recovery	72.3% at 3 nM, 74.8% at 50 nM
Linearity	1–100 nM
Detection limit	0.25 nM
Precision	1.9–2.9% for intra-assay ($n = 6$), 3.0–3.9% for inter-assay ($n = 6$) (at 80–2.5 nM)
L-tyrosine procedure	
Recovery	87.9% at 25 μM, 88.2% at 200 μM
Linearity	10–500 μM
Detection limit	2.5 nM
Precision	1.6–2.4% for intra-assay, 3.9–4.5% for inter-assay (at 400–25 μM)

[a]All data from Ref. 12.

are carried out at room temperature. The total analysis time for L-DOPA analysis, including alumina adsorption, is ~3 h.

A few important remarks should be made concerning the L-DOPA procedure. For alumina adsorption (alumina activity grade I), glass tubes should never be used. We used 17 × 100 mm conical propylene tubes. To improve recovery, it is also important to desorb in a minimum volume of perchloric acid. The eluate (in propylene tubes) should then be directly injected into the column or kept at 4 °C until injection. The detailed extraction procedure (at 4 °C) is fully described in Ref. 12. Minimal changes in the experimental conditions of the L-DOPA procedure strongly influence the quality of the analytical process. These include methanol concentration (an increase will decrease the capacity factor for all compounds without affecting the order of elution), and counterion concentration (an increase will increase the capacity factor, except for DOPAC). The pH of the mobile phase is the most important factor to be controlled. As the pH increases, L-DOPA elution times decrease significantly with a risk of co-elution with DHBA, DOPAC or catecholamines (Figure 4.2). Changes in pH of less than one-tenth of a unit could produce shifts of 1 min in the L-DOPA retention time.

The high selectivity of HPLC coupled with electrochemical detection greatly reduces drug-induced interference, as tested for acetaminophen, acetylsalicylic acid, dihydroxycaffeic acid and α-methylDOPA. None of the endogenous compounds found in plasma interfered with the retention time of L-DOPA or internal standard.

4.4.2 Clinical results

Five papers have been published on the value of the L-DOPA/L-tyrosine ratio as a marker of melanoma progression; the main biological and clinical results are summarized in Table 4.3.

The reference range was established at 4.6–9.4 nmol L^{-1} for L-DOPA, 32–88 μmol L^{-1} for L-tyrosine and 6.0–16.0 × 10^{-5} for the L-DOPA/L-tyrosine ratio in a group of 35 healthy subjects.[12] No seasonal variations (winter/summer) were observed in a group

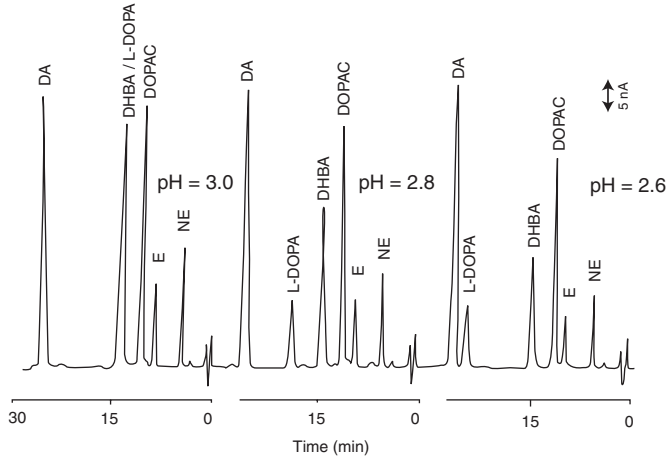

Figure 4.2 Variation of retention time of L-DOPA as a function of pH of the mobile phase. As the pH increases, the retention time of L-DOPA decreases. NE, norepinephrine; E, epinephrine; DA, dopamine; DHBA, internal standard. Reprinted from *Journal of Chromatography B*, **696**, Letellier S, Garnier JP, Spy J, Bousquet B. Determination of the L-DOPA/L-tyrosine ratio in human plasma by high-performance liquid chromatography. Usefulness as a marker in metastatic malignant melanoma, pages 9–17, Copyright 1997, with permission from Elsevier

of 11 healthy subjects;[13] additional controls consisting of 17 non-melanoma metastatic cancer patients (mostly breast cancers) did not exceed the 16.0×10^{-5} upper reference limit.[13]

Table 4.3 L-DOPA/L-tyrosine ratio in plasma/serum of melanoma patients

Study (*n* patients)	Biological results	Clinical results
Letellier *et al.*[12] (1997) ($n = 46$)	Normal range defined ($n = 39$)	Elevated in patients with distant metastasis vs other stages
Letellier *et al.*[13] (1999) ($n = 90$)	Specificity confirmed (in non-melanoma cancers) No seasonal variations	Elevated in distant metastasis vs other stages Relation with the number of distant metastases
Le Bricon *et al.*[14] (1999) ($n = 50$)	Poor concordance with tyrosinase mRNA	Sensitivity for distant metastasis: 41% (vs local: 30%)
Stoitchkov *et al.*[15] (2002) ($n = 89$)	No correlation with S100B (47% discordant results)	Elevated in distant metastasis Elevated in progressive patients with local metastasis (sensitivity/specificity: 78/67%)
Stoitchkov *et al.*[16] (2003) ($n = 60$)	Less variable than S100B	Decreased by chemotherapy (in responders) Prognostic value in distant metastasis

When compared with tyrosinase mRNA (by in-house nested RT-PCR),[14] and S100B (immunoluminometric LIA-mat Sangtec100; AB Sangtec Medical, Sweden),[15,16] there was no correlation ($r = 0.149$ with S100B) or little agreement (tyrosinase mRNA) with the L-DOPA/L-tyrosine ratio. Tyrosinase mRNA positivity by RT-PCR was associated with rapid disease progression and death within 2 months. By contrast, some cases of very low L-DOPA/L-tyrosine ratio levels have been reported shortly before death.[14] It was not sensitive to lymph node dissection (LND), whereas surgery decreased S100B by 52% in patients with elevated S100B.[16] These differences are probably due to the different respective mechanisms of production and release of these markers. The L-DOPA/L-tyrosine ratio reflects the overall metabolic activity of the melanocyte population (although an extracellular conversion of L-tyrosine to L-DOPA by tyrosinase during the loss of tumor cell integrity cannot be excluded). A local decrease in tyrosinase activity in lymph nodes or in some metastases might be also present.

Using the L-DOPA/L-tyrosine ratio, rather than L-DOPA alone, reduces inter-individual variability, at least in metastatic patients (K. Stoitchkov, unpublished data). A weak positive correlation was found between L-DOPA and L-tyrosine (+0.296, $p < 0.01$, Spearman correlation) in a group of 98 melanoma patients from the National Center of Oncology in Sofia (Bulgaria). Disease stage influenced plasma L-DOPA and L-DOPA/L-tyrosine ratio ($p < 0.001$), but not L-tyrosine concentration (data not shown). In patients with distant metastases, the 25th–75th percentile range represented 66% of the median value (L-DOPA/L-tyrosine ratio) vs 80% (L-DOPA alone). A similar effect was noted for the 5th–95th percentiles (124 vs 182%), a phenomenon also observed in patients with localized metastases.

Concerning individual variability of the L-DOPA/L-tyrosine ratio, some elements can be derived from a retrospective analysis of our database with a total of 255 patients diagnosed and/or followed in St Louis Hospital between 1994 and 2003 (S. Letellier, unpublished data). Apparent disease stability (no change to higher stage) was confirmed in 44 out of 116 patients without metastases (stage I–II) (median follow-up: 6 months from inclusion). The median difference between the first and the second samples was $+0.4 \times 10^{-5}$ (or +2.0%, NS); the 75th percentile (reflecting intra-individual variability) represented 17% of the median value. Twelve patients exceeded the 75th percentile (up to +143% increase). The inter-individual variability (inclusion values) was 21%, as previously found in healthy subjects.[12]

4.4.3 Future directions

A large multi-marker study [including LDH, melanoma antigens S100B and melanoma inhibitory activity (MIA) and the L-DOPA/L-tyrosine ratio for serological tumor markers] has been initiated in Paris in a multi-center approach. The aims of this long-term (10 years) prospective study are to optimize the biological follow-up of melanoma patients and to select the most pertinent marker(s) or combination of markers. Identifying the most appropriate category of patients (AJCC stage or group defined, for example, by a given cut-off) who would benefit from the use of these markers is also of great interest. When analyzing inclusion values in our homogeneous group of 255 patients, the L-DOPA/L-tyrosine ratio was, for the first time, significantly higher in stage III (local metastases)

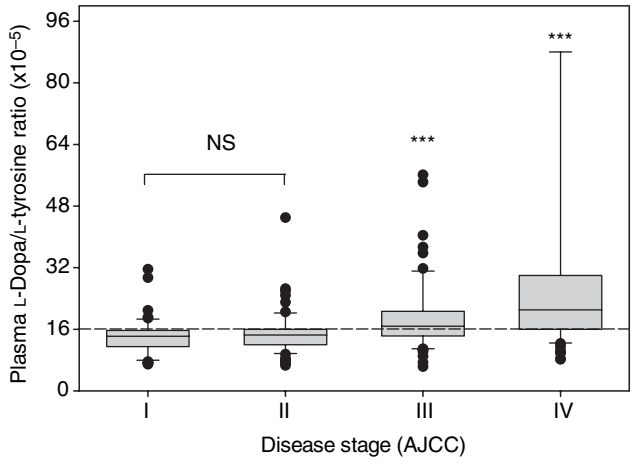

Figure 4.3 Plasma L-DOPA/L-tyrosine ratio and AJCC disease stage. A total of 255 melanoma patients were included: stage I, 54; stage II, 62; stage III, 67; and stage IV, 72 (AJCC staging using Ref. 2). *** Significantly different from other stages (Kruskal–Wallis ANOVA and Dunn's multiple comparison procedure). Box, 25th percentile/median/75th percentile; error bars, 5th percentile/95th percentile. There was 14 upper outliers: 7 in stage III, including a patient with Dubreuilh melanosis (184.8×10^{-5}), and 7 in stage IV (values up to 310×10^{-5})

than lower stages (no metastases) (Kruskal–Wallis ANOVA and Dunn's multiple comparison procedure, $p < 0.05$) (Figure 4.3). In stage III, 57% of patients were above the 16.0×10^{-5} upper cut-off[12] for the L-DOPA/L-tyrosine ratio (vs 22–24% in stage I–II). Patients with clinically localized disease, but with elevated L-DOPA/L-tyrosine ratio (above 16.0×10^{-5} or other cut-off to be defined by ROC analysis), might be considered as being at 'higher risk' for disease progression. Accordingly, they might deserve to be followed more closely and to be candidates for alternative or more aggressive therapies. An example of a longitudinal follow-up of a melanoma patient with the L-DOPA/L-tyrosine ratio is presented in Figure 4.4.

4.5 CONCLUSION

L-DOPA and L-tyrosine can be efficiently measured in human plasma by reversed-phase HPLC with electrochemical (L-DOPA) or fluorimetric (L-tyrosine) detection. Careful attention should be paid, however, to the entire analytical process. The specificity of the L-DOPA/L-tyrosine ratio for melanoma and its sensitivity (even in some patients with early disease) have been established. In advanced melanoma, it possesses a prognostic value, a relation with disease progression and treatment efficacy (chemotherapy). Studies on a large number of patients and with a long follow-up period are needed to refine the use of the L-DOPA/L-tyrosine ratio.

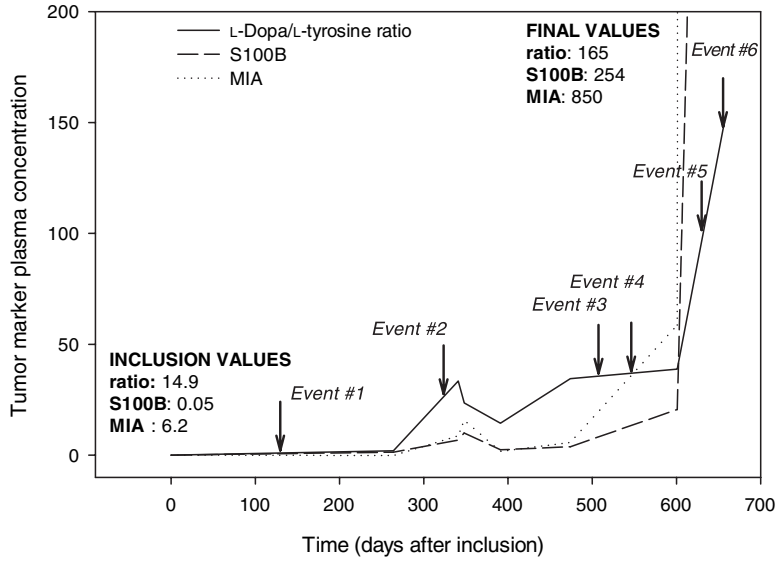

Figure 4.4 Example of longitudinal follow-up by plasma L-DOPA/L-tyrosine ratio. A 52-year-old male (initial melanoma: right arm) was monitored by the L-DOPA/L-tyrosine ratio, S100B and melanoma inhibitory activity (MIA) for 21 months. Six major clinical events are reported: 1, second local recurrence, 3 years after initial diagnosis; 2, bone and pulmonary metastases (stage IV); 3, evolution of subcutaneous metastases; 4, evolution of cutaneous and pulmonary metastases; 5, metastases to axillary nodes; 6, death

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5

Hypersensitive Measurement of Proteins by Capillary Isoelectric Focusing and Liquid Chromatography-Mass Spectrometry

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5.1 INTRODUCTION

In the post-genomic era, research in proteomics has moved to the forefront of attention as proteins play a central role in biological processes. The ability to profile proteins and peptides in cells and tissues is an important step in understanding how a biological activity progresses.[1–5] In a proteomic sample, there are usually a large number of proteins that vary widely in physicochemical properties, including molecular weight (MW), isoelectric point (p*I*), solubility, acidity/basicity and hydrophobicity/hydrophilicity. Moreover, the protein concentrations can extend over six orders of magnitude, and it is often the least abundant proteins that are of the greatest interest in systems biology.[6] For these reasons, there is a continuing need to develop and evaluate new platforms for protein separation and analysis. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is the core technology for profiling protein expression.[7,8] This method can separate proteins by their p*I* in the first dimension and eletrophoretic mobility in a polyacrylamide gel in the second dimension. As a multidimensional separation method, 2D-PAGE can resolve >1000 proteins in a single run.[8] However, there are two main drawbacks, sensitivity and throughput, that ultimately limit its use in proteomics research. 2D-PAGE usually employs Coomassie Brilliant Blue or silver staining to visualize separated proteins in the gel. Silver staining is the more sensitive of

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these, detecting 0.1–10 ng amounts of protein, which correspond to 20–200 fmol for a 50-kDa protein.[7] The maximum dynamic range of 2D-PAGE is 10^4, making it inadequate for the study of proteins in low abundance.[9] Throughput is another limitation, since the entire process of gel preparation to protein separation and analysis is slow and difficult to automate fully. More importantly, the reproducibility of both the position and intensity of a protein spot are limited, which makes it difficult to compare the results from different runs.[1]

In principle, multidimensional liquid-based separations could overcome these problems. Liquid-based methods facilitate direct coupling with mass spectrometry (MS) for protein characterization. If the separation mode is analogous to 2D-PAGE, then comprehensive and powerful databases built from 2D-PAGE data can be effectively utilized.[10] Several liquid-based pH fractionation methods have been developed to replace the first dimension of 2D-PAGE. By coupling these with reversed-phase liquid chromatography (RPLC) and MS, an equivalent multidimensional separation to 2D-PAGE (p*I* vs MW under denaturing conditions) can be achieved. Lubman and co-workers reported the coupling of a Rotofor with non-porous RPLC for intact protein profiling.[11–13] The Rotofor is a relatively simple device that is the liquid analog of an isoelectric focusing (IEF) gel. The chamber is filled with liquid containing protein and ampholyte. A high voltage is then applied across the chamber and the proteins migrate to locations based on their p*I*. After separation, each protein fraction is transferred from chamber into a small vial for further analysis by RPLC-MS. Shang *et al.*[14] used a Rotofor system for intact protein separation in which the chambers were separated by Immobiline-impregnated polyacrylamide gel membranes. With this modification, the use of ampholyte was not necessary, which eliminated the possibility of ampholyte interference in MS analysis. Speicher and co-workers reported a similar liquid-based isoelectrofocusing system to fractionate protein mixtures.[15–18] Lubman and co-workers have developed a system consisting of chromatofocusing (CF) coupled with RPLC-MS.[19–21] CF is an ion-exchange-based separation in which proteins are bound to an anion exchanger and then eluted by a continuous decrease of the buffer pH.[22–24] CF can easily be coupled with RPLC-MS to build an automated two-dimensional separation system.

Capillary isoelectric focusing (CIEF) coupled with MS is another promising approach to sensitive protein separation and analysis.[25–33] In CIEF, the fused-silica capillary contains both the protein mixture and ampholyte. When an electric potential is applied, the negatively charged acidic components in the ampholyte migrate toward the anode and decrease the pH in that end of the capillary, whereas the positively charged basic constituents migrate toward the cathode and increase the pH. These pH changes continue until each ampholyte species reaches its p*I*, at which point its charge is neutral and it ceases to migrate. The ampholyte buffering capacity provides a continuous pH gradient along the capillary. Protein analytes are amphoteric macromolecules that also focus in narrow zones based on their p*I* values in the same way as the individual ampholytes. The focusing effect of CIEF permits the analysis of dilute protein samples with a typical concentration factor of 50–100-fold.[6,34,35] CIEF provides a high-resolution separation based on p*I*, whereas subsequent MS analysis provides a mass measurement with high precision and accuracy. A display of p*I* vs MW can be obtained from CIEF-MS data with higher throughput and sensitivity than 2D-PAGE.[27,28]

Unfortunately, substantial problems remain with the CIEF-MS approach. Most importantly, the ampholyte used in CIEF co-elutes with the analyte and perturbs the electrospray ionization (ESI) process in a manner that suppresses analyte signal intensity and degrades mass resolution.[36,37] In addition, the resolution of CIEF alone is not sufficient to separate proteins completely in complex samples. When proteins co-elute from an ESI source, some

high-concentration species may quench the ionization of other low-concentration species. In an extreme case, the signal of these low concentration species may be lost.[38] Finally, it is difficult to add reagents that enhance the solubility of hydrophobic proteins because their presence, like ampholyte, degrades ESI performance. For these reasons, it is desirable to insert an additional separation step between CIEF and MS such as RPLC. RPLC is easily coupled with ESI-MS and provides the opportunity to remove ampholyte and further separate proteins that are not sufficiently resolved by CIEF. A CIEF-RPLC-MS system for peptides has been reported by Lee and co-workers.[39,40] In this system, an injection loop attached to a microselection valve is placed between the anodic and cathodic cells to transfer peptide fractions into the RPLC system. Ampholyte is removed from the peptide fraction with a C_{18} trap column prior to RPLC. This system is capable of detecting thousands of peptides in a single run with high sensitivity. This system was also applied recently for intact yeast protein profiling.[41]

In our work, an improved CIEF-RPLC-MS system has been developed for protein separation and characterization.[42] A microdialysis membrane separates the cathodic cell from the capillary column. During the focusing step, catholyte is able to traverse the membrane but protein molecules cannot. After focusing, proteins are hydrodynamically pushed past the membrane to a microselection valve that collects and transfers fractions to the RPLC system for further separation. This configuration permits a linear pH gradient to be maintained in the separation capillary with no voltage applied to the microselsection valve. The cathodic cell and microselection valve can easily and safely be retrofitted into commercial instruments.

5.2 A ROBUST CIEF-RPLC INTERFACE

Figure 5.1 shows a schematic of the microdialysis membrane-based cathodic cell for CIEF. The cathodic cell is built around a PEEK tee (Valco, Houston, TX, USA). One collinear port is connected to a 55-cm CIEF capillary (50 μm i.d., 375 μm o.d.). The opposite collinear port is connected to a 20-cm fused-silica capillary that leads to a waste vial. The perpendicular

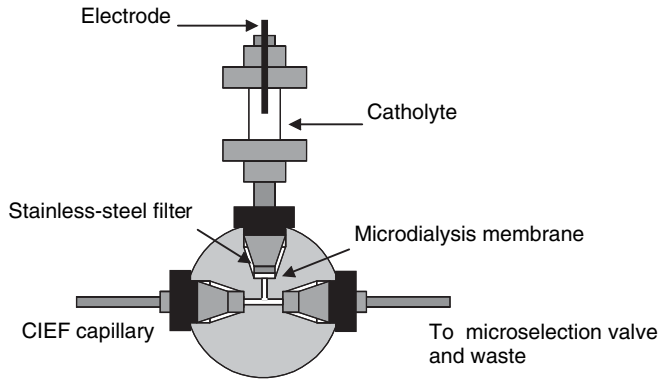

Figure 5.1 Diagram of the microdialysis membrane-based cathodic cell. Reprinted with permission from F. Zhou and M. Johnston, *Analytical Chemistry*, **76**, 2734–2740, Copyright 2004 American Chemical Society

port is connected to the cathodic vial. The cathodic vial is built from two reducing unions and a 5-cm length of Dayco polyethylene tubing (1/4 in o.d., 3/8 in i.d.). The first reducing union connects the polyethylene tubing to the PEEK tee through a 3-cm length of PEEK tubing (1/16 in o.d., 400 μm i.d.) that attaches to the perpendicular port. A microdialysis membrane (MW cutoff 3500 Da) cut to a 1/16-in diameter is inserted into the perpendicular port and supported by a 0.5-μm stainless-steel filter. The second reducing union connects the other end of polyethylene tubing to another 3-cm length of PEEK tubing (1/16 in o.d., 400 μm i.d.) containing a 6-cm Pt wire as the cathodic electrode. This cathodic 'vial' is filled with ~1.5 mL of catholyte and any air bubbles are carefully removed.

CIEF is performed with the microdialysis membrane-based cathodic cell mounted inside a commercially available automated capillary electrophoresis system (Beckman P/ACE MDQ with a UV detector). The CIEF capillary is filled with ampholyte solution (1:80 ampholyte:water). Protein and peptide test mixtures are injected from the anodic side for 2 min by pressure. Focusing is performed in an electric field of 300 V cm^{-1}; the anolyte is 91 mM H_3PO_4 and the catholyte (sealed in the cathodic cell) is 20 mM NaOH. The platinum wire in cathodic cell is connected to the negative electrode of the instrument and set to 0 V. After a focusing period of 4 min, a small pressure is applied to the anodic end of the capillary to push the focused bands hydrodynamically through a UV absorption detector located 10 cm upstream of the cathodic cell. The 300 V cm^{-1} electric field is maintained across the capillary during hydrodynamic mobilization. Absorption is monitored at 280 nm. Sample losses to the cathodic cell are studied by repositioning the UV detector 10 cm downstream of the cathodic cell. All capillary lengths and sizes are identical for each detector location. Figure 5.2a shows the separation achieved with the microdialysis membrane-based cathodic

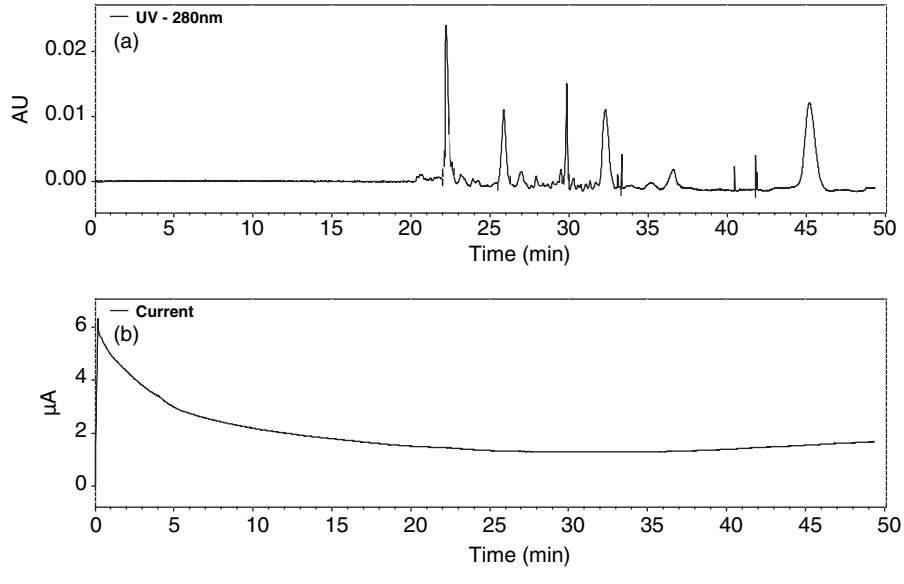

Figure 5.2 CIEF separation with the microdialysis membrane-based cathodic cell. (a) UV absorption (280 nm) vs time; (b) current profile. Reprinted with permission from F. Zhou and M. Johnston, *Analytical Chemistry*, **76**, 2734–2740, Copyright 2004 American Chemical Society

cell for a sample containing 0.1 nmol of ribonuclease A, myoglobin, carbonic anhydrase II, β-lactoglobulin, trypsin inhibitor and 1 nmol of CCK flanking peptide. In this experiment, the absorbance detector was placed upstream from the cathodic cell as described above. The current profile in Figure 5.2b shows a rapid decrease in the first few minutes with little change afterwards. A slight upturn in current is observed at very long times and is caused by hydrodynamic movement of phosphoric acid into the capillary. The current profile in Figure 5.2b is virtually identical with that obtained from a separation using the conventional cathodic cell, which indicates free movement of hydroxide ions across the microdialysis membrane. A plot of p*I* vs migration time with the membrane-based cathodic cell is also identical with that obtained with the commercial cathodic cell. In both cases, a slight non-linearity is observed, the origin of which origin has been discussed in detail elsewhere.[43] Although hydroxide ions can move across the microdialysis membrane by both diffusion and electrophoresis, diffusion is slow and a single fill of the cathodic cell can be used for over 1 month. Sample loss to the membrane-based cathodic cell was studied by comparing the peak area of ribonuclease A when the UV detector was located upstream vs downstream of the cell. Within experimental error, no change in peak area was observed between the two detector locations.

5.3 FIRST-GENERATION CIEF-RPLC-MS SYSTEM FOR PROTEINS

A diagram of the first-generation CIEF-RPLC-MS system is shown in Figure 5.3. The membrane-based cathodic cell assembly is connected to a six-port electronically controlled microselection valve with a 5-cm length of fused-silica capillary (50 μm i.d., 375 μm o.d.). The ampholyte solution (1:80 ampholyte:water) and protein–peptide test mixtures are injected and separated by CIEF as described above. The focused bands are then hydrodynamically pushed past the cathodic cell into a 1-μL stainless-steel injection loop attached to the microselection valve. The valve position is then switched, and proteins fractioned by the loop are transferred to a C_{18} reversed-phase trap column (5 mm × 300 μm i.d.) attached to a second six-port valve located on a capillary LC system. Figure 5.3 illustrates the valve positions during the transfer step. After trapping, both six-port valves are switched back to their original positions. Fractionated proteins are eluted from the trap column and separated by RPLC. Meanwhile, another fraction is collected on the 1-μL loop and the sequence is repeated. During the entire time, the 300 V cm^{-1} electric field is applied to the CIEF capillary to maintain the pH gradient. Typically, some electroosmotic flow occurs from the anodic end of the CIEF capillary during the course of the experiment. This flow is counterbalanced hydrodynamically by placing the waste vial ∼10 cm above the anodic end of CIEF capillary.

In order to collect and analyze protein fractions sequentially, hydrodynamic flow must be cycled on and off. In the 'go' mode, pressure is applied to the anodic end to push focused bands into the injection loop. In the 'stop' mode, the pressure is removed so that the analytes remaining in the capillary do not move. After the collected fraction has been transferred to the trapping column and washed, the microselection valve switches back to its original configuration and pressure is reapplied to the anodic end to collect a new fraction. While the new fraction is being collected, the fraction loaded on the trap column is eluted and analyzed by RPLC-MS.

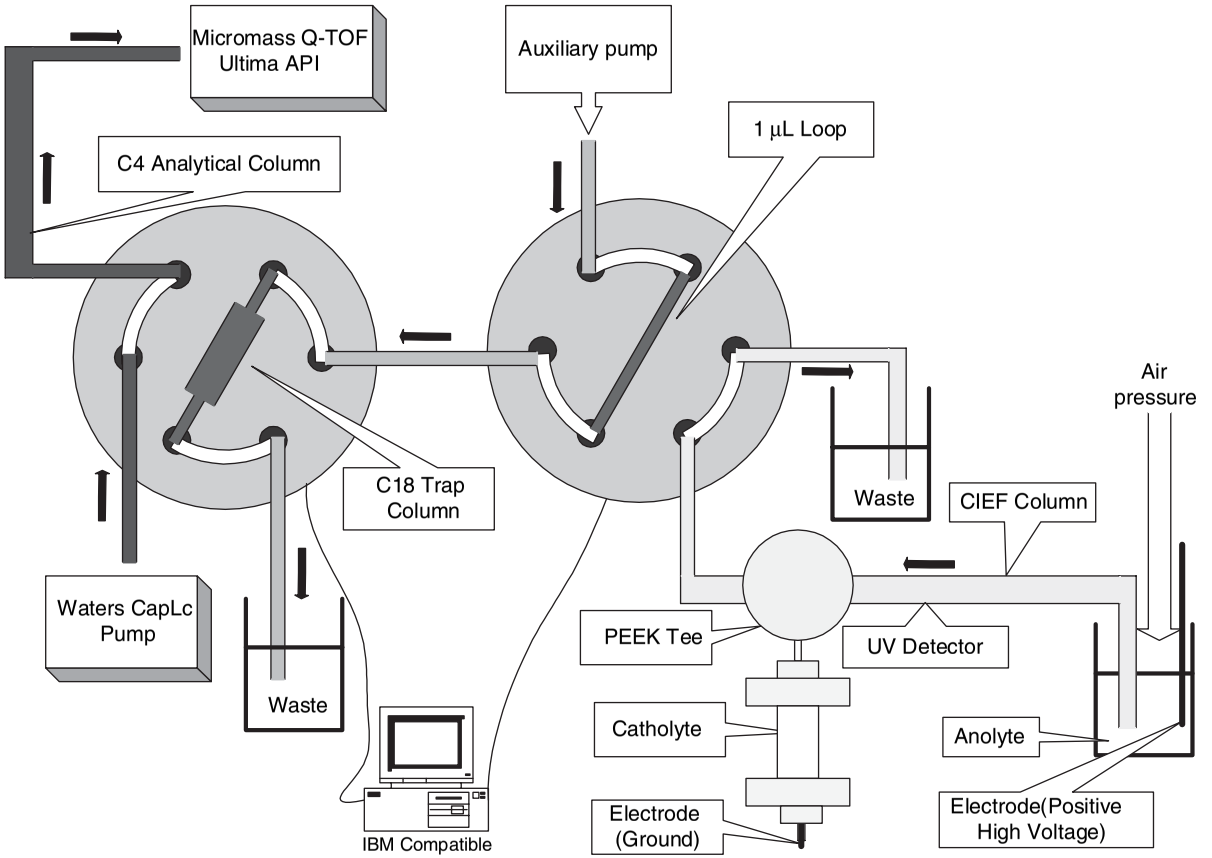

Figure 5.3 Diagram of the first-generation CIEF-RPLC-MS system. Reprinted with permission from F. Zhou and M. Johnston, *Analytical Chemistry*, **76**, 2734–2740, Copyright 2004 American Chemical Society

The protein fraction sampled by the injection loop is loaded on the trap column and washed with a solution of water–acetonitrile–acetic acid (94.9:5.0:0.1 v/v/v) at a flow-rate of 20 μL min^{-1} for 3 min. Most ampholyte is removed during this time. The protein fraction is then eluted from the trap column with mobile phase and further separated on a C_4 reversed-phase column (5 cm × 300 μm i.d.). Mobile phase A [water–acetonitrile–acetic acid (94.9:5.0:0.1)] and mobile phase B [acetonitrile–water–acetic acid (94.9:5.0:0.1)] are delivered at a flow-rate of 20 μL min^{-1} using a two-step gradient of 5 min (phase B from 25 to 60%) and 2 min (phase B from 60 to 90%). Proteins are mass analyzed with an ESI-QTOF mass spectrometer (Waters-Micromass, Beverly, MA, USA). Data processing is performed with MaxEnt1, which converts the raw spectrum of multiply charged ions into a deconvoluted display of singly charged ions.

Figure 5.4 shows how ampholyte can significantly degrade protein detection by MS. Figure 5.4a and b show the mass spectrum of 2 μM cytochrome *c* in H_2O, water–methanol–acetic acid buffer (50:49:1) with and without 1:80 ampholyte. In the presence of ampholyte, no cytochrome *c* signal is observed, highlighting the need to remove ampholyte prior to mass analysis. Figure 5.4c and d show the mass spectra of 2 pmol and 2 fmol, respectively, of cytochrome *c* loaded on the capillary and analyzed by CIEF-RPLC-MS. In each case, mass spectra were obtained with little interference from ampholyte. The high sensitivity demonstrated in Figure 5.4d may be further improved by optimizing the RPLC step.

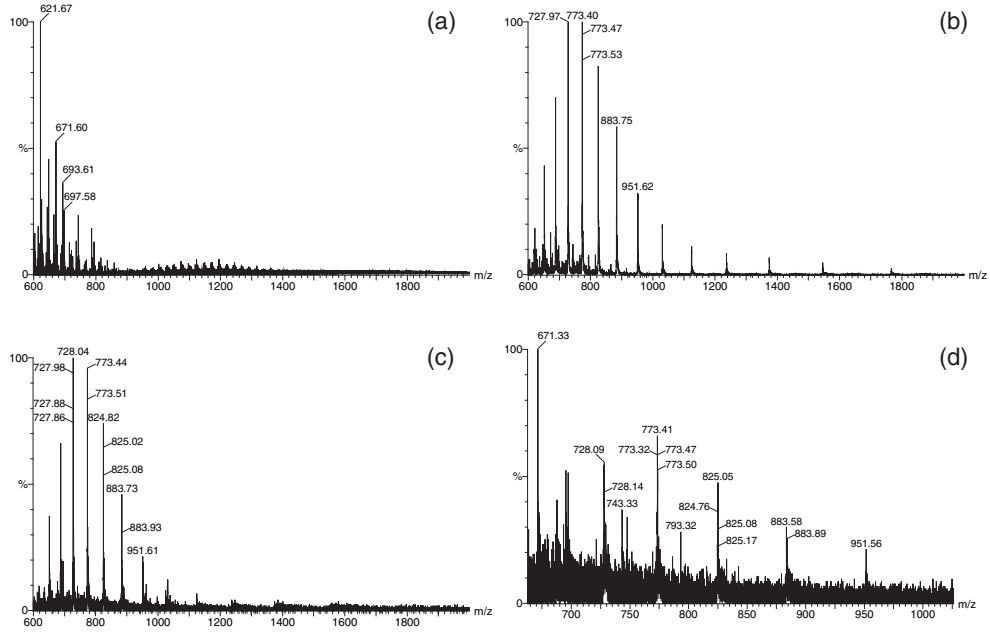

Figure 5.4 (a) ESI mass spectrum of 2 μM cytochrome *c* with 1:80 ampholyte in water–methanol–acetic acid (50:49:1); (b) ESI mass spectrum of 2 μM cytochrome *c* without ampholyte in the same buffer as in (a); (c) ESI mass spectrum of 2 pmol cytochrome *c* injected and analyzed by CIEF-RPLC-MS; (d) ESI mass spectra of 2 fmol cytochrome *c* injected and analyzed by CIEF-RPLC-MS. Reprinted with permission from F. Zhou and M. Johnston, *Analytical Chemistry*, **76**, 2734–2740, Copyright 2004 American Chemical Society

This system is then tested with a standard protein mixture containing 0.1 pmol of ribonuclease A, cytochrome *c*, myoglobin, insulin and β-lactoglobulin, 20 pmol of carbonic anhydrase II and bovine serum albumin and 1 pmol of CCK flanking peptide. The proteins and peptide are focused by CIEF and then divided into seven p*I* fractions by stop and go operation. Figure 5.5 shows the RPLC-MS analysis of these fractions. The labels A–G

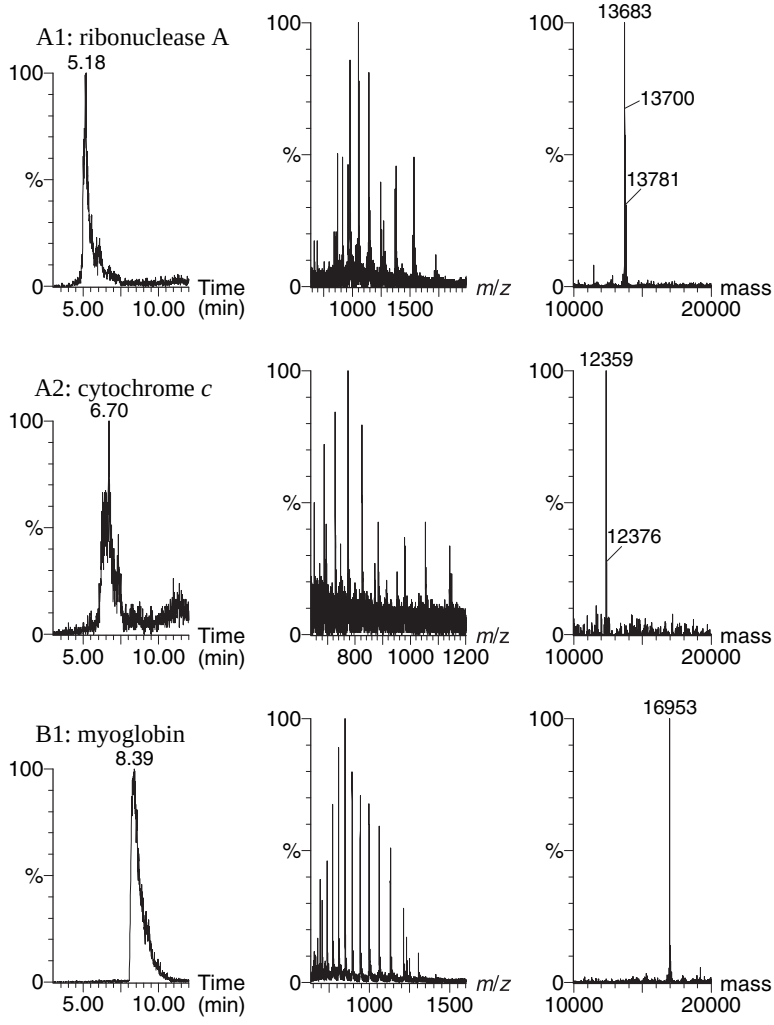

Figure 5.5 RPLC-MS of p*I* fractions. A–G represent p*I* ranges of 9.2–8.1, 8.1–7.0, 7.0–5.9, 5.9–4.8, 4.8–3.7, 3.7–2.6 and 2.6–1.5, respectively. Left panel, extracted mass chromatogram of each protein in the fraction; middle panel, raw spectrum of each protein averaged over the top 80% of the LC peak; right panel, deconvoluted spectrum (singly charged) by MaxEnt1. Only the total ion chromatogram is shown for fraction F; no deconvoluted spectrum is shown for fraction G. Reprinted with permission from F. Zhou and M. Johnston, *Analytical Chemistry*, **76**, 2734–2740, Copyright 2004 American Chemical Society

represent the seven protein fractions analyzed. Two fractions (A and D) contained two proteins, one fraction (F) contained no proteins or peptides and the remaining fractions contained just one protein/peptide. Three separate plots are shown for each protein/peptide: an extracted ion chromatogram, a raw mass spectrum averaged over the top 80% of the peak in the extracted ion chromatogram and the Maxent1 deconvoluted spectrum. For fraction F, just a total ion chromatogram is shown as the only signal observed is due to residual ampholyte. For proteins of molecular mass <20 kDa, 100 fmol are generally sufficient to obtain a high-quality spectrum. Larger proteins require more sample because the ion signal is distributed over many *m*/*z* values.

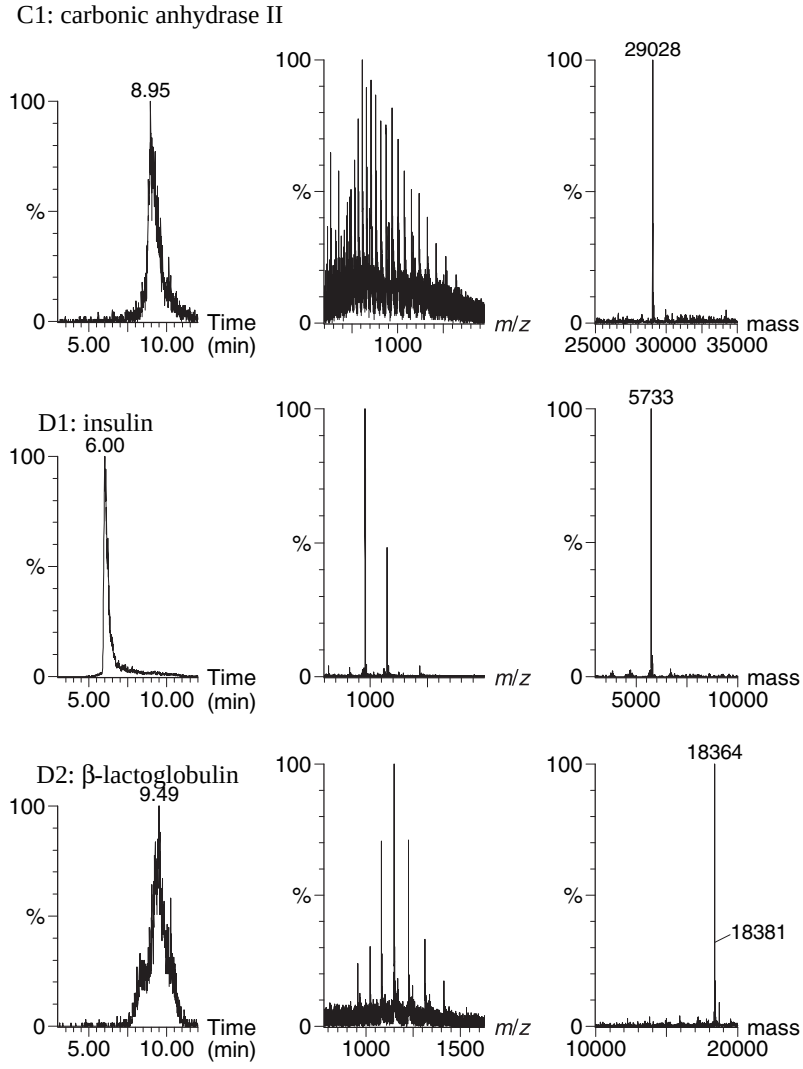

Figure 5.5 (*continued*)

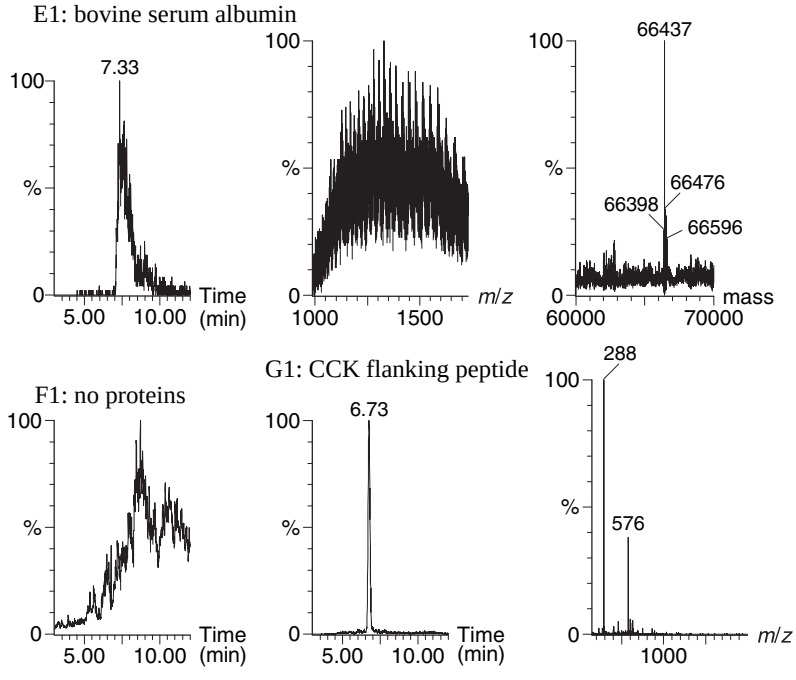

Figure 5.5 (*continued*)

5.4 SECOND-GENERATION CIEF-RPLC-MS SYSTEM

Although the first-generation system demonstrates the potential use of CIEF-RPLC-MS for protein separation and identification, it also has a disadvantage: the time allowed for RPLC is so short that the separation has to be run with a very fast gradient, giving poor resolving power. An improved CIEF-RPLC-MS system has been designed and constructed to solve this problem.[44] A schematic diagram of this second-generation system is shown in Figure 5.6. Compared with the system described above, the major difference is that there is an array of 10 storage loop located between the CIEF and RPLC instruments, and these loops retain the protein fractions, allowing more time for the RPLC step. With this setup, a shallower gradient is possible for RPLC separation, resulting in higher resolving power. In addition, a wider bore CIEF capillary (100 μm i.d.) is used so that more sample can be loaded. This system is used to profile proteins from a complex yeast enzyme concentrate, $11\,\mu g\,\mu L^{-1}$, in ampholyte solution (eCAP cIEF 3-10; diluted 1:100, ampholyte:water) that also contains 6 M urea and 100 mM dithiothreitol (DTT). A total of 50 μg of protein is loaded in each run. The sample solution is injected from the anodic side by pressure. Focusing is performed in an electric field of $500\,V\,cm^{-1}$; the anolyte is 0.4 M acetic acid and the catholyte (sealed in the cathodic cell) is 0.5% NH_4OH. The CIEF capillary is contained within a continuously flowing coolant at 20 °C to remove the heat generated during focusing. The initial current is around 6 μA and after ~6 min of focusing it drops to a steady-state value around 2 μA. Since the maximum sample volume that can be loaded onto the CIEF capillary is limited (only 4.3 μL in this experiment), the sample concentration is relatively

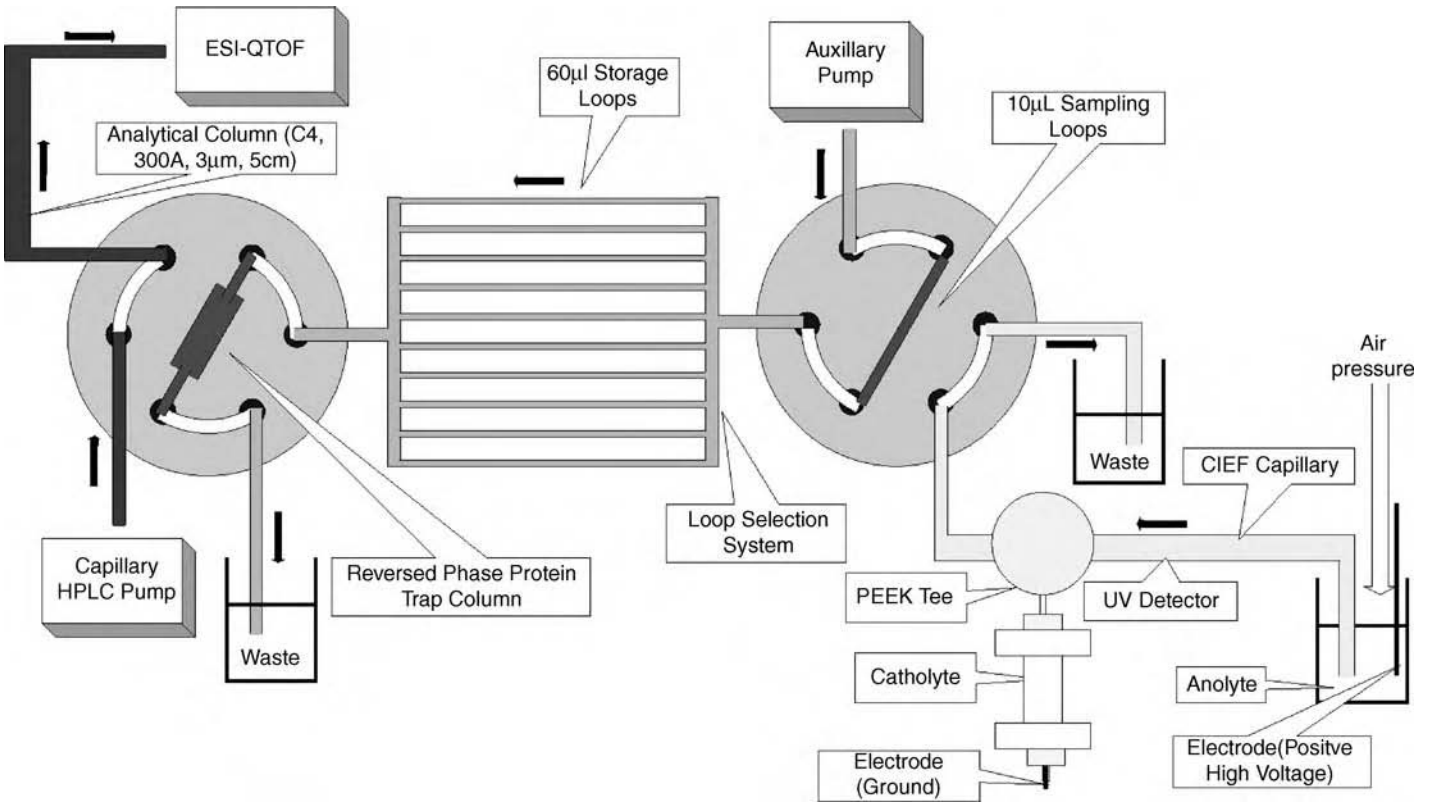

Figure 5.6 Schematic diagram of the second-generation CIEF-RPLC-MS system. Reproduced from F. Zhou and M. Johnston, *Electrophoresis*, **28**, 1383–1388 (2005), with permission from Wiley-VCH

high (11 $\mu g\,\mu L^{-1}$) in order to increase the chance of detecting low abundance proteins. The current vs time profile is smooth and spikes in the UV trace are rarely observed, indicating that protein precipitation is not significant. The focused bands are then hydrodynamically pushed past the cathodic cell into a 10-μL stainless-steel sampling loop attached to a microselection valve. The valve position is then switched, and the proteins in the sampling loop are transferred to a storage loop constructed with 20 cm of PEEK tubing (1/16 in o.d.; 360 μm i.d.; Alltech) attached to a 10-port loop selection system. During the sampling and storage steps, other proteins are held in the CIEF capillary under voltage to maintain focusing. The microselection valve position cycles back and forth to sequentially load 10 fractions into the 10 storage loops.

Each stored sample is then loaded on a reversed-phase protein trap column attached to a second six-port valve located on the capillary LC system. Proteins and peptides in the stored sample are captured on the trap column and washed with a solution of water–acetonitrile–fomic acid (94.9:5.0:0.1) at a flow-rate of 20 $\mu L\,min^{-1}$ for 3 min. Most of the ampholyte, urea and DTT are removed during this washing step. The protein fraction is then eluted from the trap column with mobile phase and further separated on a C_4 reversed-phase column (5 cm × 300 μm i.d.). Mobile phase A [water–acetonitrile–fomic acid (94.9:5.0:0.1)] and mobile phase B [acetonitrile–water–acetic acid (94.9:5.0:0.1)] are delivered at a flow-rate of 10 $\mu L\,min^{-1}$ using a two-step gradient of 40 min (phase B from 20 to 60%) and 2 min (phase B from 60 to 90%). The sample eluted from the RPLC column is sent directly into the ESI-QTOF mass spectrometer.

Figure 5.7 shows total ion chromatograms of the 10 p*I* fractions. The p*I* ranges shown are based on a linear gradient assumed in the CIEF capillary. The protein bands can become wider in the low pH range and it is possible for some proteins with higher p*I* to diffuse into a lower p*I* fraction. Each chromatogram in Figure 5.7 results from the co-elution of many proteins/peptides. This aspect is illustrated in Figure 5.8, where mass spectra from three different time segments in the chromatogram of fraction 8 (p*I* 5.1–4.4) are shown. Each mass spectrum displayed in Figure 5.8 is the combination of 25 successive scans from the time period shown the chromatogram. The mass spectra are clearly different and indicate the presence of numerous proteins/peptides. The insets of the mass spectra in Figure 5.8 show expanded regions of individual peaks. These particular peaks exhibit isotopically resolved ions that allow the precise monoisotopic masses (i.e. masses for molecules containing exclusively ^{12}C, ^{1}H, ^{14}N, ^{16}O and ^{32}S) to be determined directly from the raw mass spectra. However, many peaks in the mass spectra are not isotopically resolved as the molecular mass is above the resolving power of the mass spectrometer. Therefore, the deconvolution software MaxEnt1, supplied by the mass spectrometer vendor (Waters), is used to determine the molecular masses that give rise to the various *m/z* values observed in the mass spectrum. MaxEnt1 is based on a maximum entropy algorithm that assumes that if a molecular mass is observed in charge state z, it also should be observed to some extent in charge states $z + 1$ and $z - 1$, $z + 2$ and $z - 2$, etc.[45–47] The main parameters input by the user are the expected range of molecular masses (3000–30 000 Da for this work) and peak width at half-height (i.e. mass spectrometer resolving power; 0.15 Da for the QTOF spectrometer used in this work). The output is an isotopically averaged mass spectrum which contains only singly charged peaks ($z = 1$) of the molecular masses observed.

The raw and deconvoluted mass spectra from scans 2000–2025 of the eighth protein fraction are shown in Figure 5.9a and b, as an example of proteins that are not isotopically resolved. The most intense feature of the deconvoluted spectrum in Figure 5.9b, 16 293 Da,

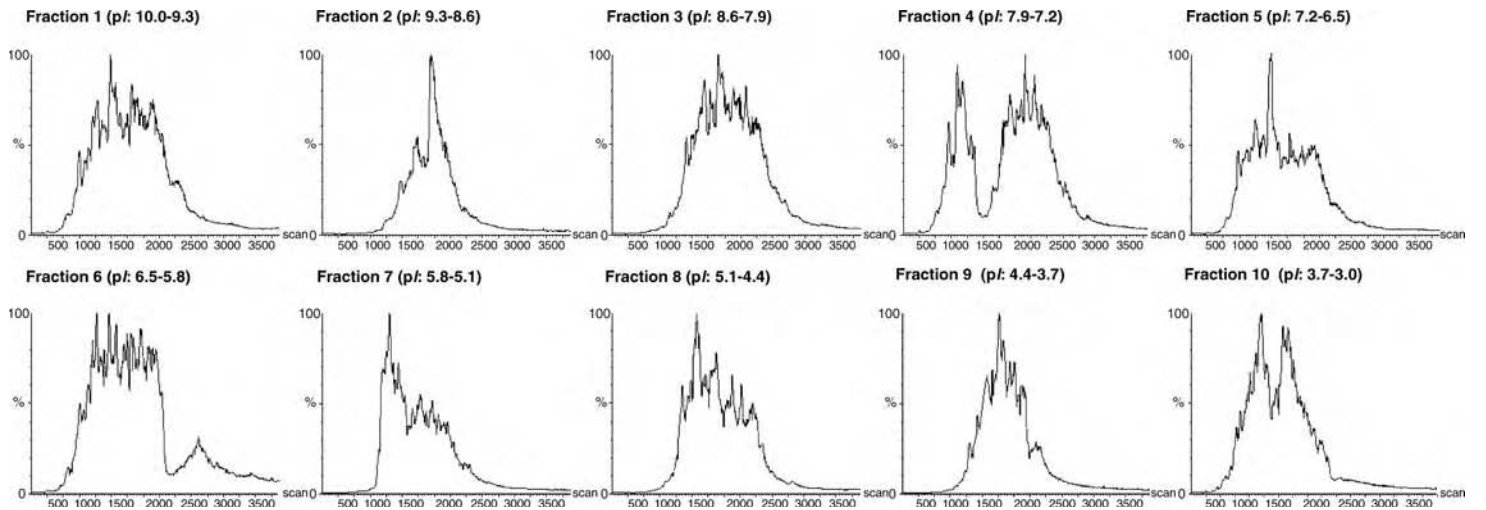

Figure 5.7 Total ion chromatograms of 10 p*I* fractions of soluble yeast proteins. Reproduced from F. Zhou and M. Johnston, *Electrophoresis*, **28**, 1383–1388 (2005), with permission from Wiley-VCH

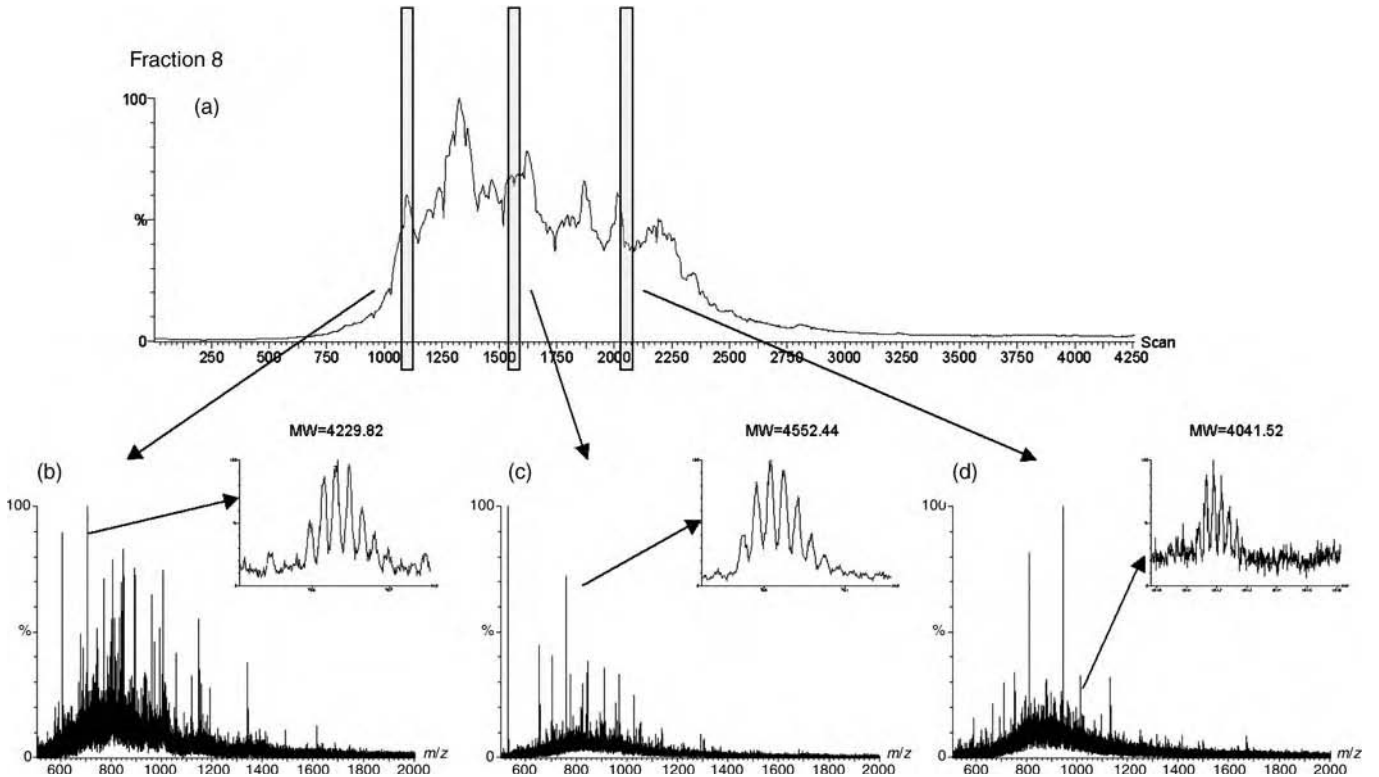

Figure 5.8 (a) Total ion chromatogram of the eighth fraction. Mass spectra corresponding to three segments of the chromatogram are shown in (b), (c) and (d). (b) Mass spectrum from scans 1150–1175. The +6 peak of a protein (monoisotopic mass = 4229.82) is shown in the inset. (c) Mass spectrum from scans 1510–1535. The +6 peak of a protein (monoisotopic mass = 4552.44) is shown in the inset. (d) Mass spectrum from scans 2050–2075. The +4 peak of a protein (monoisotopic mass = 4041.52) is shown in the inset. Reproduced from F. Zhou and M. Johnston, *Electrophoresis*, **28**, 1383–1388 (2005), with permission from Wiley-VCH

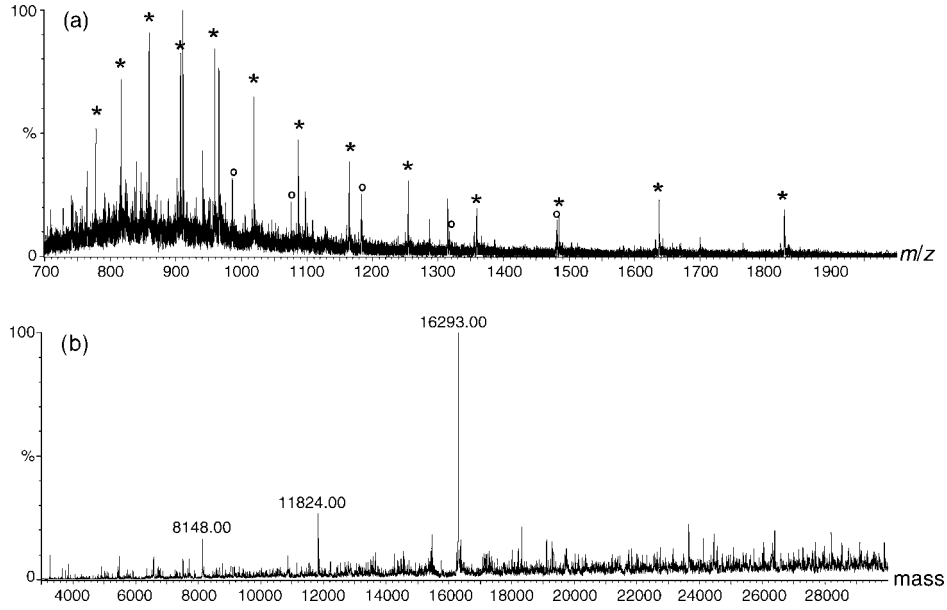

Figure 5.9 (a) Raw mass spectrum from scans 2000–2025 of the eighth fraction. Stars indicate the charge state distribution of 16 293 Da. Circles indicate the charge distribution of 11 824 Da. (b) The deconvoluted mass spectrum of (a) obtained by MaxEnt1. Reproduced from F. Zhou and M. Johnston, *Electrophoresis*, **28**, 1383–1388 (2005), with permission from Wiley-VCH

corresponds to the +9 to +21 charge state distribution indicated by stars in the raw spectrum in Figure 5.9a. The second most intense feature of the deconvoluted spectrum, 11 824 Da, corresponds to the +8 to +14 charge state distribution indicated by circles in the raw spectrum. Other features in the deconvoluted spectrum are not as easily matched with extended charge state distributions in the raw spectrum and may or may not represent real proteins in the sample. The baseline in Figure 5.9b increases with increasing mass, and it is possible that the increasing baseline and the greater number of low-intensity peaks in the higher mass range of the deconvoluted spectrum simply reflect the elevated baseline and background peaks (from artifacts associated with MaxEnt1 processing and also signal from residual additives, cone-induced fragmentation of protein molecular ions, etc.) in the low *m/z* range of Figure 5.9a. Also, the prominent peak at 8148 Da in the deconvoluted spectrum is most likely an artifact of the 16 293 Da peak – the MaxEnt1 algorithm is known to produce artifact peaks at half and twice the mass of the main peak,[10] in this case 32 586 Da (beyond the range of deconvolution) and 8148 Da.

The large number of proteins and peptides observed in Figures 5.8 and 5.9 is also characteristic of other spectra in this fraction and of most spectra in the remaining nine fractions. In all, there are over 1600 spectra in the dataset, each representing the average of 25 individual scans within the chromatogram of a single fraction. In the present work, manual interpretation (similar to the preceding paragraphs) was required for each combination of raw and processed spectra as automation software was not available. To shorten the analysis time, spectra corresponding to low total ion current (defined as less than 20% of the

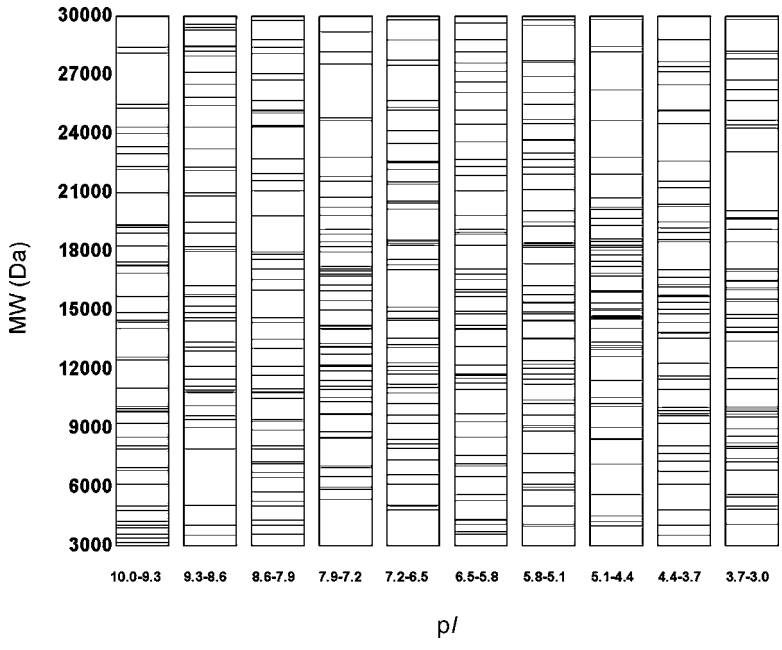

Figure 5.10 p*I* vs MW from CIEF-RPLC-MS data. The 50 most intense proteins/peptides as determined by MaxEnt1 are shown for each fraction. Reproduced from F. Zhou and M. Johnston, *Electrophoresis*, **28**, 1383–1388 (2005), with permission from Wiley-VCH

highest signal in the chromatogram) were excluded, leaving about 600 spectra for detailed interpretation. To simplify the analysis further, deconvolution was performed only over a mass range of 3000–30 000 Da. This mass range was appropriate for the present work as 2D-PAGE analysis of the yeast enzyme concentrate showed very few proteins larger than 30 kDa. Furthermore, the background and spurious peaks illustrated in Figure 5.9b increased substantially above 30 000 Da.

Figure 5.10 summarizes the results of this analysis in part by displaying the 50 most intense peaks derived by MaxEnt1 from each fraction, 500 protein 'bands' in total, as a plot of p*I* vs MW. This figure shows only the p*I* vs MW values, not the relative abundances, because the relationship between peak intensity in the raw mass spectrum and relative height in the MaxEnt1 output is still under investigation. The MW values displayed in Figure 5.10 were selected in the following way. First, the five most intense peaks in each spectrum were tabulated, taking care to exclude artifact peaks at half or twice the mass of intense peaks. Using Figure 5.9b as an example, the peaks at 16 293 and 11 824 Da were included among the five most intense peaks but that at 8148 Da was excluded. Next, the combined list of five peaks from each spectrum in a single fraction (on the order of 300 in total) was reduced to the 50 most intense peaks while removing redundant values (i.e. the same protein in adjacent spectra). Again using Figure 5.9b as an example, 16 293 Da was included in the list of 50 peaks, but 11 824 Da was not. This procedure eliminated the spurious peaks from Figure 5.10, but it also excluded many proteins. For this reason, the number of 'bands' shown in Figure 5.10 does not reflect the ultimate capability of the technique, nor does it

reflect the fact that more proteins are present in some p*I* fractions than others. Several proteins are observed in adjacent p*I* fractions, presumably because the p*I* is at the interface between the two or because several isoforms exist with similar molecular masses.

5.5 FUTURE IMPROVEMENTS

Two specific experimental improvements are on the horizon. First, a two-loop set-up will replace the existing one-loop set-up for the collection of p*I* fractions. In the new set-up, one loop will collect the fraction from the CIEF capillary while the contents of the second loop are transferred to the storage loop. In this way, CIEF can run in a continuous mode with improved reproducibility of p*I* measurement over the stop and go mode. Second, a monolithic column will replace the standard packed column in RPLC. Since monolithic columns typically achieve efficiencies of 25 000, chromatographic resolution can be increased while significantly shortening the analysis time.[48,49]

Finally, an important need is to identify the separated proteins 'on the fly'. The CIEF-RPLC-MS approach potentially provides a seamless interface to tandem mass spectrometry for protein identification using top-down approaches to proteomics such as ECD (electron-capture dissociation),[50–52] ETD (electron-transfer dissociation)[53,54] and the pseudo-MS^3.[55] In top-down proteomics, individual proteins are identified through a sequence tag derived from molecular ion fragmentation. The coupling of one or more of these top-down techniques with a multidimensional separation that is based in part on p*I* will provide a fully automated alternative to 2D-PAGE that has the ability to increase significantly the throughput of proteomics investigations.

ACKNOWLEDGMENT

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6

Chromatographic Measurement of Transferrin Glycoforms for Detecting Alcohol Abuse and Congenital Disorders of Glycosylation

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6.1 INTRODUCTION

Transferrin is a glycoprotein with an M_r of about 80 000 that is synthesized mainly in the hepatocytes and secreted into the blood. Transferrin is the major iron-transport protein and plays an important physiological role in iron homeostasis and also in the regulation of cellular growth.[1] Transferrin exists in many different forms, which are determined by the glycosylation pattern, genetic polymorphism and the degree of iron saturation.[2] The total transferrin concentration in human serum is normally in the range 2.0–3.5 g L^{-1}. Changes in the transferrin concentration commonly occur in a number of pathological conditions, for example, increased serum levels are seen in iron deficiency anemia whereas malnutrition and infections cause decreased levels.

Qualitative modifications in the glycosylation of this protein occur after prolonged heavy alcohol consumption and also in patients with rare congenital disorders of glycosylation (CDG).[3] Therefore, measurement of transferrin microheterogeneity has been used both as a biomarker for detection and follow-up of alcohol abuse [known as carbohydrate-deficient transferrin (CDT)][4] and for diagnosis of CDG.[5] This chapter will review the microheterogeneity of human transferrin and the methods used for the measurement of transferrin

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glycoforms to detect alcohol abuse (CDT) and CDG, with the primary focus on chromatographic methods.

6.2 TRANSFERRIN MICROHETEROGENEITY

The transferrin molecule consists of three subunits: a single polypeptide chain containing 679 amino acid residues, a maximum of two asparagine-linked oligosaccharide units (*N*-glycans) of complex structure in the C-terminal domain at positions 413 and 611, and two binding sites for iron, one each within the N- and C-terminal domain.[1] Normally, serum transferrin is only partially (~30%) iron-saturated. The *N*-glycans may be biantennary, triantennary or even tetraantennary in structure. The different transferrin isoforms, or glycoforms, traditionally have been named depending on the number of terminal, negatively charged sialic acid residues on the glycans. The major glycoform under normal conditions, accounting for about 75–80% of total serum transferrin,[6,7] contains two disialylated biantennary glycans (i.e. a total of four terminal sialic acid residues) and was accordingly named tetrasialotransferrin (Figure 6.1). Other glycoforms that can be detected in blood from healthy individuals are pentasialo- (~15% of serum transferrin), trisialo- (~5%), hexasialo- (~2%) and disialotransferrin (<2%). The remaining glycoforms, asialo-, monosialo-, heptasialo- and octasialotransferrin, normally occur at <1% or in only trace amounts.[6,7]

Human transferrin displays genetic polymorphism with transferrin C being the most common phenotype in all populations, whereas allelic B (lower isoelectric point, p*I*) and D (higher p*I*) variants, with a different primary structure but a normal set of glycans, occur at lower frequencies.[8]

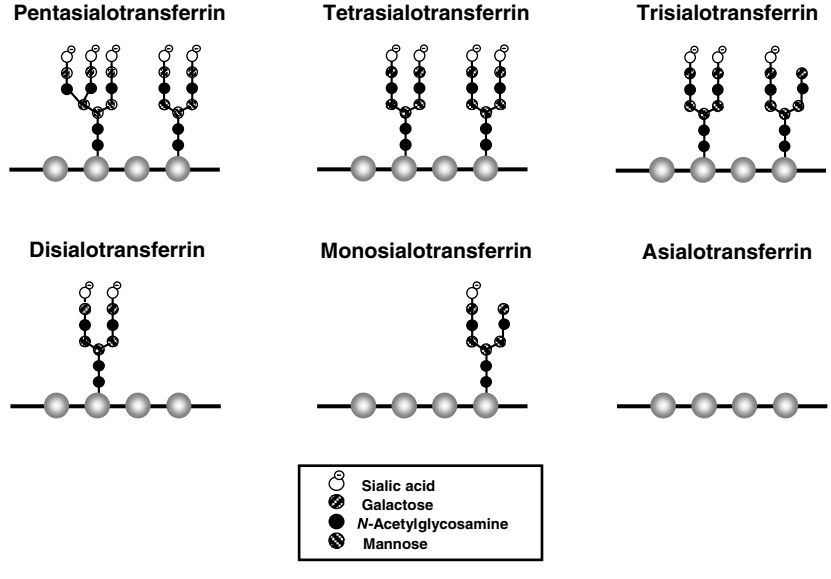

Figure 6.1 Structural illustration of the major normal glycoforms of human serum transferrin and those being influenced by chronic alcohol abuse (carbohydrate-deficient transferrin; CDT) and in congenital disorders of glycosylation (CDG)

6.3 CARBOHYDRATE-DEFICIENT TRANSFERRIN (CDT)

The abnormal glycoform pattern of serum transferrin that occurs as a result of prolonged heavy alcohol consumption, CDT, has emerged as a useful biochemical marker for identifying persons chronically abusing alcohol and for monitoring abstinence from alcohol during treatment.[4,9] Regular high alcohol intake averaging at least 50–80 g of ethanol per day for two or more weeks typically results in an altered transferrin glycoform profile. The biological mechanism by which alcohol causes changes in the carbohydrate composition of the molecule has not yet been conclusively identified, but likely involves interferences with the systems responsible for glycosyl transfer.[4,10,11] When drinking is discontinued, the glycoform pattern of serum transferrin slowly normalizes with a half-life of ~1.5–2 weeks,[4,12] and the time to reach a stable baseline level may require abstinence for 1 month or longer.[13]

The transferrin glycoforms were originally separated, identified and quantified by isoelectric focusing (IEF) based on the differences in net charge of the molecule. The glycoforms with isoelectric points at or above pH 5.7 after complete iron saturation, corresponding to disialo-, monosialo- and asialotransferrin, were collectively named CDT.[4] These glycoforms were first believed to contain truncated glycans deficient only in the terminal saccharide(s), but more recent studies have demonstrated that the major modification observed is instead a loss of one or both of the entire glycans (i.e. disialo- and asialotransferrin, respectively) and, consequently, also of the terminal negatively charged sialic acid residues.[14–18]

The major benefit of CDT compared with other laboratory tests used in routine clinical medicine to indicate chronic alcohol abuse, such as the liver enzyme γ-glutamyltransferase (GGT) and the mean corpuscular volume of erythrocytes (MCV), is its high specificity for alcohol and resulting low rate of false-positive results.[9,19,20] The limitations of CDT as a clinical marker include the lack of standardized procedures for the measurement and definition of CDT. Several different causes for falsely high or low CDT values have been reported over the years, mostly resulting from the use of inaccurate analytical methods, and/or expressing the CDT concentration in absolute (e.g. units or mg L^{-1}) instead of relative (%CDT) terms.[2,21,22] These limitations prompted the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) to convene a working group for the standardization of CDT measurement that addressed such issues as definition of the analyte, establishment of reference methodology and standardized calibration.

6.4 CONGENITAL DISORDERS OF GLYCOSYLATION (CDG)

Congenital disorders of glycosylation (CDG), formerly named carbohydrate-deficient glycoprotein syndrome, are a rare but increasingly recognized group of inherited multisystem disorders caused by mutations in the genes encoding the enzymes involved in the glycosylation of proteins and other glycoconjugates.[5,23,24] Glycosylation is a ubiquitous form of post-translational modification that has considerable impact on numerous biological processes.[25] CDG are divided into two subgroups, CDG-I and CDG-II. CDG-I comprises defects in the assembly and transfer of the dolichol-linked oligosaccharides (*N*-glycans) to the protein, whereas CDG-II refers to defects in the downstream processing of the protein-linked glycans. As a result, glycans are either completely missing (CDG-I) or become structurally abnormal (CDG-II). Cases in which the underlying defect has not been identified are called CDG-x.

The clinical symptoms of CDG are highly variable between individuals, and also within individual subtypes, but typically include psychomotor, growth and mental retardation from early childhood. CDG-Ia is by far the most frequent subtype with roughly 500 patients reported worldwide to date, while <100 have been diagnosed with type Ib-I*L* and only a few with type II (IIa–IIe).[24,26,27] However, it is generally assumed that CDG is largely underdiagnosed, partly due to the limited awareness of these disorders, but also to the variable and non-specific clinical presentation.[28–30] Another reason for underdiagnosis of this disorder may be that methods for CDG testing are not generally available.[31]

The diagnosis of CDG is based on a combination of clinical symptoms and biochemical tests, the latter including the presence of a defective glycosylation pattern of glycoproteins and enzymatic assays.[5,32,33] Confirmation and subtyping of CDG are established by molecular identification of the underlying genetic defects (i.e. detection of specific mutations). If CDG is suspected, initial diagnosis is currently based on the identification of alterations of the *N*-linked glycosylation of serum transferrin. Transferrin is a very convenient marker for this purpose, partly because of its relative abundance in serum, and also because assays for transferrin microheterogeneity are routinely available (i.e. the CDT assays for detection of alcohol misuse).

6.5 ANALYTICAL METHODS FOR TRANSFERRIN MICROHETEROGENEITY

Over the years, many different methods for carbohydrate-modified transferrin have been used for the routine determination of CDT. This has sometimes created confusion, related to discrepancies between methods (e.g. test values are given in various absolute and relative amounts), and also their ability to distinguish between normal and elevated values (i.e. the sensitivity and specificity for heavy alcohol consumption). For example, a very high total transferrin concentration might render falsely high results, when expressing the CDT content as an absolute amount and not in relation to total transferrin.[21,34,35] Moreover, changes in the definition of the analyte (e.g. heterogeneous mixtures of the asialo-, monosialo-, disialo-, and/or trisialotransferrin glycoforms in the 'CDT' fraction)[2,21,36] and the lack of traceability of reference intervals have hampered the clinical implementation and acceptance of this laboratory test.

Separation of transferrin glycoforms is based on variations in the carbohydrate structure and mainly the number of terminal, negatively charged, sialic acid residues, yielding molecules with different net charges. Originally this was achieved by IEF, but several other laboratory techniques have also been used for the investigation and diagnosis of CDT and CDG, including high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), mass spectrometry (MS), western blotting, lectin analysis and immunoassays.[2,7,12,37–43] So far, the most common methods for detection of these analytes have been IEF and immunoassays. A limitation of IEF is that there is not a linear relationship between the concentration and staining intensity of tetrasialotransferrin, the major band as determined by densitometric analysis. This limitation makes accurate relative quantification of the different glycoforms impossible. Many immunoassays for CDT (CDTect, %CDT TIA and %CDT) are limited because they require an initial minicolumn separation of the CDT fraction from the non-CDT glycoforms prior to quantification, and genetic transferrin variants may cause falsely high or low values.

6.6 CHROMATOGRAPHIC METHODS FOR CDT

HPLC measurement of CDT is routinely used in hospital and research laboratories as a way to detect chronic alcohol misuse. The first HPLC method for the quantification of iron-saturated transferrin glycoforms was introduced in 1993,[12] and was based on anion-exchange chromatographic separation of the different glycoforms by salt gradient elution followed by photometric detection. Since then, several variations of the original method have been published[7,44–48] and a number of commercial HPLC assays for CDT have been introduced.[49]

6.6.1 HPLC conditions and potential interferences

A common feature of the HPLC methods for transferrin glycoforms is that quantification relies on the selective absorbance of the iron–transferrin complex at ~460–470 nm.[50] This represents an analytical advantage over CE methods, which use detection at ~214 nm where all proteins absorb. For example, C-Reactive Protein (CRP) has been demonstrated to interfere with the CE measurements by co-eluting with monosialotransferrin in the electropherograms.[51] In contrast, CRP does not interfere with the quantification of transferrin glycoforms by HPLC, even at very high concentrations (>200 mg L^{-1}).[7] Likewise, bilirubin and hemoglobin added in concentrations up to 150 μmol L^{-1} and 0.7 g L^{-1}, respectively, did not cause any significant interference with the quantification of transferrin glycoforms.

Using HPLC, the relative amount of any single transferrin glycoform, or combination of glycoforms, to total transferrin (all glycoforms) can be calculated from the area under the curve (%AUC). In recent studies, baseline or valley-to-baseline integration for all transferrin peaks has replaced the original valley-to-valley integration mode, because the results obtained in this way were found to be less sensitive to variations in peak resolution.[7] Another improvement is the use of ferric nitrilotriacetate (FeNTA), a well-known transferrin iron donor,[52] instead of $FeCl_2$ for complete saturation of serum transferrin. Because the FeNTA complex gives almost instant iron saturation, no additional incubation time is necessary. Preparation of serum without addition of FeNTA results in a very different HPLC profile (Figure 6.2a and b). This occurs because the net charge of the transferrin molecule depends on the level of iron saturation, and the non-saturated (Fe_0) or partially saturated [transferrin saturated at the N-terminal (Fe_{1N}) or C-terminal (Fe_{1C}) domain] glycoforms will thus elute differently from the completely iron-saturated (Fe_2) forms.[2] However, the high reproducibility of test results observed under standard HPLC conditions[7] indicate that the transferrin iron load is complete and stable, with no risk for loss of iron during the chromatographic separation.

Both serum and the pretreated samples are relatively stable upon storage, and also during normal handling in the laboratory. In addition to serum, the HPLC method has also been tested for use with plasma.[7] With lithium heparin and sodium heparin–fluoride plasma, the method allowed the separation and measurement of all transferrin glycoforms similarly to those for serum. However, with EDTA plasma, a high, asymmetric peak was always observed with a similar retention time as asialotransferrin (Figure 6.2c). This peak was also observed with blank samples (i.e. EDTA tube containing water; Figure 6.2c, inset), confirming that it is an artefact peak and not the result of iron removal from transferrin by complexation with EDTA. With citrated plasma, a shoulder can be observed on the tetrasialotransferrin peak (Figure 6.2d), but this was not observed with blank citrate samples.

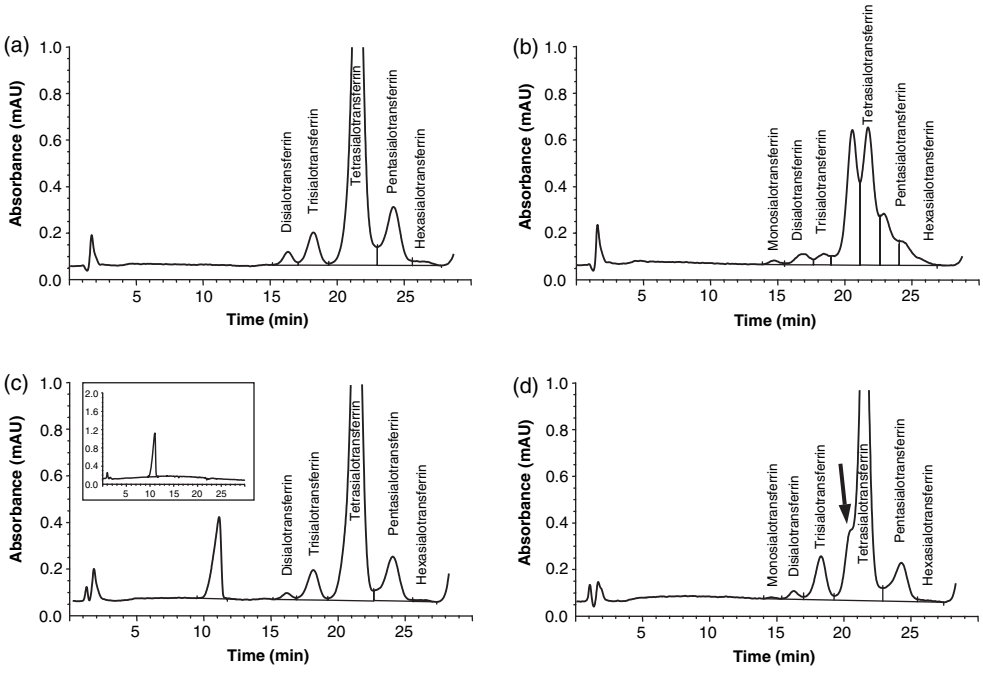

Figure 6.2 Transferrin glycoform patterns for serum and plasma samples with the HPLC method. HPLC traces for (a) a serum sample of the predominant transferrin C homozygote variant from a heavy drinker showing a slightly elevated disialotransferrin (2.1% of total AUC for transferrin), (b) the same serum sample but without treatment with FeNTA (i.e. no complete iron saturation of transferrin), (c) EDTA plasma from a light drinker showing an asymmetric EDTA peak eluting at about 11 min (inset: EDTA blank sample) and (d) citrated plasma from a light drinker showing an additional peak (at arrow) partially co-eluting with tetrasialotransferrin. Reproduced from A. Helander, A. Husa and J.-O. Jeppsson, *Clin. Chem.*, **49**, 1881–1890 (2003), by permission of the American Association of Clinical Chemistry

6.6.2 Chromatographic separation of transferrin glycoforms

The improved HPLC methods provide good separation between the different iron-saturated glycoforms of serum transferrin. A typical HPLC elution profile obtained for a serum sample from a control subject by a candidate HPLC reference method for CDT,[7] using a SOURCE 15Q PE 4.6/100 anion-exchange chromatographic column (Amersham Biosciences), is shown in Figure 6.3a. The peaks representing disialo-, trisialo-, tetrasialo-, pentasialo- and hexasialotransferrin were readily identified from their characteristic positions in the chromatogram. Most importantly, disialo- and trisialotransferrin are almost baseline separated. Because the trisialotransferrin level normally exceeds the disialotransferrin level by ~4-fold,[6,7] partial co-elution of these neighboring peaks may otherwise cause overestimation of disialotransferrin, the major CDT glycoform, and hence increase the risk of false-positive identifications of alcohol abuse.[2] In sera showing a high relative amount of trisialotransferrin (>6%), a peak representing monosialotransferrin is sometimes measurable usually at <0.5% of total transferrin (Figure 6.3c), eluting immediately ahead of disialotransferrin.

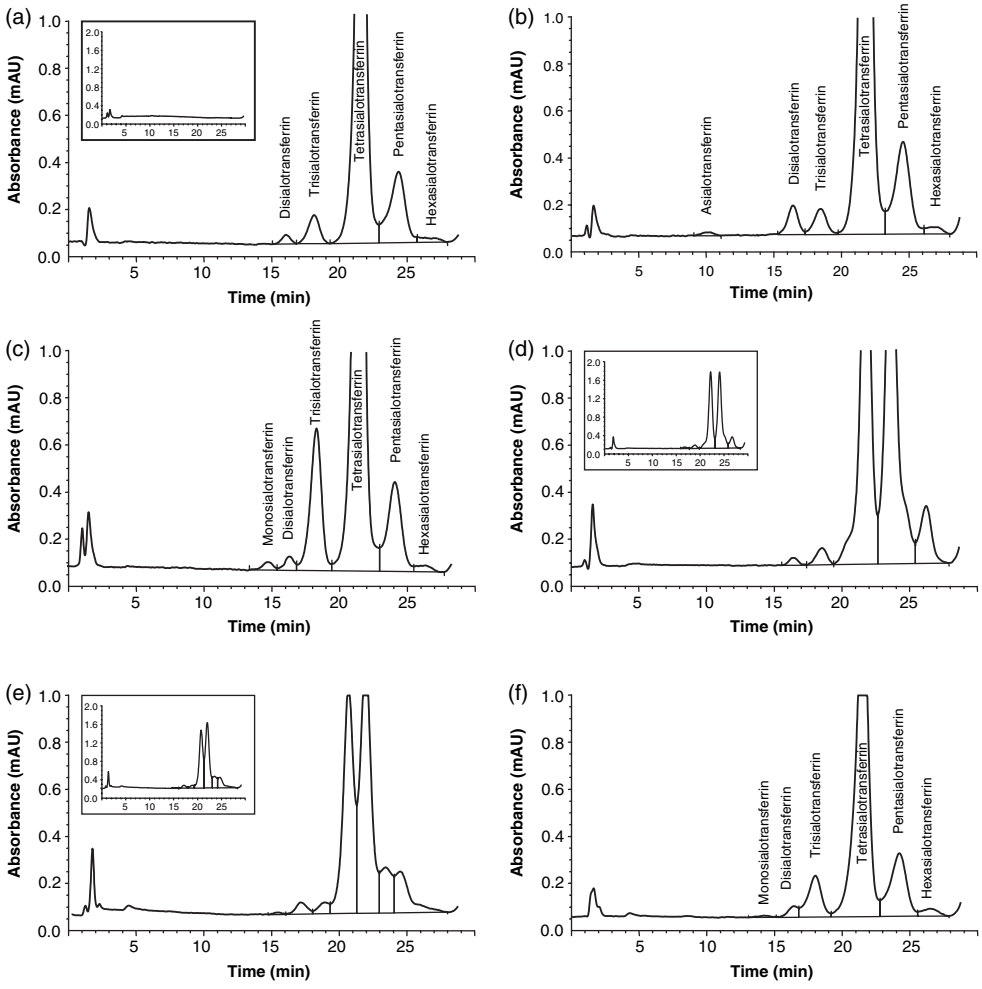

Figure 6.3 Transferrin glycoform patterns for different serum samples with the HPLC method. HPLC traces for (a) a transferrin C serum from a light drinker (inset: the same sample after immunosubtraction of transferrin), (b) serum from a heavy drinker with elevated disialotransferrin (3.2%) and a detectable (~0.4%) asialotransferrin, (c) serum from a person with high trisialotransferrin (8.4%) and a measurable (~0.5%) monosialotransferrin, (d) serum from a transferrin BC heterozygote (inset: the same chromatogram at full scale), (e) serum from a transferrin CD heterozygote (inset: the same chromatogram at full scale) and (f) serum from transferrin variant tentatively identified as a 'C2C3' heterozygote. Reproduced from A. Helander, A. Husa and J.-O. Jeppsson, *Clin. Chem.*, **49**, 1881–1890 (2003), by permission of the American Association of Clinical Chemistry

In healthy individuals, tetrasialotransferrin normally accounts for about 75–80% of serum transferrin, whereas trisialo- and pentasialotransferrin make up ~5 and ~10–15%, respectively, and disialotransferrin ~0.5–2% (mean 1.16%).[7] Patients who admitted drinking alcohol in the previous week (range 15–780 g day^{-1}) typically showed elevated values of disialotransferrin (mean 4.25%). Asialotransferrin, which is not detectable in serum from

social drinkers, was also detected at 0.15–4.38% of total transferrin, in 57% of chronic heavy drinkers and in 62% of the sera with disialotransferrin >2%.[7] A typical HPLC elution profile for a serum sample collected from a chronic alcohol consumer is shown in Figure 6.3b. Small gender differences have been observed for the higher glycoforms but not for disialotransferrin.[7]

When transferrin in serum was adsorbed with rabbit anti-human transferrin antibody, no peaks were detectable in the HPLC trace (Figure 6.3a, inset), thus confirming that all peaks observed in the chromatogram at 460–470 nm are transferrin glycoforms.[7] Using the proposed candidate HPLC reference method,[7] the separation of transferrin glycoforms was achieved within 30 min but, for routine applications, the total run time can be reduced markedly by applying a steeper salt gradient. A recent commercial HPLC assay allows reproducible separation within only about 6 min.[49]

6.6.3 Genetic transferrin variants and glycoform types

HPLC traces of sera for some of the most common genetic transferrin variants, the transferrin BC and CD heterozygotes, and a transferrin C heterozygote tentatively identified as 'C2C3',[22] are shown in Figure 6.3d–f. In addition to these, a number of very rare variants, including the transferrin B homozygote, have been identified (unpublished work).

6.6.4 Sensitivity and reproducibility

The limit of detection (LOD) of the candidate reference HPLC method for CDT[7] in %AUC for asialo- and monosialotransferrin was approximately 0.05% of total serum transferrin in the normal concentration range (~2.0–3.5 g L^{-1}). For routine use, a limit of quantification (LOQ) of ~0.1% is applied. It should be noted that the LOD and LOQ depend in part on the transferrin concentration in the serum sample. Furthermore, as all glycoforms except tetrasialotransferrin occur at low concentrations,[6] and the absorbance of the transferrin–iron complex at 460–470 nm is only ~10% compared with its absorbance at 280 nm,[12] the performance of the photometric detector is very important. However, in dilution studies, using a serum sample containing a very high transferrin concentration and water as diluent (tested range 5–0.5 g L^{-1}), the AUC for total transferrin was linear and the calculated relative amount of disialotransferrin was not markedly changed at the lower total transferrin concentrations (CV 6.0%).[7] The intra- and inter-assay imprecision for serum samples containing normal and elevated disialotransferrin levels (range 1–5.6%) were below 5% (CV) and the retention times for the transferrin glycoforms were also stable over time.

6.7 CHROMATOGRAPHIC METHODS FOR CDG

IEF has long been the most common and reference procedure for CDG, but methods based on CE,[53] HPLC[31] and LC–MS[39] have also been introduced. The CDG-I pattern is characterized by an increase in transferrin glycoforms missing one or both of the entire *N*-glycans (i.e. disialo- and asialotransferrin, respectively),[39,54] whereas CDG-II is associated with an increase in trisialo- and monosialotransferrin, indicating the presence of truncated

glycans.[55–57] A limitation of the HPLC methods has been that they require large sample volumes (0.1–1.0 mL) compared with IEF (<10 μL) and were therefore less suitable to screen for CDG in children, where the amount of sample available for determination is often limited. However, an improved method allowed for reproducible detection and relative quantification of the different transferrin glycoforms in approximately 10 μL of serum or plasma.[31]

6.7.1 HPLC testing for CDG

Using the improved HPLC method,[31] sera from CDG-I patients showed increased relative amounts of disialo- and asialotransferrin and concomitant reductions in tetrasialotransferrin (Figure 6.4). Patients with the CDG-Ia subtype showed the highest amounts of disialo- (range, 19–42%) and asialotransferrin (3.4–26%), whereas the patients with type Ib and Ig had less asialotransferrin (<3%). After the CDG-Ib patient had been treated with low-dose mannose,[58] the abnormal transferrin pattern improved, as indicated by marked reductions in disialo- (from 18 to 6%) and asialotransferrin (from 2.6 to 0.7%) (Figure 6.4e).

Sera from patients with undefined CDG-II defects showed the type II pattern with typical increases in trisialo- (range, 7.1–32%) and monosialotransferrin (5.7–15%) (Figure 6.5), indicating the presence of truncated glycans. By comparison, trisialotransferrin levels were normally <8% in controls and heavy drinkers, and monosialotransferrin was measurable only in individuals with a high trisialotransferrin level.[22] In all four CDG-II sera, two unidentified peaks were also observed (labeled A and B in Figure 6.5), and these peaks most likely represent other truncated variants.[31] This was supported by the observation that they showed similar retention times to two of the peaks that appeared after control serum was treated with neuraminidase (Figure 6.5d), which removes the terminal sialic acids of the *N*-glycans.[16,51] These observations suggest that the HPLC method allows for the separation of transferrin glycoforms not only based on the net charge of the molecule (i.e. sialic acid content), but also on structural differences of the glycans.

The CDG-I pattern resembles that observed after chronic alcohol consumption, albeit the relative increases were much higher in CDG-Ia (Figure 6.4). However, because CDG testing is typically performed at young age, there is usually no risk of false-positive results due to alcohol misuse. Furthermore, the CDG patterns are clearly distinguishable from those observed with the most common genetic transferrin variants,[22] which may cause falsely high or low values with the minicolumn immunoassays for CDT.

6.7.2 LC-MS testing for CDG

In addition to HPLC with photometric detection, an automated immunoaffinity LC method with electrospray MS detection of the transferrin glycoforms has been published.[39] This method focuses on identifying the glycoforms lacking one or both of the *N*-glycans (called 'mono-oligosaccharide' and 'a-oligosaccharide',[39] respectively, but corresponding to disialo- and asialotransferrin) and calculating relative ratios between the different forms (mono-/di-oligo and a-/di-oligo; i.e. disialo-/tetrasialotransferrin and asialo-/tetrasialotransferrin). Because increases in asialo- and disialotransferrin are characteristic for the type I pattern (Figure 6.4), this method should primarily be useful for the diagnosis of patients with CDG-I, which is the most common variant of the disease.

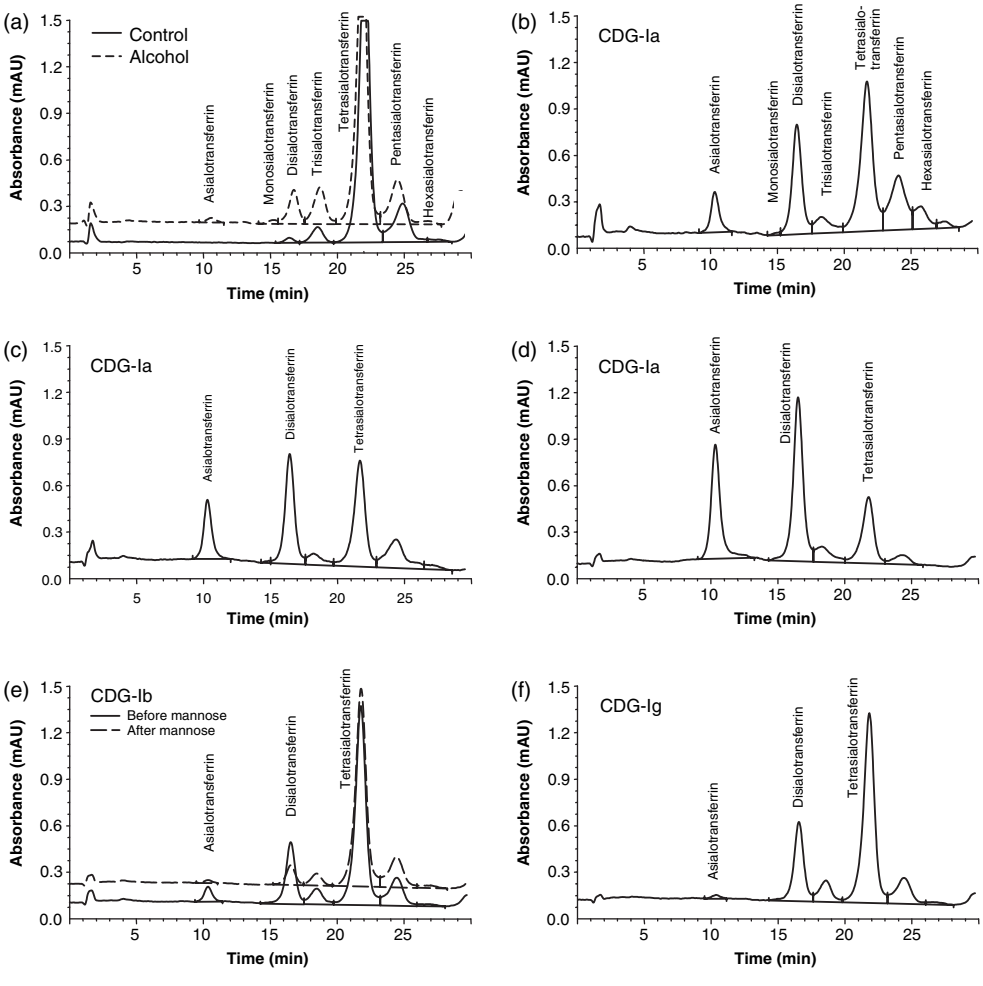

Figure 6.4 Transferrin glycoform patterns for serum samples from CDG-I patients by HPLC. HPLC traces for (a) a control serum sample from a light drinker (solid line) and from a heavy drinker with elevated asialo- and disialotransferrin and a measurable monosialotransferrin (broken line), (b–d) serum samples from three CDG-Ia patients showing elevated asialo- and disialotransferrin and reduced tetrasialotransferrin, (e) a serum sample from a CDG-Ib patient showing elevated asialo- and disialotransferrin (solid line) and another sample from the same patient after mannose therapy (broken line) and (f) a serum sample from a CDG-Ig patient showing elevated asialo- and disialotransferrin. Parts (a), (d), (e) and (f) reproduced from A. Helander, J. Bergström and H.H. Freeze, *Clin. Chem.*, **50**, 954–958 (2004), by permission of the American Association of Clinical Chemistry

6.8 SUMMARY AND CONCLUSIONS

Improved HPLC methods for the separation and quantification of transferrin glycoforms, based on anion-exchange chromatographic separation followed by selective photometric detection at 460–470 nm or MS detection, are useful for the detection and monitoring of

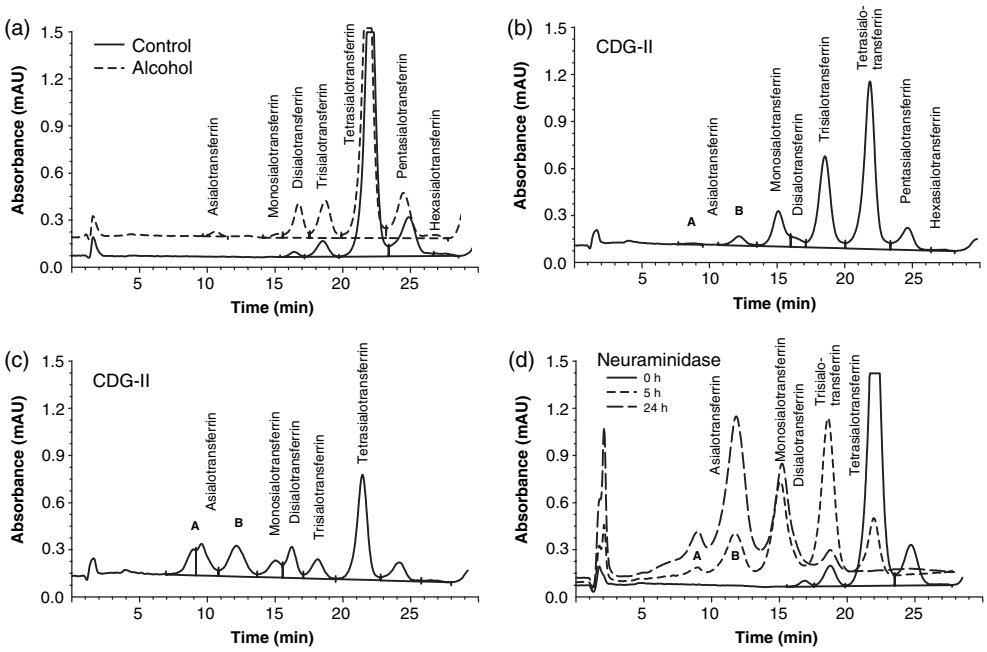

Figure 6.5 Transferrin glycoform patterns for serum samples from CDG-II patients by HPLC. HPLC traces for (a) a control serum sample from a light drinker (solid line) and from a heavy drinker with elevated asialo- and disialotransferrin and a measurable monosialotransferrin (broken line), (b, c) serum samples from two CDG-II patients with uncharacterized defects showing elevated mono- and trisialotransferrin and a reduced tetrasialotransferrin, and also two unknown peaks (A and B) and (e) a control serum before, and at different times after, treatment with neuraminidase. Parts (a), (b) and (d) reproduced from A. Helander, J. Bergström and H.H. Freeze, *Clin. Chem.*, **50**, 954–958 (2004), by permission of the American Association of Clinical Chemistry

alcohol abuse. These methods can also be used for preliminary diagnosis of CDG and for distinguishing between CDG types I and II. For CDT standardization, HPLC has been proposed as a possible reference method since it allows for reproducible and visible detection of the different glycoforms, and genetic variants and glycoform types that may cause incorrect determination of CDT with some assays are readily identified from a unique chromatographic pattern. In this respect, HPLC should be applicable to any combination of transferrin glycoforms, making it a useful diagnostic tool for clinical applications to CDT and CDG.

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7

Chromatographic Measurements of Catecholamines and Metanephrines

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7.1 BACKGROUND

Pheochromocytoma is a tumor of neuroectodermal origin that arises from the chromaffin cells (pheochromocytes) of the sympathoadrenal system. The term pheochromocytoma is derived from the chemical and pathological characteristics of a cell; 'pheochromocyte' means a cell that takes on a 'dusky' color when exposed to chromium salts (Greek: *phaios and chroma* mean dusky and color, respectively), and 'cytoma' means tumor. Almost 90% of these tumors are found in one or both adrenal glands, but they may be located anywhere along the sympathetic chain and, rarely, in aberrant sites. The 'rule of 10' has been used to describe pheochromocytoma: 10% are extraadrenal and, of those, 10% are extra-abdominal; 10% are malignant; 10% are found in patients who do not have hypertension and, finally, 10% are hereditary.[1] Those functioning tumors arising outside the adrenal medulla are termed extraadrenal pheochromocytomas, whereas those non-secreting extra-adrenal tumors are called paragangliomas.[2]

Many patients do not spontaneously report certain symptoms and signs that are particularly valuable clues to the physician in suggesting the diagnosis of pheochromocytoma. It is therefore important to obtain a detailed history from any patients suspected of having this tumor. However, routine screening of pheochromocytoma in the workup of every hypertensive is not recommended because this tumor is involved in only 0.1% of cases of hypertension. Testing should be reserved for those patients with features suggestive of the tumor, or those with incidentally discovered adrenal masses. The diagnosis of pheochromocytoma is established by the demonstration of elevated 24-h urinary excretion of free

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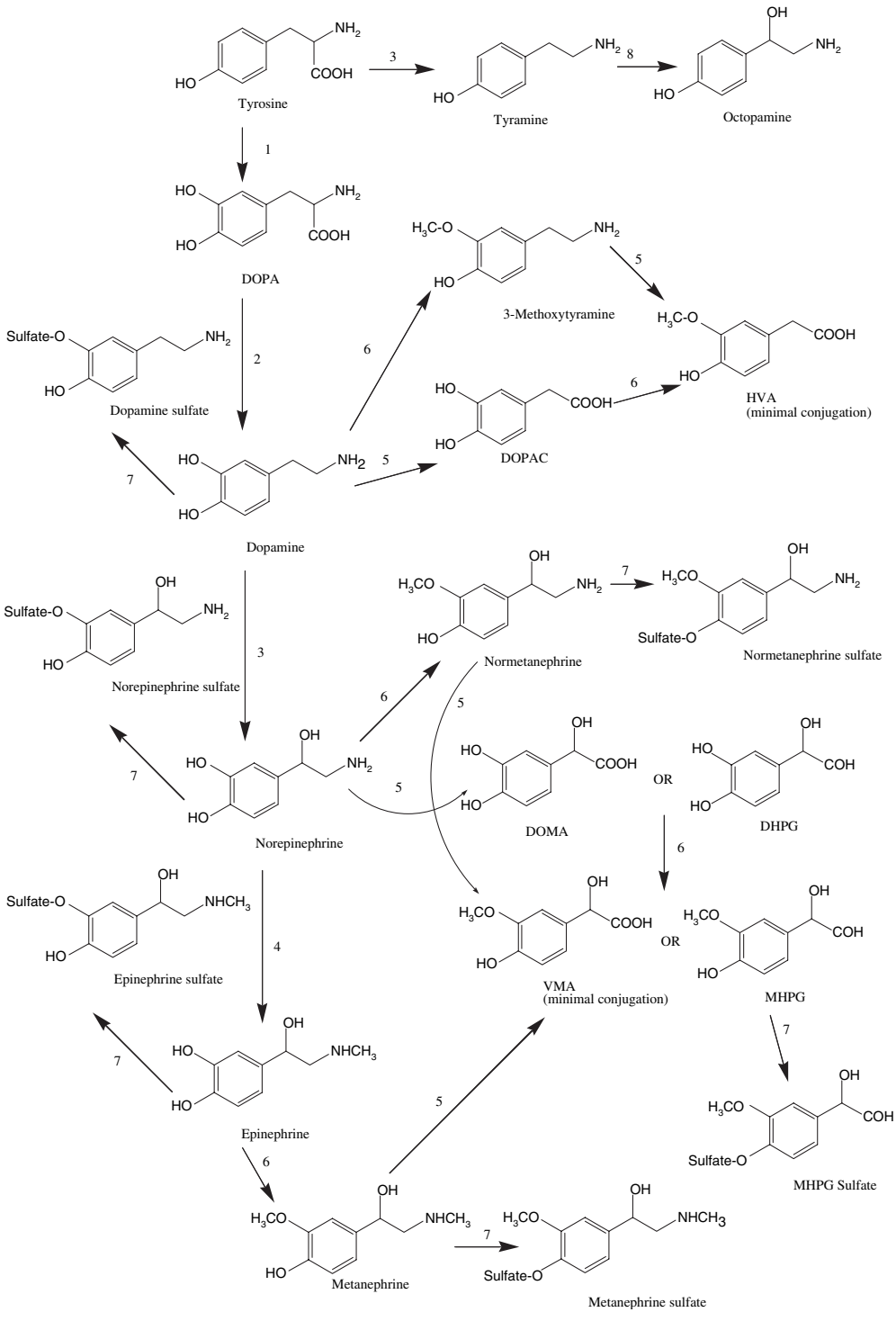
catecholamines and total metanephrines. Surgical resection of the tumor is the standard treatment.[3] If the primary tumor is localized to the adrenal gland and is benign, then survival is the same as that of a normal age-matched population.[2] In patients with unresectable, recurrent or metastatic disease, long-term survival is possible; however, the overall 5-year survival is less than 50%. Pharmacological treatment of the catecholamine excess is essential and surgery, radiation therapy or chemotherapy may provide palliative benefit.

The catecholamines are biosynthesized from the amino acid L-tyrosine as outlined in Figure 7.1. The known biochemical events leading to catecholamine metabolism and urinary excretion are also summarized in Figure 7.1. There is evidence that functioning pheochromocytomas have the various enzymes necessary for the conversion of tyrosine to catecholamines, and the level of activity of some of these enzymes (tyrosine hydroxylase, Dopa decarboxylase and dopamine β-hydroxylase) is higher than in normal adrenal tissue.[4]

In order to explain the enormous capacity of some pheochromocytomas to synthesize catecholamines in the presence of high tyrosine concentrations in many of these tumors, it has been postulated that feedback inhibition of tyrosine hydroxylase may be lacking or inefficient.[5] Most pheochromocytomas secrete a combination of norepinephrine (NE) and epinephrine (E); however, NE is usually liberated in a considerably larger concentration than E. Of diagnostic importance is the fact that pheochromocytomas that secrete at least some E are located in the adrenal gland.[6] The enzyme phenylethanolamine-*N*-methyltransferase (PNMT) was only detected in tumors when E was one of the catecholamines being secreted; non-functioning pheochromocytomas did not contain any catecholamine-synthesizing enzymes.[4] Eisenhofer *et al*.[7] reported the presence of catechol-*O*-methyltransferase (COMT), the enzyme responsible for conversion of catecholamines to metanephrines in pheochromocytomas. These results indicated that the elevated plasma levels of free metanephrines in pheochromocytoma patients are derived from catecholamines that are produced and metabolized within the tumors. Only rarely do pheochromocytomas secrete dopamine (DA), Dopa and serotonin (5-HT). It has been suggested that the elaboration of Dopa can occur only in malignant pheochromocytoma;[8] however, benign pheochromocytomas have also been reported to secrete both catecholamines and Dopa.[9] John *et al*. evaluated 86 patients with pheochromocytoma, and reported that the preoperative 24-h urinary DA was in the normal range for benign pheochromocytomas but increased in malignant pheochromocytomas.[10] They concluded that high preoperative 24-h urinary DA levels, among some other factors, might be used to predict the malignant potential of pheochromocytoma. It is noteworthy that, although increased urinary excretion of DA and its metabolite 4- hydroxy-3-methoxyphenylacetic acid [homovanillic acid (HVA)] rarely occurs in patients with pheochromocytoma, a rise in excretion of these substances occurs frequently in patients with other neural crest tumors (e.g. neuroblastoma and melanoma).

Figure 7.1 Pathways of synthesis and metabolism of catecholamines with enzymes catalyzing various reactions. (1) Tyrosine hydroxylase; (2) aromatic amino acid decarboxylase; (3) phenylamine-β-hydroxylase; (4) phenylethanolamine-*N*-methyltransferase; (5) monoamine oxidase plus aldehyde dehydrogenase; (6) catechol-*O*-methyltransferase; (7) conjugating enzymes: about 95% phenolsulfotransferase and 5% phenolglucuronatetransferase (in human). DOPA, dihydroxyphenylalanine; DOMA, dihydroxymandelic acid; DHPG, dihydroxyphenylglycol; DOPAC, dihydroxyphenylacetic acid; HVA, homovanillic acid; MHPG, methoxyhydroxylphenylglycol; VMA, vanilmandelic acid



The determination of urinary metanephrines [normetanephrine (NM) and metanephrine (MN)] is the best urine test available for screening patients suspected of having pheochromocytoma. This view seems to be shared by most investigators who have had the most experience in studying such patients. A study by Rosano *et al*., assessing the diagnostic accuracy of identifying patients with pheochromocytoma by determining 24-h urinary catecholamines [using high-performance liquid chromatography (HPLC)], total metanephrines and VMA, revealed that measurement of total metanephrines was the best discriminator between essential hypertension and pheochromocytoma.[11] These results were in agreement with those of Samaan *et al*., who found urinary metanephrines to be the most sensitive (97% positive) test for pheochromocytoma, whereas increased 24-h urinary catecholamines and VMA were detected in 76 and 88% of these patients, respectively.[12] Lucon *et al*. reviewed case histories of 50 patients in Brazil with pheochromocytoma and reported that urinary metanephrines were the best indicators with a diagnostic sensitivity of 97%.[13] Levels of NE in the urine and blood and urinary VMA showed sensitivities of 93, 92 and 90%, respectively. The lowest sensitivity was noted in plasma (67%) and urinary (64%) E and plasma DA (63%). In the Singapore General Hospital, the urinary free catecholamine and catecholamine metabolite levels of four pheochromocytoma patients were compared with the corresponding levels from 12 non-pheochromocytoma patients.[14] It was found that although the urinary VMA test was sensitive (100%), its specificity was only 31%, whereas the urinary total metanephrine test was both sensitive (100%) and specific (100%). As mentioned earlier, increased 24-h urinary DA level is one of the factors that indicates the likelihood of malignant pheochromocytoma.[10] Hence, patients with this elevated urinary DA should have more frequent follow-up evaluations to identify malignancy at earlier stages.

7.1.1 Total or individual assays

It has been suggested that evaluating M and NM excretions separately could increase the diagnostic sensitivity of these tests, because in some cases pheochromocytoma can secrete predominantly E, whereas in others the predominant catecholamine is NE, DA or Dopa.[15] In a retrospective study of 372 patients with sustained hypertension, for whom the determination of 24-h urinary metanephrines or VMA was ordered, Kairisto *et al*. found that evaluating NM excretion has better diagnostic specificity at 100% diagnostic sensitivity than did the sum of NM and M excretions. They also found that the determination of VMA in addition to NM and MN did not yield additional diagnostic information for any of the patients.[16] Similar findings were reported by Gerlo and Sevens after reviewing the measurement of catecholamines and their metabolites in 19 patients with pheochromocytoma.[17] They commented that assays of total catecholamines and total metanephrines are diagnostically inadequate, and that NE/E and NM/MN should be measured separately. Specific assays for E or MN are essential for identifying pure E-secreting tumors. Combined assays diminish the impact of E or MN and may cause false negatives. The specific measurement of E or MN is also valuable in the diagnosis of multiple endocrine neoplasia type 2 (MEN-II)-associated pheochromocytoma. It is also important to note that hypertension may not be manifested in patients harboring predominantly E-secreting tumors.[18] Therefore, measuring NM and MN with a specific procedure is the single most reliable diagnostic test for all patients suspected of having a pheochromocytoma, for patients with

suggestive symptoms and for asymptomatic patients in whom a 'clinically silent' unsuspected adrenal mass has been incidentally detected. The assay of urinary VMA does not yield additional diagnostic information to urinary metanephrines and may fail to detect predominantly E-secreting tumors.[13,17,19]

This brief review of the literature pertaining to the diagnosis of pheochromocytoma indicates that 24-h urinary measurements of individual NE, E, NM and MN might be most useful for the diagnosis of pheochromocytoma in patients with suggestive symptoms such as headache, perspiration and palpitations. Urinary DA level could also be measured if malignancy is suspected. The traditional assay of urinary VMA may not be necessary if metanephrines are quantitated. There had also been much discussion with regard to the use of plasma metanephrines for the diagnosis of pheochromocytoma.[7,20–23] These studies reported that tests for plasma metanephrines are more sensitive than tests for plasma catecholamines or urinary metanephrines for the diagnosis of this tumor, particularly in paroxystic forms between crises and in patients with suspected MEN-II. Eisenhofer *et al*. demonstrated that measurement of plasma free metanephrines not only provided information about the likely presence or absence of a pheochromocytoma, but also, when a tumor was present, helped predict tumor size and location.[24]

7.2 ANALYTICAL MEASUREMENTS OF CATECHOLAMINES AND METANEPHRINES

Owing to the immense importance of catecholamines and metanephrines for the diagnosis and follow-up of pathologies such as pheochromocyotma, researchers have endeavored to improve the analytical methods used for their measurements, and substantial progress has been made. Nowadays, many of the old techniques of measuring catecholamines and metanephrines are obsolete, as new methods have greatly improved the analytical sensitivity, accuracy and clinical suitability for measuring these biogenic amines in biological fluids and tissues. To appreciate the developments of these analytical techniques, some of the earlier and current methods developed for the measurement of urinary catecholamines and metanephrines will be reviewed.

7.3 EARLY METHODS

7.3.1 Catecholamines

Traditional methods for the quantitation of catecholamines [norepinephrine (NE) and epinephrine (E)] relied on the production of detectable fluorophores. In the early fluorometric methods, NE and E were oxidized and rearranged under alkaline conditions to fluorescent trihydroxyindole products called adrenolutins.[25–27] Inclusion of ascorbic acid, along with rapid measurement of fluorescence, was required because the adrenolutins are easily oxidized. Drugs such as α-methyldopa, isoproterenol, quinidine, propranolol, labetalol and tricyclic depressants, which also show fluorescent properties, may interfere with this test. Modifying the original methods to include iodine as the oxidizing agent allows for the additional measurement of DA.[28] Although they are still used in some clinical laboratories, fluorescent methods have been mostly replaced by chromatographic methods.

7.3.2 Metanephrines

As with the catecholamines, fluorescence methods have also been reported for urinary metanephrine analysis. Fluorescent derivatization of the metanephrines NM and MN by chemical oxidation was based on modification of the trihydroxyindole reaction used for catecholamines. The individual metanephrines were measured following chromatographic separation and fluorescent derivatization[29,30] or through the formation of differential fluorescent compounds by oxidation at different pH levels.[31–34] Since the stability of the fluorescent products was variable, with some products decomposing within 10 min,[29] this method has limited application in current practice. Other early methods for analysis of NM and MN included electrophoresis[35,36] and paper[37,38] and thin-layer chromatography.[39] These assays were technically complex and had poor analytical sensitivity.

Measurement of total urinary metanephrines using spectrophotometric methods has also been reported.[40,41] After sample hydrolysis and isolation of metanephrines by ion-exchange chromatography, the hydroxyl- and amino-containing side-chains were oxidatively cleaved by periodate. Oxidation of both NM and MN resulted in the formation of a common end product, vanillin, which was measured spectrophotometrically at 360 nm. This method was, however, susceptible to interferences from other compounds such as methylglucamine[42] and the 4-hydroxylated metabolite of propranolol.[43] Furthermore, the method did not differentiate between NM and MN, and results were only semi-quantitative for grossly increased concentrations of total metanephrines.[44]

Radioenzymatic methods had also been used to quantify urinary NM.[45,46] Phenylethanoleamine-*N*-methyltransferase and [^{3}H]*S*-adenosylmethionine convert NM to its [^{3}H]*N*-methylated derivative, [^{3}H]MN. The main advantages of this method were its high sensitivity and freedom from interference by common antihypertensive drugs such as propranolol, guanethidine, reserpine, hydrochlorothiazide, labetalol and methyldopa. Since measurements of both NM and MN are imperative for the diagnosis of pheochromocytoma, this radioenzymatic assay for NM is not commonly used for that purpose.

7.4 CURRENT CHROMATOGRAPHIC METHODS

The use of high-performance liquid chromatography (HPLC) as an analytical tool for the life sciences has become increasingly important and the techniques and instrumentation involved are being developed to higher levels of sophistication. Significant improvements in the specificity of the measurements of catecholamines and metanephrines have resulted from the coupling of HPLC separation with fluorescence, electrochemical, chemiluminescence or mass spectromectric detection.

7.4.1 Chemistry of catecholamines

The catecholamines all contain a 3,4-dihydroxyphenyl group (catechol unit) as common structure element, but differ with respect to the 1-substituent (Figure 7.1). The enzyme phenylamine-β-hydroxylase or dopamine-β-hydroxylase introduces the hydroxyl group stereospecifically and converts the achiral DA into chiral NE in the *R* stereoisomeric

configuration. The same configuration is present in E, as the enzyme phenylethanolamine-*N*-methyltransferase is only involved in the transformation of the primary amino group of NE into a secondary amine by methylation.[47] Based on their chemical structures, it is evident that the catecholamines and metanephrines possess some common physical and chemical properties. Their highly polar nature and amphoteric character due to the presence of amino and phenolic groups limit the use of conventional extraction procedures for isolation purposes. Another complicating factor is the ease with which all catechol-type compounds are oxidized in air to form quinones and secondary products, especially in alkaline media. These properties have to be considered in all work-up and handling procedures, which may include the use of antioxidants as protecting agents and avoidance of high pH or exposure to light. The solution chemistry of the catecholamines is largely dominated by the nucleophilic amino group that serves as an excellent target for various derivatization reagents. As will be discussed later, however, some important derivatives used in fluorescence detection techniques require a primary amino group and therefore cannot be applied to E and MN. The ease of oxidation of the catechol ring is another property that has also been utilized for analytical purposes. The technique of direct electrooxidation at an anodic surface (electrochemical detection) is well established for the analysis of these compounds.

7.4.2 Specimen preparation

Only a few methods for urinary catecholamines have been published that do not require pre-extraction prior to analysis.[48,49] These methods minimized sample preparation by making use of different precolumn derivatization procedures. The selection of a suitable method for sample preparation prior to analysis by HPLC depends on a number of factors, such as the biological source, the type of column used and the selectivity of the detection method. In cases where the analyte concentration is very low and the analyte is present in a complex matrix (urine or plasma) with interfering compounds, an exhaustive pretreatment may be unavoidable. Sample pretreatment is also essential to ensure the sensitivity and specificity of the assay and protect the analytical column from contamination.

Liquid- or solid-phase extraction methods have been adopted for the isolation of catecholamines and their metabolites from urine samples. The liquid extraction system is ordinarily based on the formation of a complex, in alkaline medium, between diphenylborate and the diol group in the catecholamines. However, the liquid extraction methods reported in the literature are relatively tedious and often involved multiple extraction steps.[50–52] For the more widely used solid-phase extraction methods, catecholamines may be selectively isolated from the urine sample by adsorption with activated alumina,[53,54] phenylboronic acid[55,56] or cation-exchange resins.[57–65] All the specimen preparative procedures are specific for the free catecholamines, i.e. the extracted catecholamines do not include the conjugated fraction.

The procedure for extraction of catecholamines with activated alumina was developed by Anton and Sayre,[66] and has subsequently been used in a number of studies. Alumina extraction has not been popular, although automated purification with alumina micro-columns was studied closely by Tsuchiya *et al.*[67] The sample preparation scheme includes increasing the pH of the alumina to >8.5 and vigorous shaking of the sample with the alumina, resulting in adsorption of the catecholamines by attraction of the hydroxyl groups of the catechol nucleus. The alumina can then be washed with water or buffer, and finally the catecholamines are eluted with acid, such as 0.3 M acetic acid.[53] Since catecholamines are

unstable at high pH, the sample should not stay in the Tris–EDTA (pH 8.5) buffer for long periods. Tsuchiya *et al*. also pointed out that attention must be paid to catecholamine instability in alkaline solutions, especially when using an alumina procedure.[67] The alumina extraction scheme has also been applied to in-line sample pretreatment using small alumina precolumns. Using this method, the recovery of E, NE and DA was found to be approximately 100%, and for 3,4-dihydroxybenzylamine (DHBA, internal standard) the recovery was 76%.[54] Despite the strong affinity of alumina for catecholamines (NE, E and DA), the adsorption or retention of the metanephrines (NM and MN) on alumina was found to be negligible.[54] Therefore, solid-phase extraction with alumina could not be applied for the simultaneous extraction of catecholamines and metanephrines from urine. This limitation, and the fact that alumina needs chemical activation (which can be difficult to reproduce), may be reasons why alumina extraction has not been widely used.

In 1977, an affinity gel with boric acid covalently bound to a resin matrix was developed for the adsorption of catecholamines via the formation of a boric acid complex with the vicinal hydroxyl groups at increased pH.[68] The adsorbed compounds can be eluted by decreasing the pH. Determination of catecholamines in urine and plasma by in-line sample pretreatment using an internal surface boronic acid gel has been reported.[56] Phenylboronic acid columns are commercially available, with specified adsorbing properties, simplifying the specimen clean-up procedure and improving column-to-column reproducibility, The catecholamines were selectively adsorbed on the material and subsequently separated (in-line or off-line) on a reversed-phase or cation-exchange column. As with alumina extraction, a drawback of the boric acid gel is that it cannot selectively retain metanephrines since they lack the vicinal hydroxyl groups.

Cation-exchange resins have also been used to extract catecholamines from urine. One type of resin that is commonly adopted is Bio-Rex 70. Acidic metabolites were not retained by Bio-Rex 70 cation-exchange resin and therefore did not interfere with the assay. Similar results were obtained for the alcoholic metabolites and free amino acids. Under the conditions used in one study, both catecholamines and metanephrines were retained by the resin. However, only those compounds that contained a vicinal hydroxyl group (NE, E and DA) could be eluted with boric acid.[58] It was subsequently reported that formic acid at high concentration could be used to elute both catecholamines and metanephrines adsorbed on Bio-Rex 70 cation-exchange resin, thereby allowing the simultaneous extraction of catecholamines and metanephrines for HPLC analysis.[59] Using a solid-phase extraction (SPE) copolymer sorbent of *N*-divinylpyrrolidone and divinylbenzene, Vuorensola *et al*.[69] reported the extraction of dopamine and metanephrines from urine, and Sabbioni *et al*.[70] extracted 4-hydroxy-3-methoxyphenylethylene glycol and catecholamines from plasma. However, the simultaneous extraction of both catecholamines and metanephrines was not demonstrated in these studies. In the development of methods to extract both catecholamines and metanephrines, the cation-exchange cartridges described by Chan and co-workers provide a helpful starting point.[49,71–73] They reported several studies that evaluated measurements of catecholamines and metanephrines in urine samples using simultaneous extraction with a weak cation-exchange resin.

In general, minimal sample handling offers many advantages and if the HPLC-conditions are well chosen for a particular analytical problem, very crude samples can often be analyzed without off-line manipulation. Assays involving direct injection of the specimen may eliminate the necessity for an internal standard if recoveries are high and reproducible. In some cases of catecholamine analysis, the compounds may also be injected directly for

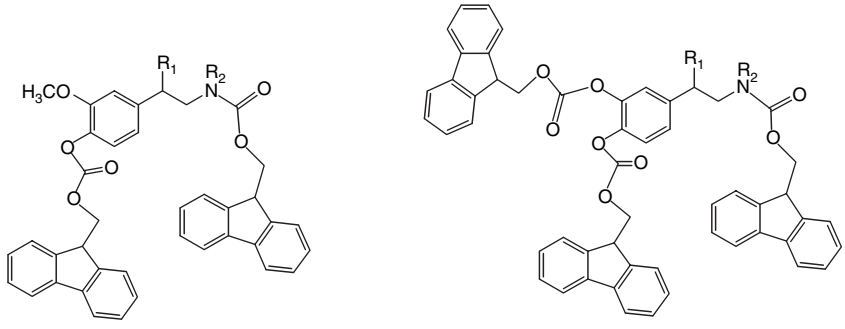
HPLC analysis without prior sample clean-up.[74–76] Gamache *et al.*[74] utilized the specificity of the coulometric array detection method for the analysis of urinary NM and MN without prior extraction. The urine sample was simply acid hydrolyzed, diluted and filtered prior to isocratic separation on a reversed-phase ion-pair chromatographic column. The assay was specific for the metanephrines with negligible interference from anti-hypertensive drugs. Yamaguchi *et al.*[75] derivatized NE, E, serotonin and 5-hydroxyindoleamines with benzylamine in the presence of potassium hexacyanoferrate(III) under mild conditions. The resulting fluorescent derivatives were then injected directly for HPLC with fluorescence detection. Panholzer *et al.*[76] utilized a column-switching HPLC system for the simultaneous analysis of E, NE, DA, serotonin, MN, NM, DOPAC, HVA and 5-hydroxyindoleacetic acid (5-HIAA) in human urine. The sample was injected directly on to a C_{18}-alkyl-diol silica precolumn that extracted the analytes from the urine matrix. The analytes were then eluted from the precolumn on to the analytical column by the use of column-switching techniques and were separated by ion-pair reversed-phase HPLC. The analytes were then oxidized to the corresponding quinones and converted into fluorescent derivatives by reaction with *meso*-1,2-diphenylethylenediamine.

7.4.3 Fluorescence detection

The catecholamines have native fluorescence, therefore it is possible to detect these biogenic amines by measuring the fluorescence of the effluent from an HPLC separation. HPLC with detection of native fluorescence provides sufficient sensitivity (5 $\mu g\, L^{-1}$) after catecholamine or metanephrine isolation from urine by cation-exchange chromatography[77–79] or concentration with alumina.[80] Many of the drug interferences in the traditional fluorescence procedures are eliminated by the selectivity of the chromatographic separation, but some compounds such as the α-methyldopa metabolite α-methylnorepinephrine may elute close to NM and MN, depending on the chromatographic conditions.[77] This technique has not been widely used in bioanalytical work.[47] Derivatization may also be used to enhance further the sensitivity and specificity. Postcolumn derivatization methods include ethylenediamine condensation,[81] trihydroxyindole (THI),[53,82] glycylglycine[83] and 2-cyanoacetamide.[84] The THI method is the most sensitive for HPLC measurements of NE and E but is insensitive with regard to DA and requires rather complicated instrumentation for the postcolumn derivatization reaction.[82]

Precolumn derivatization methods include 1,2-diphenylethylenediamine treatment,[51,85] dansylation of E, NE and DA,[86] derivatization of NE and DA by *o*-phthalaldehyde and mercaptoethanol[87] and derivatization of catecholamines with 9-fluorenylmethyloxycarbonyl chloride (FMOC-Cl).[88] Derivatization with *o*-phthalaldehyde increases the sensitivity of NE and DA, but E is not measured because only primary amines are derivatized. Co-analysis of catecholamines, metanephrines and other related compounds by combined electrochemical oxidation and fluorescence derivatization had also been reported.[60,89] This approach involves sequential chromatographic separation, coulometric oxidation and final chemical derivatization with 1,2-diphenylethylenediamine to fluorescent products.

Chan *et al.*[49] developed an assay for the simultaneous determination of catecholamines and metanephrines using FMOC derivatization. The assay is convenient for the simultaneous analysis of NM, MN, E and DA in human urine sample without prior extraction procedures. In this study, urine was directly derivatized and subjected to a simple extraction step with



(a) (b)

Derivative	Structure	R_1	R_2	Exact mass
FMOC-NM	a	OH	H	627.68
FMOC-MN	a	OH	CH_3	641.24
FMOC-NE	b	OH	H	835.28
FMOC-E	b	OH	CH_3	849.29
FMOC-DA	b	H	H	819.28

Figure 7.2 Chemical structures and molecular masses of the FMOC derivatives of catecholamines and metanephrines

chloroform prior to injection. With this assay, the requirement for time-consuming solid-phase extraction of these compounds in urine samples is therefore circumvented. The potential errors involved during extraction and recovery would also be reduced. After derivatization, the samples were found to be stable at ambient temperature for at least 3 days. The enhanced stability of these FMOC derivatives is imparted by the reaction of the unstable phenol or catechol functional groups with FMOC-Cl (Figure 7.2). The latter reaction was confirmed in this study using atmospheric pressure chemical ionization mass spectrometry (APCI-MS). However, this assay does not measure urinary NE accurately due to poor resolution.

In summary, these derivatization methods coupled with fluorescence detection have not gained widespread use in clinical laboratories. One possible reason for their limited use may be the preference of clinical laboratory analysts towards the use of the electrochemical detection, since it is highly selective and sensitive for these catecholic compounds and does not require pre- or postcolumn derivatization.

7.4.4 Electrochemical detection

Electrochemical detection is based on the principle that electroactive compounds are oxidized or reduced at a certain potential and the resulting flow of electrons creates a

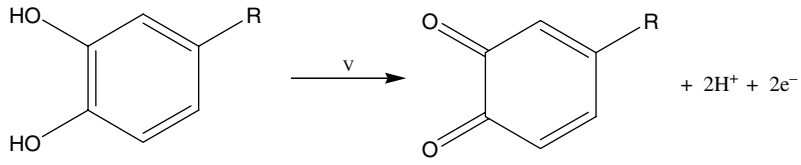


Figure 7.3 Principle of electrochemical oxidation of catecholamines

measurable current. Catecholamines and metanephrines are easily oxidized to form quinones, as illustrated in Figure 7.3, and therefore are suitable for electrochemical detection. Because these biogenic amines are more easily oxidized than many other compounds, it is possible to obtain a certain degree of selectivity with electrochemical detection if the oxidizing potential is kept as low as possible. Two types of electrochemical detectors exist. The coulometric detector oxidizes 100% of the catecholamine molecules in the effluent and therefore creates a larger current than the amperometric detector, which oxidizes less than 10% of these molecules. It has been reported that the higher yield of the coulometric detector does not seem to increase its sensitivity compared with the amperometric detector because the background noise level increases in proportion to the increase in signal, so the two detectors have a similar signal-to-noise ratio.[90] Nonetheless, the wide use of the coulometric detector for the trace analysis of free catecholamines and/or metanephrines in urine reflects its high sensitivity in practical applications.[62,74,91,92] This high sensitivity and selectivity of coulometric detection may be a result of the oxidation of the analytes in the guard cell followed by reduction at successively higher reduction potentials in the analytical cell.[93] The response of the electrochemical detector is influenced by ionic strength and pH of the mobile phase, which must be taken into account when optimizing the HPLC assay. For all their simplicity, though, electrochemical detectors require considerable attention to troubleshooting and minimization of disturbances in order to obtain optimal sensitivity.[94]

Electrochemical detection with ion-pairing adaptations of reversed-phase chromatography are the most common methodologies, and many techniques for measuring urinary catecholamines and metanephrines have been published.[57,59,65,71,72,74,95–108]

Ion-pairing with alkyl sulfonates or sulfates enhances the retention of cationic amine moieties on the lipophilic stationary phase. The effects of mobile phase composition (ion-pairing agents, ionic strength or organic components) on the chromatographic and electrochemical behavior of catecholamines and their metabolites has been reviewed previously.[93.109]

The coupling of ion-exchange chromatography with electrochemical detection has been applied to the measurement of urinary catecholamines.[62,110–113] Early applications of ion-exchange chromatography were hampered by the lack of column reproducibility, resulting in the loss of catecholamines during the analytical process.[110] Mefford reported that semi-irreversible loading of C_{18} columns with lauroylsarcosine resulted in separations that are dependent on ion-exchange mechanisms.[112] Subsequently, *N*-methyloleoyl taurate was applied for the ion-exchange chromatographic separation of catecholamines. This latter resin facilitated the elution of E prior to NE and, therefore, allowed enhanced sensitivity for the normally lower concentration of E.[112] This enhanced sensitivity may be of particular value in detecting small tumors that preferentially secrete E. Sarzanini *et al*. reported an ion

chromatographic procedure for the simultaneous purification and determination of urinary NE, E and DA.[62]

7.4.5 Chemiluminescence detection

Chemiluminescence occurs when an energy-releasing reaction produces a molecule in an electronically excited state and that molecule, as it returns to the ground state, releases its energy as a photon of light. The chemiluminescent process is distinctly different from the photoluminescent processes such as fluorescence and phosphorescence. The latter processes occur after the excited state of a molecule is produced from its ground state by the absorption of light energy. Thus fluorescent species emit light only while being irradiated. Phosphorescent species may appear to emit light without being irradiated, but this emitted energy had to have been absorbed at an earlier time. Chemiluminescent reactions, however, produce light without any prior absorption of radiant energy. As such, chemiluminescence requires no excitation source (as does fluorescence and phosphorescence) but only a single light detector such as a photomultiplier tube. Furthermore, a monochromator or filter is often not needed. Most chemiluminescence methods involve only a few chemical components to generate light. Luminol chemiluminescence,[114] which has been extensively investigated, and peroxyoxalate chemiluminescence[115] are both used in bioanalytical methods and have been adapted to the analysis of catecholamines and their metabolites.

Bis(2,4,6-trichlorophenyl) oxalate (TCPO) is an example of the peroxyoxalate reaction sequence. It involves the fuel (TCPO) plus the oxidant (H_2O_2) reacting to produce an intermediate, which in this example is shown as a dioxetane (Figure 7.4) although the reaction probably produces many intermediates, and others, such as hydroperoxyoxalate,

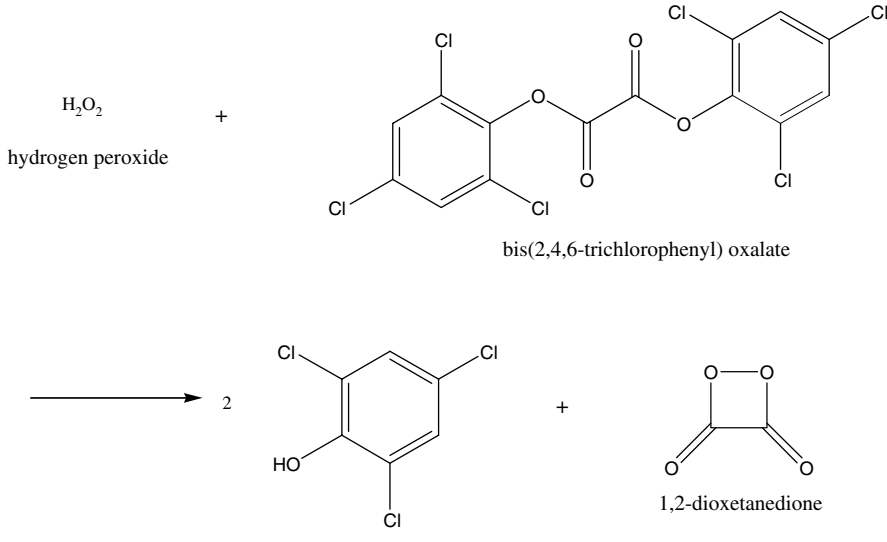


Figure 7.4 Reaction between hydrogen peroxide and TCPO

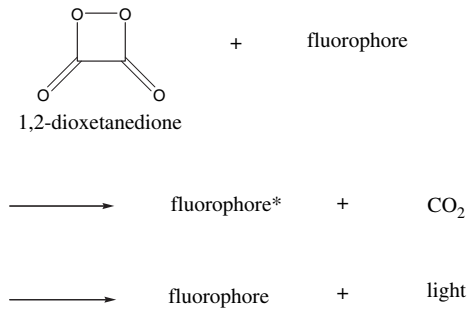


Figure 7.5 Excitation of a fluorophore by intermediate produced by the reaction between hydrogen peroxide and TCPO

have been proposed.[116] The intermediate, 1,2-dioxetanedione, excites a fluorophore, 9,10-diphenylanthracene (DPA). The process of transferring the energy of the initial reaction, the chemical reaction of hydrogen peroxide with TCPO, to light emission from the excited-state fluorophore (fluorophore*) facilitates the analytical determination of these compounds (derivatized as flurophores) (Figure 7.5).

Chemiluminescence detection following HPLC separation of derivatized catecholamines (NE, E and DA) and isoproterenol using the peroxyoxalate reaction has been reported.[117] The amines and isoproterenol, derivatized with 1,2-diarylethylenediamines, were separated on a reversed-phase HPLC column with isocratic elution with imidazole buffer (pH 5.8, 120 mM)–methanol–acetonitrile (6:2:9, v/v/v). The eluate was detected by a post-column chemiluminescence reaction using bis[4-nitro-2-(3,6,9-trioxadecyloxycarbonyl)phenyl] oxalate and hydrogen peroxide. The chromatographic detection limits for catecholamines were approximately 40–120 amol for an injection volume of 100 μL (signal-to-noise ratio of 3). A highly specific and sensitive automated HPLC–peroxyoxalate chemiluminescence method for the simultaneous determination of catecholamines (NE, E and DA) and their 3-*O*-methyl metabolites (NM, MN and 3-methoxytyramine) has also been reported.[118] Automated precolumn ion-exchange extraction of diluted plasma is coupled with HPLC separation of these compounds on an octyldecylsilica (ODS) column, postcolumn coulometric oxidation, fluorescence derivatization with ethylenediamine and finally peroxyoxalate_chemiluminescence reaction detection. Although these methods may be applied for the quantitation of catecholamines and metanephrines, the complexity associated with the multiple steps such as the pre- or postcolumn derivatization of catecholamines and the postcolumn chemiluminescent reaction needs to be considered. All these steps have to be manipulated and controlled prudently to ensure the accurate measurement of these analytes.

Luminol is also widely used as a chemiluminescent reagent, but unlike the peroxyoxalate systems, it does not require an organic/mixed solvent system. The chemiluminescent emitter is a 'direct descendent' of the oxidation of luminol (or an isomer such as isoluminol) by an oxidant in alkaline solution. Probably the most useful oxidant is hydrogen peroxide, which is also used in peroxyoxalate chemiluminescence. If the fuel is luminol, the emitting species is 3-aminophthalate (Figure 7.6).

The use of luminol as a chemiluminescent reagent for the detection of catecholamines has been reported.[119–121] In a typical scheme (Figure 7.7), imidazole was used to catalyze the

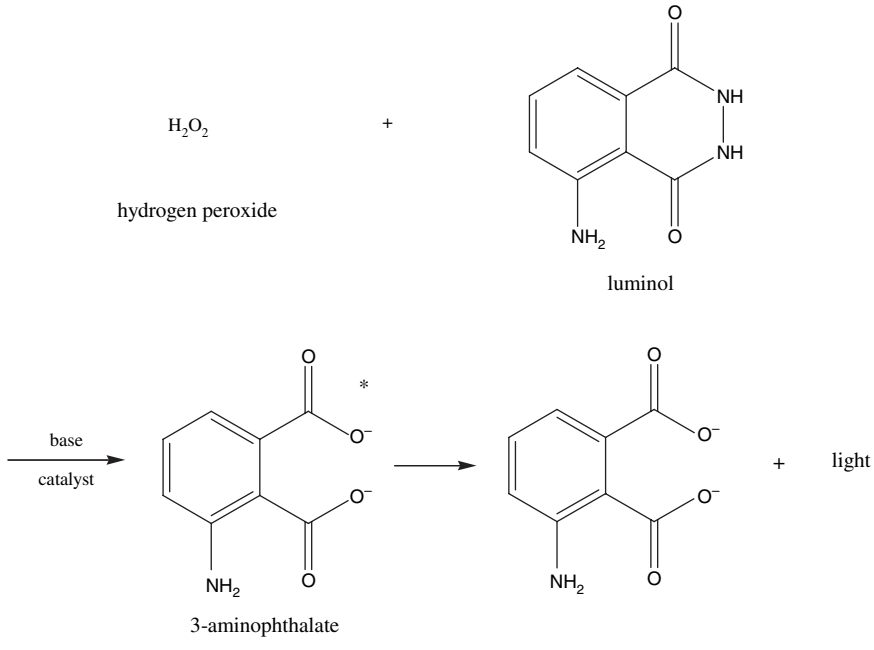


Figure 7.6 Reaction between hydrogen peroxide and luminol in the presence of peroxidase producing the excited 3-aminophthalate and the emitted light at a wavelength of 430 nm

decomposition of catecholamines to generate hydrogen peroxide, then the hydrogen peroxide was detected by luminol chemiluminescence with horseradish peroxidase as the catalyst.[119,120] However, as these assays were not coupled to HPLC separation, only total free catecholamines were measured. Israel and Tomasi[121] developed a chemiluminescent procedure for measuring catecholamines based on the observation that lactoperoxidase catalyzes both the oxidation of catecholamines and the chemiluminescent reaction of luminol with their oxidation product (hydrogen peroxide). The assay had been adapted for continuously monitoring the release of catecholamines from adrenergic tissues, from cell suspensions and from cells loaded in culture with dopamine. Nonetheless, this assay measured total catecholamines and could not be applied for measuring individual NE, E and DA.

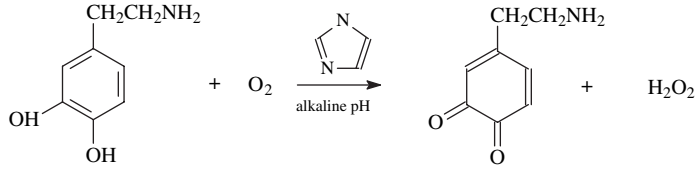


Figure 7.7 The phenolic hydroxyl group of catecholamine reacts with oxygen in aqueous solution. The reaction was catalyzed by imidazole to produce hydrogen peroxide at alkaline pH

7.4.6 Mass spectrometry

Tandem mass spectrometry is becoming an important analytical technology in the clinical laboratory environment. Applications already exist for tandem mass spectrometry in toxicology and therapeutic drug monitoring and many new applications are being developed. The combination of tandem mass spectrometry with sample introduction techniques employing atmospheric pressure ionization has enabled this technology to be readily implemented in the clinical laboratory. Although mass spectrometry has been traditionally used in other industries and applications, it is now being used for a great variety of analytes, including steroids, peptides and catecholamines.

As with other analytes, the earlier mass spectrometric measurements of urinary amines, particularly the metanephrines, were developed using gas chromatography–mass spectrometry (GC-MS), as the coupling of GC to MS technology was technically more straightforward than LC coupling to MS.[122,123] Nonetheless, the number of published GC-MS methods developed for biogenic amines is limited, probably because of the chemical manipulations required to produce volatile derivatives suitable for GC. Crockett *et al*. reported a rapid GC-MS assay for urinary metanephrine and normetanephrine using simultaneous derivatization with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide and *N*-methylbis(heptafluoro)butryamide at room temperature.[124] The method performance compares well with that of a standard HPLC assay and avoids drug interferences that commonly affect HPLC assays for urine metanephrines.

The simultaneous LC-MS analysis of urinary catecholamines and metanephrines was first reported by Chan and Ho.[73] They developed a rapid assay (6.5 min) for the quantitation of urinary NE, E, DA, MN and NM using HPLC coupled to atmospheric pressure chemical ionization mass spectrometry (APCI-MS) (Figure 7.8). Numerous chromatographic separation techniques can be coupled with APCI-MS. The key to achieving separations and optimal MS performance in LC-MS is to find separation conditions that are compatible with atmospheric pressure ionization. Catecholamines and metanephrines are amphoteric in nature and chromatographic separations are usually performed using ion-pairing reversed-phase HPLC or weak cationic-exchange HPLC at acidic pH. However, ion-pair reagents commonly adopted for the separation of catecholamines and metanephrines, such as 1-heptanesulfonic acid and 1-octanesulfonic acid, are not suitable for APCI-MS.

In a preliminary study, a SynChropak CM 100 weak cation-exchange column was used to separate these biogenic amines in order to avoid the use of any ion-pairing reagent. One problem with this approach is that it requires the use of non-volatile buffers of higher ionic strength, such as phosphate buffer, which is not compatible with MS analysis. Subsequently, a C_{18} reversed-phase column was used and the chromatographic resolution of these biogenic amines was possible in the presence of an APCI-compatible buffer reagent consisting of ammonium formate and formic acid. Since these amines are mostly protonated at pH 3, the resolution of these compounds using reversed-phase HPLC might be attributed to the 'ion-pairing' effect of the formate ion. This finding is important because it allows the separation of biogenic amines (especially E and NM, which have the same molecular weight) within a short time of 6 min, and also allows the use of an APCI-compatible buffer reagent, both of which are important for the gas-phase ionization of the analytes in the APCI probe. Similar approaches in the LC-MS analysis of biogenic amines were reported for the screening of pheochromocytoma[125] and the analysis of catecholamines in brain tissue.[126] Tornkvist *et al*.

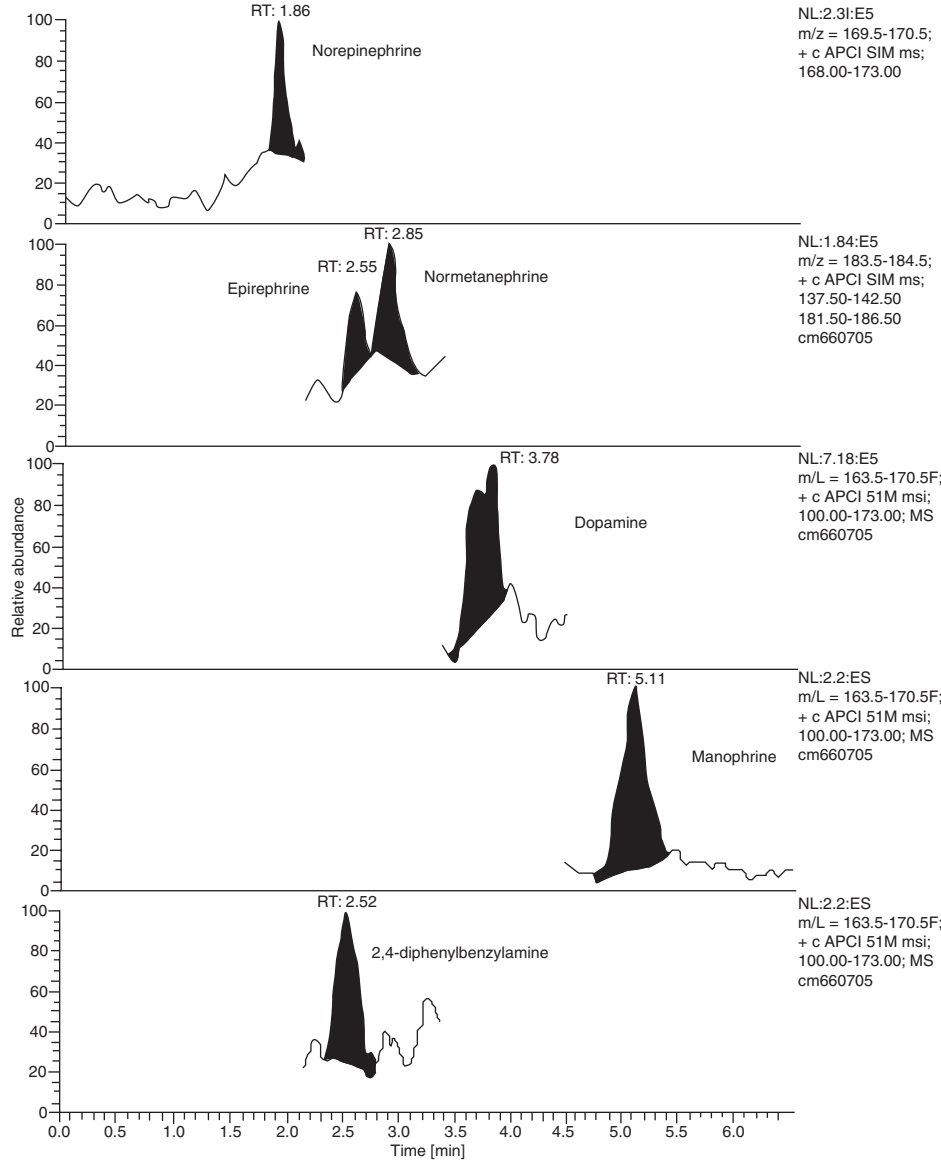


Figure 7.8 HPLC–APCI-MS SIM chromatographic profiles of an extract of a urine sample collected from a healthy male subject. From E. C. Y. Chan and P. C. Ho, *Rapid Communications in Mass Spectrometry*, **14**, 1959–1964. Copyright 2000 © John Wiley & Sons, Ltd. Reproduced with permission

pointed out that the use of mobile phase without ion-pairing agents and with high content of organic modifier facilitated the coupling of HPLC to the selective and sensitive MS detector.[126] Quantification of plasma free metanephrines is usually accomplished using HPLC with electrochemical detection, but sample preparation is labor intensive and time consuming, analytical cycle times are long and interfering substances may obscure the

relevant peaks. The LC-MS-MS assay using multiple reaction monitoring is highly selective and sensitive for the measurement of trace quantities of analyte in complex matrices. Utilizing the LC-MS-MS approach and cyano analytical column chemistry, Lagerstedt *et al.* measured nanomolar concentrations of free metanephrine and normetanephrine in plasma for the diagnosis of pheochromocytoma. The analytical method demonstrated high precision and throughput.[127]

7.5 PRACTICAL CONSIDERATIONS FOR THE STABILITY OF URINARY CATECHOLAMINES AND METANEPHRINES DURING STORAGE

Catecholamines and metanephrines are prone to oxidative conversion to their corresponding quinones,[128] hence proper storage and preservation of 24-h urine samples after collection from patients is essential for accurate quantitation of these compounds. Various groups have reported stability studies of catecholamines in biological fluids.[129–133] Boomsma *et al.* studied the optimal collection and storage conditions for catecholamines in human plasma and urine,[131] and reported that catecholamines were stable at 4 °C for 1 month in unpreserved urine and for 4 months in urine preserved with EDTA and sodium metabisulfite. In acidified urine, catecholamines were also reported to be nearly unchanged after 1 year at 4 and −20 °C. Despite the claimed stability in this study, fluctuating stability profiles of the catecholamines (NE, E and DA) were observed after various lengths of storage. It was also observed that fluctuations in catecholamine concentrations occurred throughout the storage period. The authors attributed the chaotic stability profiles of these analytes in plasma and urine to the depletion of antioxidant compounds added to the biological samples. Similar fluctuations in concentrations of these biogenic amines were also observed in unpreserved urine samples. Miki and Sudo reported in their stability study that unpreserved urine samples could be used for the measurement of catecholamines, cortisol and creatinine.[133] However, they did not comment on the increases in measured concentrations of catecholamines (>10%) in acidified urine samples (pH 1 and 0.5) during storage at room temperature (15–20 °C). Metanephrines (NM and MN) were not examined in most of these stability studies.[130–133] It should be appreciated that any fluctuating concentration profiles of catecholamines and metanephrines during storage could possibly lead to an inaccurate estimation of their actual concentrations in the biological samples. This would render the test results invalid and may eventually lead to higher laboratory costs due to the need for additional tests. More importantly, such inaccurate measurements could result in the incorrect diagnosis of the disease condition. Therefore, the reason(s) for these fluctuating concentrations are of great interest. Using a stability-indicating validated amperometric HPLC method[71] for the measurements of urinary catecholamines and metanephrines, the factor(s) that account for the fluctuating stability profiles of catecholamines in biological samples during storage were identified by Chan *et al.*[72] The detected levels of catecholamines and metanephrines in biological samples were found to be affected by the simultaneous degradation of these compounds and deconjugation of their sulfoconjugates (CA-S and MN-S) when these samples were not properly stored and preserved. These two simultaneous processes most likely contributed to the chaotic stability profiles of these amines observed in some studies. Deconjugation of urinary CA-S and MN-S could occur during the first 3 days of storage at 10 and 30 °C in both unpreserved and preserved urine

samples. Addition of preservatives (HCl or Na_2EDTA and sodium metabisulfite) was found to have no effect on the deconjugation process. Instead, deconjugation was accentuated by the presence of HCl (3 M, 5 mL L^{-1}) as a preservative in the sample and at higher temperature (30 °C). However, storing the samples at extremely low temperature minimizes the deconjugation process. Free catecholamine and metanephrine concentrations were found to be stable for at least 3 weeks in both unpreserved urine and aqueous samples stored at −80 °C.[72] From this, it was concluded that deconjugation of the sulfoconjugates and degradation of their free amines did not occur during this period in the unpreserved urine samples stored at −80 °C. It should be noted that the storage temperature of the urine during sample collection period can have a profound influence on the stability of biogenic amines. The stability of biogenic amines in biological specimens has been reviewed elsewhere.[134]

7.6 FUTURE DEVELOPMENTS

Until recently, the most common analytical technique used for the determination of catecholamines and metanephrines in urine was HPLC. Because of the minute amount of material present in the sample, methods of detection generally have to be very sensitive and selective. The detection methods used in most published studies are electrochemical[57,59,74,135] and fluorimetric.[51,60,78,79,89] Recently, chemiluminescence has also been explored as a detection method for catecholamines and their 3-*O*-methylated metabolites in rat plasma.[117]

Electrochemical detectors are sensitive and selective for the quantitation of urinary catecholamines and metanephrines. However, there are limitations to this mode of detection, which are seldom addressed in research papers. Based on our experience with the amperometric detector, we found that this detector is prone to malfunctioning due to a number of factors. The common malfunctions of the system include unstable baseline, excessive noise, transient spiking, noise corresponding to pump stroke, ghost peaks, high background current and sudden decrease in sensitivity. The common causes of these problems are fouling of the auxiliary electrode, the brass contact of the working electrode, and the carbon plate and/or spacer. Baseline drift and diminished sensitivity also result from carbon plate deterioration, defective contact between electrical connectors, bubbles in the flow cell, elution of metal ions leached from the injector body, ambient temperature changes and draught.

As mentioned earlier, in fluorimetric detection, the amines may be monitored either by natural fluorescence[78,79] or after derivatization reaction with either 1,2-diphenylethylenediamine (DPE),[51,85] trihydroxyindole (THI),[82,136,137] *o*-phthalaldehyde (OPA)[138] or fluorescamine.[138] Although the fluorescence detector is relatively easy to maintain, each of these derivatization methods faces some shortcomings. The natural fluorescence methods show low sensitivity of the catecholamines and metanephrines. DPE increases the sensitivity of all three catecholamines; however, the reaction products are stable for only 30 min after the reaction. When THI is used, HPLC requires rather complicated instrumentation for postcolumn derivatization and cannot be applied to DA. Derivatization with OPA and fluorescamine increases the sensitivity of NE and DA, but E is not measured because only primary amines are derivatized by this reaction.

These challenges observed for both electrochemical and fluorimetric modes of detection prompted the need for a more sensitive, selective and maintenance-free HPLC assay for routine, rapid, simultaneous measurements of catecholamines and metanephrines in urine. The emergence of LC-MS and LC-MS-MS[73,125–127] has provided new analytical approaches

to this field of study. Although MS-MS requires expensive hardware, it is most cost effective when groups of compounds can be measured simultaneously. As the price/performance ratio of this technology improves, it will likely become more common in clinical laboratory applications.

HPLC is a proven technique that has been adopted by laboratories worldwide during the last three decades. One of the primary drivers for the growth of this technique has been the continual improvement in the packing materials (chemistry, particle size, packing technology) used to effect the separation. The underlying principles of this evolution are specified by the van Deemter equation, which relates linear velocity (flow-rate), diffusion components (both linear and eddy) and resolution [in terms of height equivalent of a theoretical plate (HETP), a measure of column efficiency]. Based on the van Deemter equation, as the particle sizes decreases to less than 2.5 μm, not only is there a significant gain in column efficiency, but also the efficiency does not diminish at increased flow-rates. By using smaller particles, speed and peak capacity (number of peak resolved per unit time in gradient separations) can be extended to greater limits, termed ultra-performance liquid chromatography (UPLC).[139] With the need of rapid turn-around times for clinical analyses and higher sensitivity and selectivity for the analysis of biogenic amines, especially in biological matrices, the fast separation, high sensitivity and resolution of UPLC are attractive features. Applications of UPLC-MS and UPLC-MS-MS in pharmaceutical R&D, food testing, environmental analysis, proteomics and life science research and clinical laboratory medicine are likely to become more common as this technology reaches its full potential.

DEDICATION

This chapter is dedicated to the late brother-in-law of P.C.L. Ho, Mr Eddie Kwok Kuen Lee, a former patient with pheochromocytoma. His medical history encouraged and stimulated the authors to pursue research on the diagnosis of the disease.

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8

Chromatographic Measurement of Volatile Organic Compounds (VOCs)

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8.1 INTRODUCTION

Volatile organic compounds (VOCs) measured in clinical and forensic laboratories may be grouped into several categories, including ethanol and other volatile alcohols, anesthetics, alkyl nitrites, industrial solvents and chemicals and multiple volatile compounds often abused and categorized as inhalants. There is no classification system based on clinical effect or chemical structure and these compounds may exist in solid, liquid and gaseous forms. The common feature of these compounds is volatility, the property of existing in or being able to be converted to a gaseous form. This volatility affects the necessity for proper collection, handling and analysis of specimens and profoundly affects the ability to determine accurately the concentrations of these compounds in body fluids and tissues following ingestion or exposure. Reasons for measuring these compounds include confirmation of ingestion (or inhalation), stratification of severity of effects, including intoxication, detection or monitoring of exposure, and verification of administration.

8.2 GENERAL CONSIDERATIONS

The volatility of these compounds facilitates the use of gas chromatography (GC) for separation and quantitation. There are many factors to consider when choosing or implementing a chromatographic method for the analysis of one or more VOCs, including the intended use [medical diagnosis, forensic applications, general screen, quantitation of specific

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compound(s) for monitoring of exposure, verification of use, post-mortem analysis, etc.], volatility of the compound, available resources and specimen(s) in addition to the chromatographic parameters. Many of these factors will be reviewed in order to provide information to be considered when choosing or implementing a method.

8.3 INTENDED USE

The choice of a chromatographic method may be dependent on the intended use. The appropriate method when the intent is to establish a general screen for inhalant abuse may be very different from the method chosen to monitor administration of a particular anesthetic or exposure to industrial chemicals in the workplace. Likewise, forensic applications may vary from a general screen to post-mortem quantitation of particular suspected VOCs in blood and tissues. The number of methods developed for specific purposes (or compounds) is beyond the scope of this chapter, but it is hoped that the information provided will assist the analyst in finding or adapting chromatographic methods suitable for their particular situation.

8.4 VOLATILITY OF COMPOUNDS

The partitioning of a volatile compound into phases (air, blood and tissue) influences pharmacological and toxicological effects of the compound and must be considered when developing chromatographic methods. This distribution between phases is expressed by the partition coefficient (K), which may be calculated by dividing the concentration of the analyte in the sample phase by that in the gaseous phase. The lower the partition coefficient, the more readily the compound partitions into the gas phase and the greater is the risk of loss due to evaporation. For example, loss of hexane with a K of 0.14 (air–water system at 40 °C) is more likely than loss of ethanol with a K of 1355 (air–water system at 40 °C).[1] Hence preventing loss due to evaporation during specimen collection and handling is more critical for samples to be analyzed for hexane than for those containing ethanol. Analytical sensitivity can be increased during extraction and/or analysis lowering the K using techniques such as increasing the temperature or adding salts to decrease solubility. Partition coefficients of VOCs are typically listed for air–water but analysts are concerned with coefficients involving the biological samples to be analyzed. Blood–air, blood–tissue and tissue–air partition coefficients for some solvents[2] and anesthetics[3] have been determined, but the analyst may have to determine these parameters during the development of a particular method.

In addition to the partition coefficient, the analyst must be aware of the phase ratio, defined as the relative volume of the space above the sample (headspace) compared with the volume of the sample in the container, and its effect on loss of analyte. Each time the container is opened, analyte in the headspace is lost and the equilibrium of the compound's concentration in the sample and headspace is re-established. The larger the headspace in relation to the sample size, the greater is the loss due to evaporation. Collecting and storing samples in sealed containers with minimal headspace may minimize the loss of VOCs. Using partition coefficients and measuring phase ratios, the percentage loss after equilibrium for five inhalation anesthetics in closed 5-mL tubes at 37 °C has been calculated.[3] Partition coefficients and phase ratios impact both method sensitivity and loss of analyte. In general, the concentration of volatile analytes in the headspace is higher when conditions are adjusted

to give the lowest values for both the partition coefficient and the phase ratio. Practical applications to achieve these results include increasing temperature, agitation, addition of inorganic salts (sodium or ammonium chloride or sulfate, potassium carbonate, sodium citrate, etc.) to decrease the solubility of polar organic volatiles in aqueous matrices and using sample containers with minimal headspace.

8.5 SAMPLE COLLECTION, HANDLING AND STORAGE

Precautions taken in the collection and storage of the specimen affect the likelihood of detecting exposure to volatile substances. Recommended precautions include collection of blood using an anticoagulant (lithium heparin) into a glass container with a cap lined with metal foil if possible.[4] Soft rubber stoppers are permeable to toluene and other VOCs. Samples should be collected and stored in containers with minimal headspace and addition of an internal standard after collection will minimize errors due to evaporation during storage or tissue homogenization. Samples should be stored, transported and handled at temperatures between -5 and 4 °C.[5] For highly volatile compounds, additional precautions should be employed. The preparation of calibration (standard) solutions of gaseous analytes poses additional problems. Yang *et al.*[6] performed all procedures on ice and used vacuumed tubes and precooled (-20 °C) gas-tight syringes to prevent analyte loss during spiking of standard and control samples. Liquid and solid analytes can be weighed in directly. Streete *et al.*[7] prepared calibration standards of gaseous analytes by transferring a volume of vapor from a cylinder into a glass gas sampling bulb fitted with a septum port and then injecting appropriate volumes (using a gas-tight syringe) into calibrated septum bottles filled with nitrogen. Broussard *et al.*[8] described a process for the preparation of a methanolic stock standard of a VOC (difluoroethane) utilizing a cylinder of the VOC in the gaseous state. Other authors prepare calibration standards in water,[9] methanol[10] and aqueous solutions of ethanol or *n*-propanol.[9]

8.6 HEADSPACE GAS CHROMATOGRAPHIC METHODS

Although some direct injection methods for determining ethanol[11] and other VOCs have been developed, headspace (HS) techniques are most frequently used. These HS methods include static mode variations such as solid-phase microextraction (HS-SPME), cryogenic focusing (HS-CF), cryogenic oven trapping (HS-COT) and the dynamic mode method of purge and trap.[5] For HS methods, an aliquot of HS air is sampled from a sealed vial containing specimen plus internal standard. As previously discussed, VOCs in the specimen are partitioned into the HS above the sample and the sample is taken when equilibrium has been established. Evaporation may be accelerated using heating, addition of salt and/or agitation. For tissue samples, homogenization at low temperature in a closed container or treatment with a proteolytic enzyme such as subtilisin A may be used to enhance evaporation.[5]

Solid-phase microextraction (SPME) is a sampling and concentration technique used to increase the sensitivity of HS methods. This technique is utilized for arson analysis and environmental monitoring purposes and also for clinical and forensic procedures. Short, narrow diameter, fused-silica optical fibers coated with stationary phase polymers are either immersed in the sample or the HS and compounds are adsorbed or absorbed (depending on

the type of fiber). Extraction can be enhanced by adjustment of conditions (pH, temperature, time) and employing techniques such as agitation and addition of salt. Fibers are chosen based on the desired selectivity; in general, like attracts like, e.g. polar fibers are used for polar analytes and non-polar fibers for non-polar analytes. A review by Mills and Walker[12] summarizes the general principles of SPME and includes a list of SPME devices with some of their properties.

One of the most commonly used fibers for VOC analysis is coated with a 100-μm polydimethylsiloxane phase (PDMS) and is available as a unit with a stainless-steel guide rod housed in a hollow septum-piercing needle (Supelco, Bellefonte, PA, USA). The fiber can be withdrawn into the needle for protection during handling and the depth of fiber exposure can be controlled using the adjustable holder. In HS techniques, the needle can be used to pierce the septum of the sample vial and to introduce the fiber into the injection port of the gas chromatograph. In the sample vial, the extraction onto the fiber reaches equilibrium fairly quickly for volatile compounds. Mills and Walker's review[12] includes tabular summaries of HS-SPME–GC methods for the detection of alcohols, drugs, solvents and chemicals in blood and urine.

Another way to increase sensitivity is to increase the volume injected into the column. Lowering the temperature of the entire column in cryogenic oven trapping (COT) methods or for the injection port at the inlet of the column in cryogenic focusing (CF) methods allows the injection of as much as 10 times the typical sample volume. Liquid nitrogen or liquid carbon dioxide is used to lower the temperatures to well below 0 °C (−180 °C for nitrogen and −90 °C for carbon dioxide). These techniques result in better peak shapes in addition to increased sensitivity.

In dynamic HS analysis, also called purge and trap, a carrier gas constantly passes above the sample and evaporated volatiles are trapped in a cryogenic and/or adsorbent trap.[5] Analytes are released on to the column by extensive heating. This technique results in increased sensitivity, particularly for water-soluble compounds. It is a fairly difficult technique and problems include artifacts/interfering peaks due to impurities in the purge gas or water and foaming from biological samples. Procedures using an antifoam agent for biological samples and a dehydration agent have been developed. Wille and Lambert[5] have tabulated analytical data for all of the HS techniques discussed.

8.7 COLUMNS AND DETECTORS

Initially packed columns were used, but capillary columns are most frequently used today. A variety of polar and non-polar column phases have been used to determine VOCs depending on the particular compound(s) analyzed. Numerous examples were summarized by Wille and Lambert.[5] Similarly, a range of detection devices have been utilized. Flame ionization detection (FID) and mass spectrometry (MS) in the selected-ion monitoring mode (SIM) are most frequently used, but other techniques include electron-capture detection (ECD) and Fourier transform infrared (FTIR) detection.

8.8 IDENTIFICATION, QUANTITATION AND CONFIRMATION

The identification and quantification of VOCs utilizing GC is accomplished with the same techniques as used for non-volatile compounds. Most methods do not require a derivatization

step because of the volatility of the compounds of interest, but testing for some of the polar metabolites of VOCs does require derivatization. The partition coefficient and measurement of the headspace concentration could be used to determine the concentration of a VOC, but typically quantification is performed using calibration curves and an internal standard. Problems involved with the preparation of standard (calibration) and control samples and techniques used to minimize evaporation have been discussed previously (Section 8.5). Pure standards in solvents or as a gas in a cylinder may be purchased from chromatography/reference material suppliers. Because many VOCs are used for industrial purposes, standards containing multiple analytes in methanol are available for US Environmental Protection Agency (EPA) methods. For example, approximately 60 VOCs in environmental samples are detected by EPA Methods 502.1, 502.2, 524.1 and 524.2 and reference materials containing these compounds in methanol are available.

The matrix effect may cause problems in some biological samples, particularly post-mortem samples. Techniques that can be used in these cases include standard addition, multiple HS extraction and full evaporation.[5] The standard addition method, involving comparison of the signal in a sample before and after addition of known amounts of the VOC, is a relatively routine procedure. The multiple HS technique is based on the fact that each time an aliquot is removed from the HS, re-equilibrium occurs and the concentration reduces logarithmically. After multiple injections, the original concentration can be determined by extrapolation. Heating a small amount of sample at a high temperature in a closed container causes evaporation of (almost) all of the analyte. This full evaporation technique is another technique for avoiding the matrix effect.

Acceptable forensic practice recommends confirmation of the detection of an analyte (drug) by a second or definitive method. For VOCs, two acceptable GC confirmation procedures are re-sampling and re-analysis using a different column or use of mass spectrometry for absolute identification.

8.9 ETHANOL AND OTHER VOLATILE ALCOHOLS

Ethanol, the most frequently measured VOC in clinical and forensic laboratories, may be measured using enzymatic or chromatographic methods. Laboratories measuring ethanol alone almost always utilize enzymatic (alcohol dehydrogenase) methods performed on automated analyzers, but laboratories offering a broader test menu employ chromatographic methods. The National Academy of Clinical Biochemistry (NACB) laboratory medicine practice guidelines (LMPG) recommend that laboratories have the stat capability, with a turnaround time of 1 h or less, of performing quantitative assays for certain analytes including ethanol, methanol and ethylene glycol.[13] In clinical and forensic laboratories, the quantitative analysis of ethanol and methanol is frequently part of a volatiles panel, including 2-propanol and acetone, commonly performed using HS-GC. Both packed and capillary columns have been used, although currently capillary columns are more frequently used. Many types of packing materials have been used for ethanol analysis, including Carbowax, Porapak, DB-1, DB-WAX, DB-624 (Agilent Technologies, Wilmington, DE, USA) and columns developed specifically for such analysis such as DB-ALC1, DB-ALC2 (J&W Scientific, Folson, CA, USA) or Rtx-BAC1 and Rtx-BAC2 (Restek, Bellefonte, PA, USA). Commonly used internal standards are *n*-propanol, 2-butanone (methyl ethyl ketone) and *tert*-butanol.[14,15]

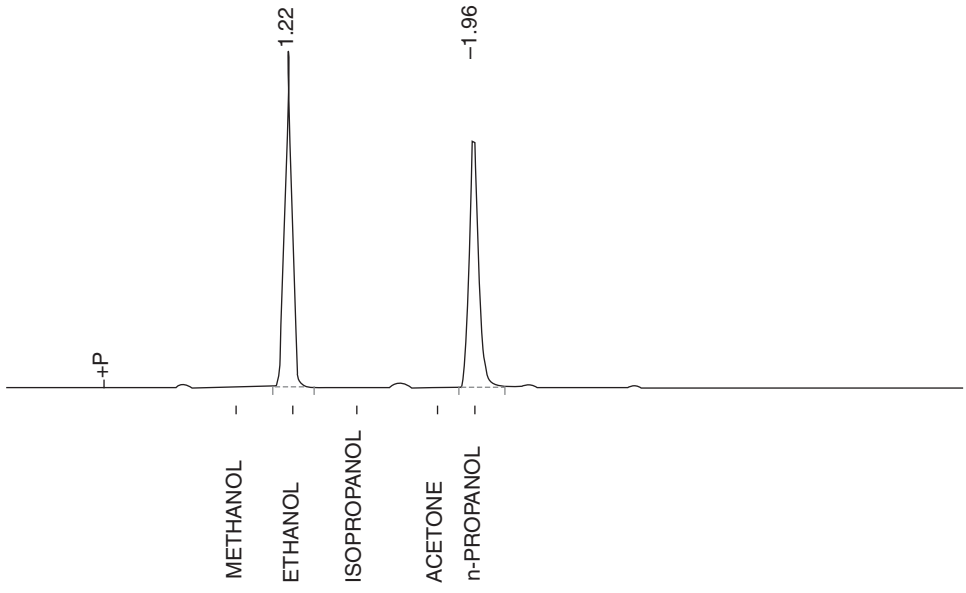

Figure 8.1 Chromatogram of ethyl chloride (retention time 1.22 min) and internal standard *n*-propanol (retention time 1.96 min.) on an Rtx-BAC1 column (30 m × 0.32 mm i.d., 1.8 µm). Static headspace sampling following a 5-min equilibration time at 70 °C and isothermal GC separation at 45 °C for 3.5-min. Note that ethyl chloride and ethanol have the same retention time.

These methods for ethanol and volatile alcohols may be modified to be used as a screening method for the detection of multiple VOCs[9,10] or to quantitate a particular volatile substance.[16] In some instances, the first evidence of inhalant abuse or solvent exposure may be the presence of an unknown peak during the analysis of ethanol and volatile alcohols. If inhalation or exposure to a particular VOC is suspected, a potential time-saving first step in method development would be HS injection of the analyte compound using the laboratory's procedure for ethanol. As mentioned previously, a second complementary analytical technique should be used for all forensic toxicological confirmation analyses,[15,17] including ethanol analysis. For example, Laferty[18] reported co-elution of ethanol and ethyl chloride on 0.3 and 0.2% Carbowax and 60/80 Carbopack C columns and Broussard *et al.*[19] reported similar results using an Rtx-BAC1 column. Figures 8.1 and 8.2 illustrate how ethyl chloride could be mistaken for ethanol in a one-column system but not in a two-column system.

8.10 INHALANTS AND SCREENING FOR MULTIPLE VOCs

The abuse of a wide range of volatile chemicals (often called inhalants) that may be inhaled accidentally or intentionally is a worldwide problem, particularly in the adolescent population. There are more than 1000 ordinary relatively inexpensive household products that may be abused via inhalation. VOCs such as toluene, chloroform, butane, propane and acetone are present in many commercial products (solvents, contact adhesives, correction fluid,

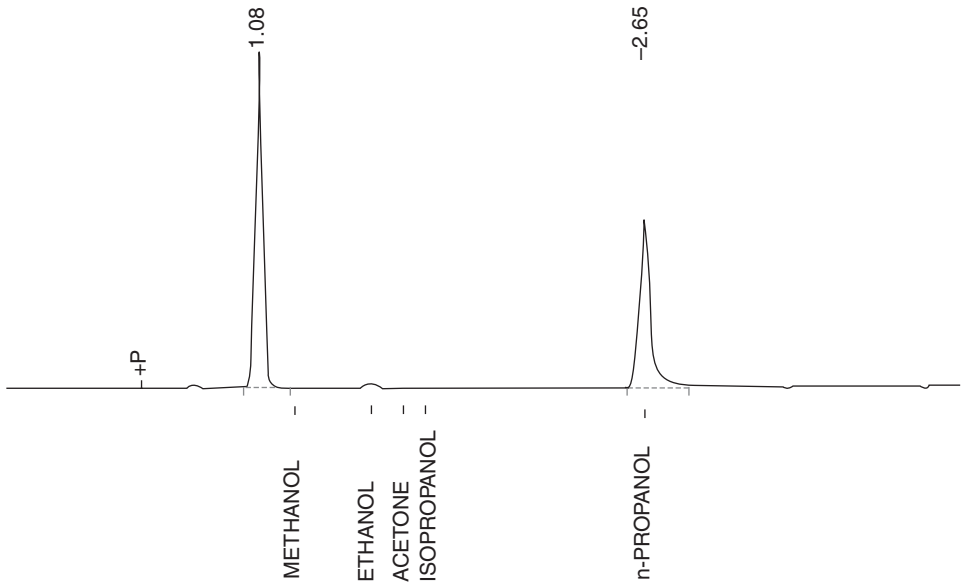

Figure 8.2 Chromatogram of ethyl chloride (retention time 1.08 min) and internal standard *n*-propanol (retention time 2.65 min.) on an Rtx-BAC2 column (30 m × 0.32 mm i.d., 1.2 µm). Static headspace sampling following a 5-min equilibration time at 70 °C and isothermal GC separation at 45 °C for 3.5 min. Note that ethyl chloride and ethanol are separated.

gasoline, lighter fluid, refrigerants, fire extinguishers, etc.) and halogenated hydrocarbons are the propellants for virtually all aerosol products. Three of the most frequently abused volatile substances or classes of substances are toluene, halogenated hydrocarbons (solvents such as chloroform, carbon tetrachloride, methylene chloride, 1,1,1-trichloroethane in correction fluid, trichloroethylene used for dry cleaning and ozone-depleting chlorofluorocarbons and their non-ozone-depleting replacements) and butane.

Inhalants in small doses produce euphoria characterized by loss of inhibition but also have the potential to cause cardiac arrhythmias ('sudden sniffing death'). Large doses produce acute problems, including convulsions, coma and death. Depending on the product, volatile substances may be inhaled directly from the container (snorting or sniffing), from a plastic bag (bagging) – particularly if the product is an aerosol or a viscous liquid such as glue – or from a saturated cloth (huffing).[16] Of these routes of administration, bagging usually results in the highest concentration, snorting the lowest and huffing an in-between concentration. In addition to screening for abuse, other reasons for detection, identification and quantitation of these compounds include monitoring of exposure in the industrial setting and post-mortem cause-of-death investigations.

For isolated cases, the first indication of the presence of a volatile substance may be the presence of an unknown peak when a sample is analyzed for ethanol and other volatile alcohols (see the previous section). In these instances, method development focuses on the compound encountered and frequently involves modification of the ethanol analysis procedure. Numerous procedures for single VOCs or classes of VOCs have been developed and summarized in review articles.[5,6,12,20,21] HS-GC remains the most frequently used

method, although high-performance liquid chromatography (HPLC) is used for the detection of the more polar and less volatile urine metabolites.[5,22] Blood is the specimen of choice, although analysis of urine metabolites sometimes extends the time frame for detection of exposure. For fatalities in which inhalant abuse is suspected, tissues (brain, lung, fat, liver, heart, kidney) should be analyzed in addition to blood. Typically the highest concentrations are found in blood and brain tissue.

Several HS-GC procedures, listing the retention data of as many as hundreds of compounds, for the screening of multiple VOCs have been published.[4,7,9,10,23–25] Although limits of detection (LODs) and linear analytical ranges vary with particular methods, most procedures have typical LODs of $0.1\ \mathrm{mg\,L^{-1}}$ and upper limits of linearity (ULOL) to concentrations as high as $5000\ \mathrm{mg\,L^{-1}}$.

Table 8.1 lists the retention times of common volatiles for the procedure described by Sharp.[9] This HS procedure utilizes dual-column elution on non-polar DB-1 (30 m × 0.32 mm i.d., 5.0-μm film thickness) (Agilent Technologies) and polar DB-WAX (30 m × 0.32 mm i.d., 0.25-μm film thickness) (Agilent Technologies) columns with flame ionization detection (FID). HS vials (20 mL) containing 0.1 mL of sample and 1.5 mL of *tert*-butanol (internal standard) are incubated for 30 min at 70 °C and aliquots are injected using a 20:1 split injection through a Y connector. The injector and detector temperatures were 150 and 220 °C, respectively. The total run time was 20 min with a temperature program of 40 °C for 9 min, then ramped at $10\ ^\circ\mathrm{C\ min^{-1}}$ to 150 °C. Method validation included comparison on a single specialty column (DB-624; 30 m × 0.53 mm i.d., 3.0-μm film thickness) (Agilent Technologies) system and confirmation of peak identities by mass spectrometry (MS). The procedure provides detection and identification of more than 40 compounds. For the few compounds that do not display unique retention times on both columns, either the use of a DB-624 column or HS-GC–MS have been shown to be acceptable confirmation procedures. For confirmation on the DB-624 column, samples were incubated for 30 min at 60 °C. The injector and detector temperatures were 150 and 250 °C, respectively. The total run time was 15 min with a temperature program of 45 °C for 5 min, then ramped at $9\ ^\circ\mathrm{C\ min^{-1}}$ to 90 °C and held 5 min. Columns for HS-GC–MS confirmation were DB-1 (30 m × 0.25 mm i.d., 0.25-μm film thickness) and DB-WAX (15 m × 0.25 mm i.d., 0.25-μm film thickness). Although this method is used as a screen for forensic purposes, it can be adapted for use in the detection and/or quantitation of compounds for workplace monitoring purposes.

8.11 INTERPRETATION

Clinical chemists and toxicologists should be aware that the detection of volatile substances in blood does not always indicate inhalant abuse or occupational exposure. Acetone and other volatile compounds may be found in ketoacidotic patients and some inborn errors of metabolism result in the accumulation of volatile compounds. Even though many studies and case reports have included concentrations of volatile substances in blood, definitive correlations between these blood concentrations and the clinical features of toxicity have not been demonstrated for any of these compounds.

The detection of urinary metabolites has also been used to detect occupational exposure or to confirm inhalation of volatile substances. Urinary metabolites such as phenol (benzene metabolite), trichloroacetic acid (tetrachloroethylene), hippuric acid (toluene) and methylhippuric acid (xylene) have been detected and measured.[16] Results are often

Table 8.1 HS-GC retention times (min) of VOCs[a,b]

Compound	DB-1 run program	DB-WAX 40–150 °C	DB-624 40–150 °C
Acetaldehyde	2.33	1.55	2.20
Acetone	4.14	1.86	3.49
Acetonitrile	3.76	3.61	3.86
Amyl acetate[c]	19.96	7.17	>15
Amyl acetate, artifact	>20	10.01	>15
Benzene	13.15	2.71	7.77
Butanol, 1-	12.94	8.99	8.84
Butanol, 3-methyl-1-[d]	15.72	11.55	11.09
Butanol, 4-chloro-1-, artifact	>20	14.29	>15
Butanol, 4-chloro-1-[c]	11.31	2.10	6.76
Butanol, iso-(2-methyl-1-propanol)	11.27	6.29	7.70
Butanol, *terti*-	5.45	2.43	4.25
Butyl acetate, *n*-	18.16	5.36	13.29
Chloral hydrate[e]	>20	>20	>15
Chlorobenzene	19.49	11.06	>15
Chlorobutane, 1-	12.51	1.96	7.32
Chloroform	10.61	3.98	6.84
Cyclohexane	13.58	1.59	7.22
Diethyl ether	5.04	1.46	3.15
Ethanol	3.39	2.75	3.02
Ethchlorvynol	>20	>20	>15
Ethyl acetate	10.44	2.27	6.42
Ethylbenzene	19.93	6.92	>15
Formaldehyde[d]	15.42	2.38	10.48
Heptane, *n*-	15.09	1.54	8.26
Hexane, isomer 2	9.46	1.36	4.52
Hexane, isomer 1	12.01	1.51	6.07
Hexane, *n*-	10.50	1.42	4.99
Hexanone, 2-	17.40	5.63	12.93
Methanol	2.33	2.36	2.31
Methyl acetate	5.60	1.93	3.94
Methyl ethyl ketone	8.88	2.36	6.28
Methyl *terti*-butyl ether	8.12	1.53	4.49
Methyl-2-pentanone, 4-	15.84	3.69	10.87
Methylene chloride	5.73	2.63	4.10
Octane, *n*-	18.35	1.78	11.55
Octanol[e]	>20	18.25	>15
Pentane	5.07	1.38	2.92
Pentanol, 1-[c]	16.77	12.77	12.29
Pentanol, 3-	14.49	7.14	9.73
Pentanone, 4-hydroxy-4-methyl-(HMP)[e]	18.94	17.24	>15
Propanol, 2-	4.46	2.68	3.66
Propanol, *n*-	6.98	4.54	5.47
Toluene	17.14	4.29	11.22
Trichloroethane, 1,1,1-	12.40	2.20	7.13

(*continued*)

Table 8.1 (*Continued*)

Compound	DB-1 run program	DB-WAX 40–150 °C	DB-624 40–150 °C
Trichloroethanol, 2,2,2-	>20	>20	>15
Trichloroethylene	14.69	3.47	8.90
Trimethylpentane, 2,2,4-	14.76	1.49	7.92
Xylene, *m*-[c]	19.93	6.86	>15
Xylene, *o*-[c]	>20	7.19	>15
Xylene, *p*-	>20	7.20	>15

[a](Columns: DB-1, 30 m × 0.32 mm i.d., 5.0-μm film thickness; DB-WAX, 30 m × 0.32 mm i.d., 0.25-μm film thickness; DB-624, 30 m × 0.53 mm i.d., 3.0-μm film thickness (Agilent Technologies, Wilmington, DE, USA).

[b]Retention time entries denote compounds readily detected at 100 mg%.

[c]Artifact peaks detected.

[d]Split peak.

[e]Primary peak is an artifact or breakdown product.

Reproduced from the *Journal of Analytical Toxicology* by permission of Preston Publications, A Division of Preston Industries, Inc. Sharp M.E., A comprehensive screen for volatile organic compounds in biological fluids, *J. Anal. Toxicol.* 2001, **25**, 631–636.

expressed as a ratio to the urine creatinine concentration in order to normalize results in relation to urine volume and fluid intake. Caution must be used when interpreting results. For example, urinary hippuric acid may be due to ingestion of benzoate preservatives in foods and not to exposure to toluene.

8.12 CONCLUSION

Compounds classified as VOCs are typically measured using HS-GC. Many methods have been developed for the screening, detection, identification and quantitation of single or multiple VOCs. Due to the volatility of these compounds, sample collection, handling and storage are extremely important. In order to choose and/or develop methods that accurately detect and quantitate VOCs, the analyst must understand and utilize the principles of phase equilibrium, including partition coefficients and phase ratios. In clinical and forensic laboratories, the occurrence of an unknown peak during the analysis of ethanol and volatile alcohols by HS-GC may be the first indication of the presence of a VOC and the substance may be identified and quantitated by modification of the method. Several screening methods for multiple VOCs have been developed and details of one such method has been presented in this chapter.

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9

Chromatographic Techniques for Measuring Organophosphorus Pesticides

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9.1 INTRODUCTION

The term 'pesticides' comprises a large number of substances belonging to many completely different chemical groups, the only common characteristic being that they are effective against pests. Pesticides may be classified based on their chemical structure, their pharmacokinetic or pharmacodynamic profile and also their formulation, application and target organism.[1–3] Some substances are not easily categorized by standard methods as they can be used against two or more targets or in different formulations, resulting in varying pharmacokinetic and/or pharmacodynamic properties. A major classification system links pesticides to the pest that is controlled, e.g. insecticides, herbicides and fungicides controlling insects, weeds and fungi, respectively (Table 9.1).

A second classification categorizes the pesticides according to their chemical nature, i.e. inorganic and organic compounds. Most pesticides used today are organic compounds, a small number of which are either derived from plant products or extracted directly from plants. However, most modern pesticides are synthetic compounds, this fact being primarily responsible for the rapid expansion of pesticides since the 1940s: in 1997, for example, 15 400 t of herbicides, 8400 t of fungicides, 3300 t of other pesticides and 900 t of insecticides were sold in Germany.[4] Although the amount of insecticides appears to be small in comparison with other substances, it has to be taken into consideration that 81 t of the total of 900 t of insecticides were used for private gardening. This means that 10% of the insecticides produced were utilized by persons without appropriate expertise.

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Table 9.1 Classes of pesticides

Class	Pest
Acaricide	Mites
Bactericide	Bacteria
Fungicide	Fungi
Herbicide	Weeds
Insecticide	Insects
Molluscicide	Snails
Nematicide	Nematodes
Rodenticide	Rodents (mouse and rat)

Today's commercially available pesticides are characterized by a vast variety of chemical structures and functional groups making their chemical classification quite complex. The major chemical groups are as follows:

- *Organophosphates*: these are esters of phosphoric, phosphonic, phosphorothionic and related acids.
- *Organochlorines*: these are hydrocarbons with one or more chlorine atoms.
- *Carbamates*: these substances are formed by salts or esters of carbamic acids.
- *Pyrethroids*: these substances are synthesized from pyrethrums, natural plant products in different kinds of chrysanthemum.
- *Triazines*: these substances are classical herbicides consisting of a number of substituted 1,3,5-triazines.
- *Substituted ureas*: this group comprises substances such as phenylureas, sulfonylureas and benzoylureas, which are mainly herbicides.

For humans, pesticides exclusively targeted against vermin hold a great toxicological risk. Others, e.g. herbicides, have only a small if any influence on the human organism, as they were developed to interfere with plant physiology. Insecticides can be subdivided into three groups based on their mechanism of action: pyrethroids, organochlorine compounds and organophosphorus pesticides together with carbamates are distinguished, the last type being acetylcholinesterase inhibitors.

To estimate the toxicological risk of a given pesticide, a knowledge of exposure levels is a crucial step in the risk-evaluation process and can be achieved by measuring the dose entering the body. However, the analytical detection of pesticides in food or different parts of the environment (e.g. air, soil, water) and detection in human biological tissues are difficult, for many reasons: First, current analytical methods are highly sophisticated and require specialized laboratories. Pure pesticide standards, and even more so standards for their metabolites, are rarely commercially available. Therefore, only a few validated methods exist that have been approved by reference organizations. In field studies on pesticide exposure, it is difficult to collect representative samples and to define a correct sampling time. Furthermore, permissible exposure limits and biological exposure indexes are available for only a limited group of compounds. For these and other reasons, a detailed discussion of

which would go beyond the scope of this chapter, biological monitoring of pesticide exposure is a very complex issue.

Based on these considerations, the following sections will focus on organophosphorus insecticides and their toxicological properties and well review various analytical methods for the qualitative detection and quantitative determination of different substances of this class.

9.2 ORGANOPHOSPHORUS PESTICIDES (OP*s*)

Substances belonging to the group of OP*s* are esters of phosphoric, phosphonic, phosphorothionic and related acids. In Table 9.2 the chemical structures of the most commonly used OPs and their LD_{50} values (rat) are shown.

9.2.1 Mechanism of action

OP*s* act through inhibition of acetylcholinesterase activity in the central nervous system (CNS). This enzyme is essential for the hydrolysis of acetylcholine (ACh), which is a neurotransmitter within the human body. ACh is crucial for most inter-cell communication: upon stimulation of nerve endings, ACh molecules are released and diffuse through the synaptic gap. The interaction of ACh with its receptor evokes a sodium and calcium influx into the downstream cell, resulting in an electrochemical stimulus. This stimulus is then either passed on using intracellular signaling pathways (nerve cells) or used to implement a specific response in the recipient cell (other organs). After transferring their information, it is necessary to remove the ACh molecules from their receptor binding sites. This is realized by an enzymatic cleavage of ACh into choline and acetic acid, thereby restoring the receptors and enabling them to process new signals.

The enzyme cholinesterase, effecting the degradation of ACh, consists of two active sites, the anionic and the esteratic site (Figure 9.1). In the first step, ACh is attached to the anionic site and the acetyl group of the ACh molecule is transferred to the amino acid serine. The resulting serine ester is then cleaved hydrolytically and the enzyme is regenerated.[5]

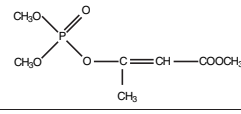
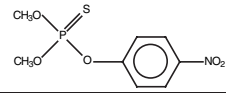
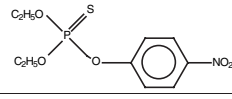
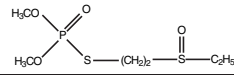
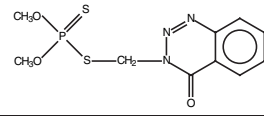
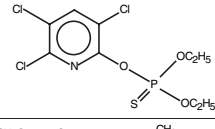
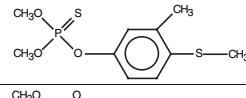
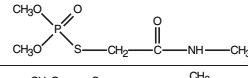
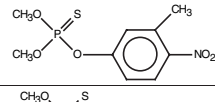
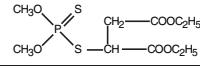
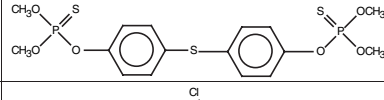
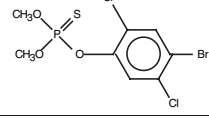
The first step of the reaction of cholinesterase with the OP*s* is similar to its interaction with ACh as its catalytic serine residue is phosphorylated and the acid component is released from the educt (Figure 9.2). This phosphorylation is nearly irreversible and the cholinesterase is no longer able to cleave ACh, causing a continuous stimulus. This uncontrolled reaction results in an intoxication.[6]

Thiophosphate insecticides are protoxins, as they are unable to phosphorylate the serine until after the first liver passage, during which the thiono group is replaced by an oxo group. Therefore, they are called indirect acetylcholinesterase inhibitors.

9.2.2 Intoxication

OP*s* are very lipophilic and are readily absorbed both enterally and percutaneously. The adsorption rate is increased by simultaneous intake/application of organic solvents or plant oils.

Table 9.2 Chemical structures of the most popular organophosphorus insecticides

Name	Chemical structure	LD_{50}, rat, oral (mg kg^{-1})
Mevinphos		3.7
Parathion-methyl		6
Parathion-ethyl		7
Oxydemeton-methyl		50
Azinphos-methyl		80
Chlorpyrifos		245
Fenthion		250
Dimethoate		300
Fenitrothion		500
Malathion		1400
Temephos		2000
Bromophos		3750

The onset of intoxication symptoms is dependent on the pathway of absorption: within a few minutes of inhalation, from 15 min to 1 h after swallowing, and 2–3 h after cutaneous resorption, a toxic concentration will be reached in the blood. With indirect acetylcholinesterase inhibitors, e.g. parathion, symptoms of poisoning occur later. No exact data exist about the extent of bioavailability in the human body.

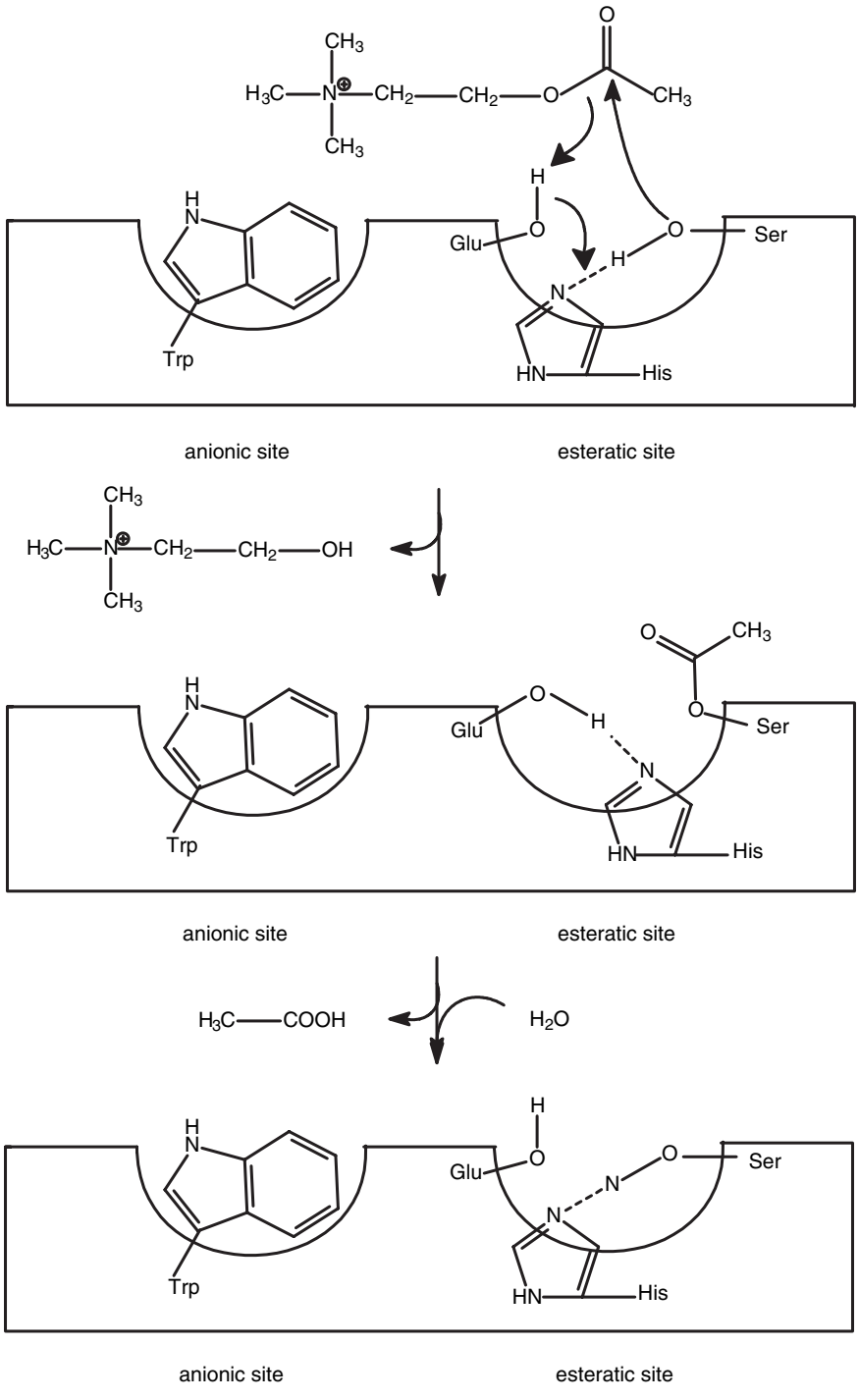


Figure 9.1 Chemical reaction of acetylcholine (ACh) with the enzyme acetylcholinesterase (Ser, serine; His, histidine; Glu, glutamate; Trp, tryptophan)

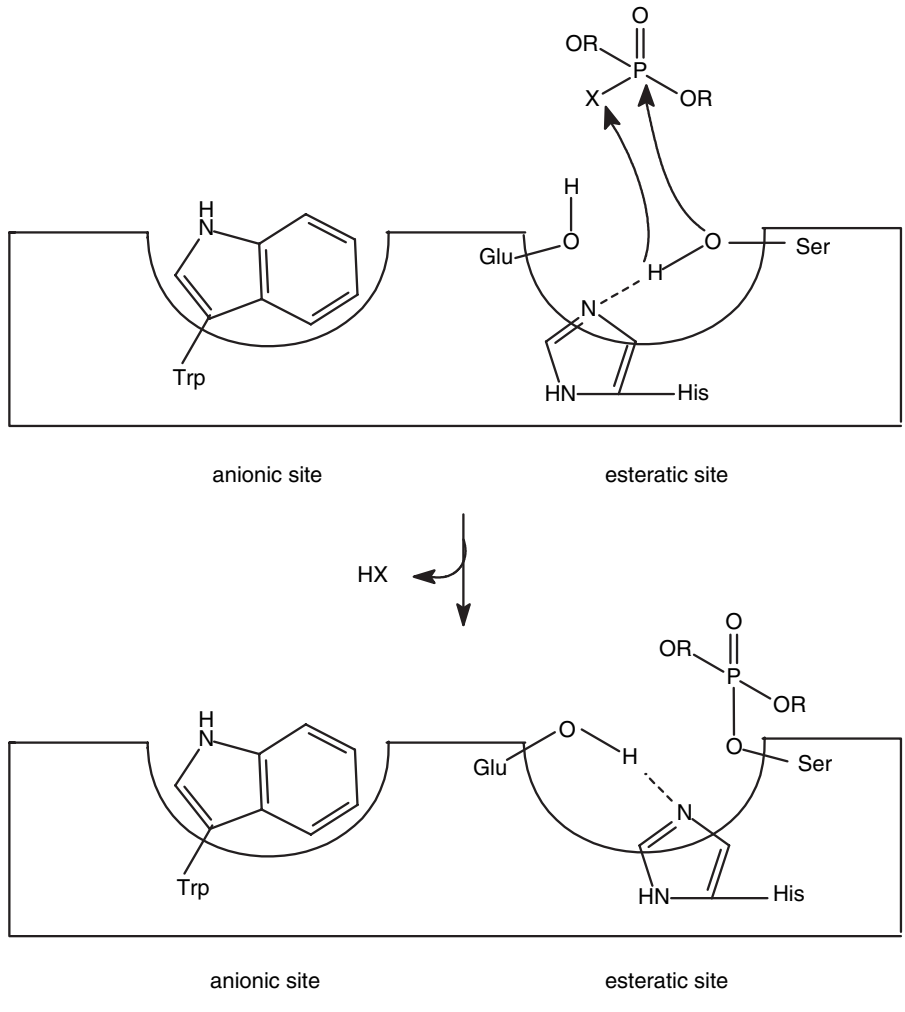


Figure 9.2 Chemical reaction of OP*s* with the enzyme acetylcholinesterase (Ser, serine; His, histidine; Glu, glutamate; Trp, tryptophan; X, acid group)

The most frequently observed symptoms of mild intoxication with OP*s* are miosis, lacrimation, increased secretion from mucous membranes, hypersalivation, nausea, emesis, gastrointestinal spasms, diarrhea and profuse sweating. Progressive and massive intoxications result in agitated melancholia, dyspnea, algospasms, coma, confusion, tremor, muscular cramps, muscle stiffening, amyasthenia and central and peripheral respiratory paralysis. Diagnostic clues for intoxication with OP*s* are blue or red coloration of the concoction, of the vomit or of residues in the mouth of the patient. Also, an intense onion-like smell is indicative of OP intoxication.[7]

The toxicity of a given OP is variable, depending on its chemical structure, lipophilic character, affinity to the esterase and the readiness with which it is hydrolyzed. With increasing lipophilicity, OP*s* are increasingly well absorbed through the skin and through the mucous membranes of the gastric and intestinal tract. Furthermore, high lipophilicity in

OP*s* facilitates their CNS penetration. In contrast, a polar character of a given OP increases its affinity to the cholinesterase molecule, i.e. to its anionic site. If the phosphoric ester bond in an OP is easily hydrolyzable, its toxicity will be low as the cleavage products are unable to interact with the enzyme. However, stable phosphate esters that contain a hydrolyzable functional group (e.g. alkyl groups) are extremely toxic. After dissociation of this cleavable group from the OP–esterase complex, the aggregate is stabilized (Figure 9.2) resulting in irreversible inhibition of the enzyme. This process is called 'aging' of the enzyme, with OP*s* optimized towards fast aging being used as chemical warfare agents.[8]

9.2.3 Progression of intoxication and longer term risks

In cases of attempted suicide, often fatal intoxications occur where, in spite of early hospitalization, patients are beyond remedy. Initially, signs of recovery may be observed, but after 2–3 days patients relapse with a measurable increase in OP plasma concentrations. This phenomenon is called intermediary syndrome (IMS), first described by Senananyake *et al.* in 1987,[9] and is possibly a consequence of a severe intoxication with permanent depolarization of the neuromuscular end-plates and constant excitation of the nicotinic acetylcholine receptors in the CNS of the patient. The IMS is clinically characterized by acute respiratory paresis, weakness of facial, palatal, external ocular, nuchal and proximal limb muscles and depressed tendon reflexes. Some authors[10,11] propose that an insufficient therapy with oximes or atropine (see Section 9.2.5) and inadequate artificial respiration in the early stages of intoxication may cause the occurrence of an IMS. It is further remarkable that only some distinct OP agents (e.g. fenthion, dimethoate, monocrotophos and methamidophos) seem capable of producing the IMS.

A third neurological manifestation, besides the acute toxicity which appears within 30 min after resorption and the IMS which develops about 2–3 days after resorption, is the so-called organophosphate-induced delayed neurotoxicity (OPIDN).[12,13] Symptoms of OPIDN appear about 2–3 weeks after acute exposure to OP compounds. OPIDN is caused by inhibition of another esterase enzyme, neuropathy target esterase (NTE), and establishes a second structure–activity relationship (SAR) in OP toxicology as a predominantly motoric, distal symmetrical polyneuropathy emerges. OPIDN generally follows acute cholinergic symptoms. Muscle cramps and pain in the legs are the usual initial complaint, sometimes followed by distal numbness and paresthesias. Progressive leg weakness is then accompanied by a depression of tendon reflexes. After several days, similar symptoms may appear in the hands and forearms. Sensory loss sometimes develops, initially in the legs and then in the arms, but is often mild or inconspicuous. The importance of this clinical picture is underlined by the fact that more than 40 000 cases of OPIDN have been documented from 1899 to 1989. Permanent damage remains mostly after a severe intoxication, and differentiation between such damage and OPIDN is often not possible.

In spite of the extensive knowledge about different kinds of consequences of an intoxication with OP*s*, data on toxic blood concentrations are non-existent for most of the substances. When known, the values vary strongly between the respective OP*s*. For example, the toxic and comatose-fatal (see case report) blood concentrations of parathion are in the ranges 10–50 and 50–80 ng mL^{-1}, respectively. In contrast, higher concentrations of

malathion are required for toxicity: doses of 500 ng mL^{-1} and 175 μg mL^{-1} were described as being toxic and fatal, respectively.[14,15]

9.2.4 Therapy

In case of intoxication, the first measure is to stabilize respiration and circulation. This can be achieved by administration of atropine, which is a non-depolarizing inhibitor of acetylcholine receptors. In the early stages of intoxication, treatment with oximes, for example toxogonin, provides the opportunity to reactivate the enzyme. In the first step of the reactivation process, the bound OP molecule undergoes a nucleophilic attack by the oxime that causes cleavage of the ester bond, thereby unblocking the catalytic serine residue of the esterase. The NO–phosphate ester bond in the oxime–OP complex is very unstable and degrades easily, releasing the hydrolyzed OP. The mechanism of the reactivation reaction is shown in Figure 9.3.[16]

9.2.5 Analytical procedures

For detection of intoxication with OP*s*, many different analytical methods have been reported. One alternative is to measure the cholinesterase activity, because an acute intoxication is characterized by a 20% decrease in acetylcholinesterase activity. Another approach includes the determination of the unmetabolized OP*s* in blood or other tissues of

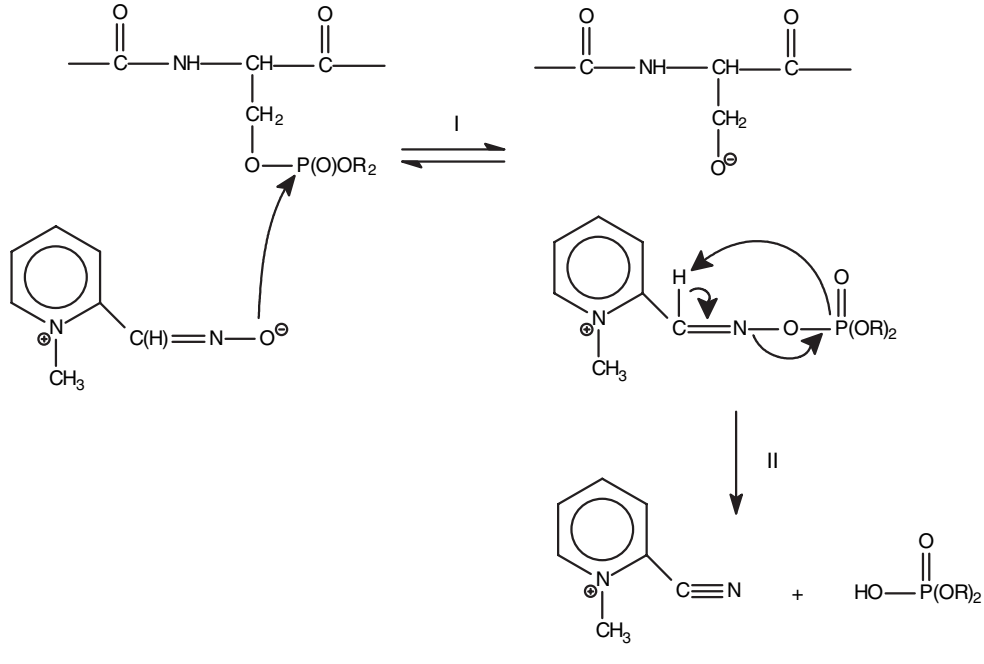


Figure 9.3 Nucleophilic attack of an oxime on the enzyme acetylcholinesterase with cleavage of nitrile and dialkyl phosphate

the contaminated person. Furthermore, the analytical detection of different metabolites of OP*s* in urine has often been described. In subsequent sections, these different detection methods for biological samples (blood, urine and tissues) with their advantages and disadvantages are illustrated.

Analytical methods concerning OP*s* do not focus exclusively on the determination or quantitation of the OP*s* themselves, the latter often being an integral part of toxicological screenings for drugs, pharmaceuticals and their metabolites: Pelander *et al.*[17] developed a toxicological screening method using 1 mL of urine sample and Turbolon spray liquid chromatography–time-of-flight mass spectrometry (LC-TOFMS) in the positive ionization mode with continuous mass measurement. The substance database consisted of exact monoisotopic masses for 637 compounds, for 392 of which an LC retention time was available. Lacassie *et al.*[3] presented sensitive and specific multiresidue methods for the determination of pesticides of various classes in human biological matrices. These methods involved rapid solid-phase extraction using polymeric material. For volatile pesticides such as OP*s* and organochlorines, GC-MS was employed, and for thermolabile and polar pesticides such as carbamates, LC-MS was used. A GC-MS method for the determination of 15 pesticides in whole blood was developed by Frenzel *et al.*[18] For analysis, whole blood was hemolyzed and subsequently deproteinized. After extraction of the supernatant, the pesticide concentration was determined using GC-MS. In addition to the above methods, a multitude of additional GC-MS and LC-MS methods for the determination of pesticides, especially OP*s*, in different matrices such as water, vegetables and fruits have been described. A discussion of these methods, however, would go beyond the scope of this chapter.

9.2.5.1 Serum and erythrocyte cholinesterase activity

In addition to the specific acetylcholinesterase that is situated in the erythrocytes, an unspecific cholinesterase called 'pseudo-cholinesterase' exists in plasma. This unspecific cholinesterase hydrolyzes the molecule ACh significantly slower than other cholinesterases, which is why the activity of this cholinesterase is of less importance for an intoxication with OP*s*. Nonetheless, the measurement of the esterase activity comprises both esterases, which results in misleading data.

Methods for the detection of cholinesterase activity can generally be divided into four groups, electrometric,[19–23] colorimetric,[24–28] titrimetric[29,30] and tintometric[31,32] methods, with the last being the one mainly used in the field. The colorimetric method developed by Ellman *et al.*[24] is the most frequently used procedure. Titrimetry is extremely accurate and precise, but rarely used because of its high cost and complexity. Electrometric methods are less sensitive than colorimetric methods and less accurate than titrimetric methods.

The colorimetric method is based on the hydrolysis of the substrate acetylthiocholine to acetate and thiocholine as performed by the cholinesterase. Thiocholine is then reacted with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) to form a yellow anion (5-thio-2-nitrobenzoate). The latter is quantitated by spectrometric analysis at 405 nm, with the concentration being proportional to the cholinesterase activity in the given sample. Also for a few days post-mortem the cholinesterase activity in different tissues is measurable.[33]

Despite the advantages of biologically monitoring the exposure to pesticides via the degree of cholinesterase inhibition, the enormous intra- and inter-individual variations in

cholinesterase activity pose a serious problem. For example, plasma cholinesterase activity may be depressed by cirrhosis, chronic hepatitis or other liver diseases and also by drug use and abuse.[34] There are no differences in cholinesterase activity associated with race in general, but plasma cholinesterase activity in North American black races has been reported to be lower than in caucasians of the same sex.[24] Any results obtained using enzyme inhibition monitoring should therefore preferably be compared with values for the innate cholinesterase activity of each subject, if possible the median of three samples obtained in a pre-exposure period.[35]

9.2.5.2 Unmetabolized OPs

In cases of poisoning, blood and/or urine or gastric contents can be tested for unmetabolized OP*s* to confirm exposure. In fatalities, the CNS and other tissues such as liver or kidney should be screened for the same unchanged substances. In the literature, different screening methods for OP*s* and analytical methods for the detection of single components have been described. A review of available analytical methods and their main characteristics is given in Table 9.3.

Screening methods

In cases where an acute intoxication with OP*s* is suspected, it is necessary to rapidly obtain information about the absorbed substance, which is why the application of a screening method is very suitable. So far a small number of different screening techniques using different principles of sample preparation and/or detection have been developed. Liu *et al.*[36] presented a simple and rapid method for the isolation of 11 OP*s* from human urine and plasma with Sep-Pak C_{18} cartridges. Detection of the pesticides was achieved by wide-bore capillary gas chromatography with flame ionization detection (GC-FID). Pesticide-containing samples were initially diluted with water before being applied to the cartridges. Elution of analytes was achieved using chloroform/2-propanol. Separation of most compounds from each other and from impurities was generally satisfactory as judged from the gas chromatograms. The recoveries were close to 100% for many compounds and none was less than 60%.

This method is a good example of the evolution in pesticide sample preparation: today, liquid–liquid extraction of aqueous samples is often replaced by solid-phase extraction (SPE), which has its commercial roots in the late 1970s. Since then, it has become a common and effective technique for extracting analytes from complex matrices. In contrast to liquid–liquid extraction, the solvent consumption in SPE is low, long evaporation times for solvent removal therefore cease to apply, interfering impurities are removed to a larger extent and, in addition, the sample is concentrated. Another advantage of SPE is the flexible application: SPE can be performed 'off-line' i.e. manually, semi-automated (e.g. using the ASPEC from Gilson), or 'on-line' (using Prospect from Spark Holland, OSP-2 from Merck, etc.), which is especially useful for large-scale operations.[1]

Despite the advantages of SPE, it has to be considered that the parallel extraction of different analytes, such as in screening procedures, can be problematic: due to the distinct physico-chemical properties of various analytes, the universal use of a single SPE matrix for all substances can result in low recoveries for individual analytes. This issue will be addressed in the context of the following examples.

Table 9.3 Analytical procedures for the determination of organophosphorous pesticides in biological samples

Analyte	Matrix	Sample preparation	Apparatus	LOD	Recovery (%)	RSD (%)	Reference
Azinphos-ethyl	Blood, urine, gastric lavage liquid	Extraction with benzene	GLC-FPD, TLC	1 μg L^{-1}	92–102	—	44
Chlorpyriphos	Serum, gastric content	Extraction with diethyl ether	GC-MS, SIM *m/z* 314	25 ng mL^{-1}	102–104	2–3	46
	Blood	Extraction with acetone	GC-FPD	—	—	—	47
Chlorpyrifos-methyl, dichlorvos	Blood, tissues	Extraction with diethyl ether	GC-FID	—	—	—	45
Diazinon	Blood, tissues	Extraction with acetonitrile and hexane	GC-FID	—	—	—	93
Dichlorvos	Blood, tissues	Extraction with ethyl acetate	GC-FID and GC-MS	—	—	—	51
Dichlorvos	Blood	SPMEM (solid-phase micro-extraction membrane)	GC-MS (SIM)	—	—	—	52
Dichlorvos and malathion	Blood, tissues	Extraction with methanol and SPE purification	GC-NPD	12 ng mL^{-1} (malathion); 14 ng mL^{-1} (dichlorvos)	>75	0.1–8.5	50
Dichlorvos and metrifonate	Blood	Extraction with toluene	GC-MS and GC-NPD	0.8 μmol L^{-1} (metrifonate); 50 nmol L^{-1} (dichlorvos)	87–99	2–9	48
Disulfoton	Plasma	Extraction with hexane[a]	GC-FID	—	—	—	94
	Blood, tissues	SPE Extrelut[b]	GC-FPD and GC-MS	0.2 ng	104 ± 2.7	—	95

(*continued*)

Table 9.3 (*Continued*)

Analyte	Matrix	Sample preparation	Apparatus	LOD	Recovery (%)	RSD (%)	Reference
Fenitrothion[c]	Blood, gastric lavage liquid	SPE Extrelut	GC-FID, GC-MS and GC-FPD	—	—	—	58
Fenitrothion	Blood, tissues	—	GC-FPD	0.005 μg mL^{-1}		5	57
Fenthion	Blood, urine, tissues	SPE C_8	HPLC–DAD and GC-MS (EI) scan[d]	0.25 μg mL^{-1} (HPLC–DAD), 0.10 μg mL^{-1} (GC-MS)	71 ± 12.5 (blood), 46 ± 8 (liver)	—	59
Malathion	Blood	HS-SPME	GC-MS (EI)	1 μg g^{-1}	86.4 ± 6.3	4	53
	Blood, tissues	Extraction with diethyl ether	GC-MS (EI)	1 μg g^{-1}	—	—	55
Methamidophos	Blood, tissues	Extraction with solvent	GC-NPD	82 ng mL^{-1}	—	—	96
	Blood, brain of exposed animals	Treatment with acetonitrile and ethyl acetate	GC-MS (EI), SIM *m/z* 94	—	75–80	—	97
Metrifonate	Serum	SPE C_{18}	GC-flame thermionic detection	2.5 ng mL^{-1}	99.4 ± 2.9	—	98
Oxydemeton-metyl	Blood, tissues	SPE C_{18}	LC-MS (ion trap)	1 ng mL^{-1}	79–88	1–6	60
Parathion, paraoxon	Plasma, tissues	Extraction with isooctane (plasma), hexane (tissues)	GC-EC	—	79.4–110.3	1–9	56

Parathion-methyl	Blood, tissues	HS-SPME	GC-NPD	1 ng mL^{-1}	46–54	0.9–5.1	54
Screening	Blood, urine,	Extraction with	GC-NPD	—	50–133	—	39, 40
(23 substances)	serum	1 mL toluene	GC-MS (EI)				
(22 substances)	Blood	HS-SPME	GC-MS (EI)	0.01–0.3 μg g^{-1}	70–95	3–31	42
(11 substances)	Plasma, urine	SPE C_{18}	GC-FID	—	>60	—	36
(11 substances)	Serum, urine	Deproteinization with acetonitrile	HPLC–DAD (230 nm)	0.05–6.8 μg mL^{-1}	97.4–101.4	0.5–6.5 (serum), 1.4–3.1 (urine)	37
(25 substances)	Serum	SPE C_{18} or extraction with *n*-hexane–ethyl acetate	HPTLC	0.12–1.1 μg mL^{-1}	27.6–101.8	1.3–14.4	38
(10 substances)	Blood	SPE C_{18}	GC-NPD	—	71.8–97.1	—	41

[a] After oxidation with potassium permanganate.
[b] After extraction oxidation with potassium permanganate and another extraction.
[c] Urine samples were analyzed with the same procedure for determination of 3-methyl-4-nitrophenol, aminofenitrothion, aminofenitroxon, acetylaminofenitroxon and *S*-methylfenitrothion.
[d] After derivatization with diazomethane.

In contrast to the above mentioned GC methods, Cho *et al.*[37] developed a simple and rapid method for measuring 11 OP*s* and one metabolite of fenitrothion in serum and urine using high-performance liquid chromatography (HPLC) with diode-array detection (230 nm). Sample preparation consisted of protein precipitation with acetonitrile. Without further cleaning, the deproteinated sample was directly injected into the HPLC system with a C_{18} column and acetonitrile/water as the mobile phase. Detection and identification of the compounds were achieved by UV spectral data analysis. Using this approach, two cases of fatal poisoning were examined: starting with the sample delivery, a period of only 2 h was needed for data acquisition and interpretation in both cases, thereby proving this method to be simple and rapid enough to be applied to biological samples in an emergency.

Futagami *et al.*[38] published a method for the identification of 25 commonly used OP*s* in human serum using high-performance thin-layer chromatography (HPTLC). The sample preparation method includes both liquid–liquid and solid-phase extraction. The OP*s* were separated on plates with three different developing systems within 6–18 min and detected by means of UV radiation and chromogenic reactions with 4-(4-nitrobenzyl)pyridinetetraethylenepentamine reagent or palladium chloride reagent. The limits of detection (LODs) of this method are within the range of those of other GC and HPLC methods (see Table 9.3). The recoveries of dichlorvos and trichlorfon using SPE were markedly lower than those using liquid–liquid extraction. As both substances are highly water soluble, these differences can be attributed to unwanted elution of the analytes during a water washing step in the SPE method.

Tarbah *et al.*[39] also used a single-step liquid–liquid extraction method with toluene for the determination of 23 OP*s* in urine, blood, serum and food samples using GC with nitrogen–phosphorus selective detection (GC-NPD) and electron ionization mass spectrometry (GC-EIMS). The recoveries for spiked human plasma ranged between 50% (demethoate) and 133% (dialifos). Akgur *et al.*[40] successfully measured OP concentrations in 28 cases of acute OP poisoning using the above application.

In contrast, Garcia-Repetto *et al.*[41] presented a GC-NPD method for the identification and quantification of 10 pesticides in human blood using SPE on C_{18} cartridges. This method, with mean recoveries between 71.8 and 97.1%, replaced an earlier method involving liquid–liquid extraction of a mixture of *n*-hexane and benzene.

Musshoff *et al.*[42] used headspace (HS) solid-phase microextraction (SPME) in combination with GC-MS for the determination of 22 OP*s* in human blood. The recoveries for spiked blood samples ranged between 70 and 95%. With this rapid HS-SPME method, no elaborate sample preparation is necessary. A carry-over of matrix compounds on to the GC column is effectively excluded in HS-SPME, hence the chromatograms did not show any interference (Figure 9.4). Advantages of this matrix-free chromatographic technique are a prolonged lifespan of the GC columns and liners and a low cleaning frequency of the ion source, as in HS-SPME these components are not burdened with analytically irrelevant contaminants. With the above procedure, four cases of intoxication were detected within 12 months, the OP*s* involved being parathion-ethyl on two occasions, malathion and bromophos-ethyl.

Recently, LC-MS techniques have become increasingly popular as an approach to analytical problems. In keeping with this development, Saint-Marcoux *et al.*[43] described a general unknown screening procedure for serum samples, using SPE and subsequent liquid chromatography–electrospray mass spectrometry (LC-ESIMS). This method allows for the detection and identification of pesticides and also other drugs and toxic compounds.

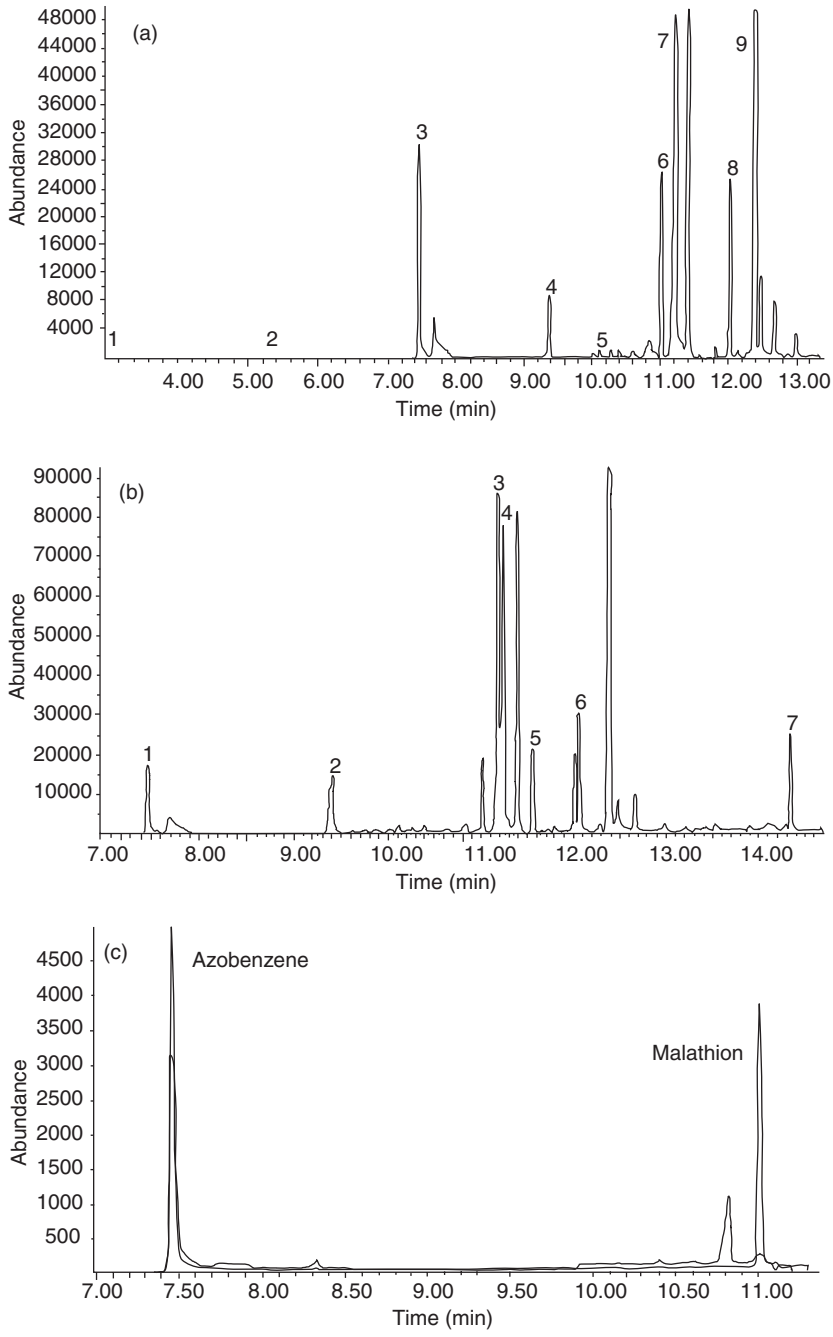


Figure 9.4 SIM chromatograms with SPME-GC-MS.[42] (a) and (b) blood samples spiked with two different mixtures of OP*s*. (a) 1-Dichlorvos; 2-mevinphos; 3-azobenzene; 4-diazinon; 5-parathion-methyl; 6-malathion; 7-chlorpyriphos; 8-chlorfenvinphos; 9-bromophos-ethyl. (b) 1-azobenzene; 2-disulfoton; 3-fenthion; 4-parathion-ethyl; 5-bromophos-methyl; 6-quinalphos; 7-edifenphos. (c) Blood sample from a person intoxicated with malathion

In summary, very different screening methods for OP*s* have been described in the literature. Although some authors prefer a conventional liquid–liquid extraction method, SPE is also often used. Sometimes the recoveries obtained are comparatively low using SPE, but this kind of sample preparation offers other advantages, e.g. low solvent consumption. Separation of analytes has been carried out with HPTLC in addition to GC or HPLC in combination with different detectors, and more recently using LC-MS. The LOD and recovery values of all methods presented are comparable, hence analytical laboratories can therefore select any of the above methods according to the equipment available.

Detection of individual compounds

In cases of acute OP poisoning, the adsorbed substance can often be identified using the label on the respective pesticide container. On these occasions, no screening but only a confirmatory test for the individual target substance has to be performed. In Table 9.3 a selection of such specific determination methods is listed. In the following section, exemplary extraction and detection methods for individual substances will be discussed in more detail.

Marques[44] detected the OP azinphos-ethyl in blood, urine and liquid from gastric lavage in 35 cases of intoxications. The concentrations determined were subsequently correlated with the degree of illness of the patients. The samples were extracted with benzene and then analyzed using GC with flame photometric detection (FPD) with a recovery of between 92 and 102%.

Chlorpyrifos is an OP with a higher LD_{50} than azinphos-ethyl (see Table 9.2) and has been detected using GC-FPD and more recently GC-MS.[45–47] All three methods (see Table 9.3) employed a liquid–liquid extraction step using different solvents (diethylether, acetone). Nolan *et al.*[47] investigated with the above approach the kinetics of chlorpyrifos and its principal metabolite in six healthy male volunteers. In contrast, Martinez *et al.*[46] reported a mild case of self-poisoning with a chlorpyrifos formulation following oral ingestion.

The determination of dichlorvos (DDVP), another OP, is often accomplished in combination with other OP*s*. For example, Villen *et al.*[48] developed an analytical method for the simultaneous quantification of metrifonate, an OP used in the treatment of *Schistosoma haematobium*, and dichlorvos in whole blood. Metrifonate is chemically unstable as in neutral and alkaline aqueous solutions it is transformed to dichlorvos and hydrolysis products.[49] The method employs a liquid–liquid extraction with toluene and subsequent GC-MS for detection. The within-assay coefficients of variation were 2 and 5% at 225 and 50 nmol L^{-1} (LOD), respectively. Garcia-Repetto *et al.*[50] analyzed DDVP and malathion in blood and tissues of rats using GC-NPD. Samples were homogenized, extracted with cold methanol and purified by SPE on a C_{18} column. The detection limit reached with this method was comparatively low (14 ng mL^{-1}). Using the above methodology, the authors were able to propose a novel approach to toxicokinetic modeling: rather than using only data from blood or plasma, as was usually done, this work categorized several different tissues into compartments. Shimizu *et al.*[51] used ethyl acetate as extraction solvent for the determination of DDVP in serum and tissues samples by GC-MS and GC-FID. No validation data were given. Yang and Xie[52] presented a new, simple and solventless preparation technique, with a solid-phase micro-extraction membrane (SPMEM), for the extraction of DVPP from blood. SPMEM merges sampling, extraction and concentration into a single step and combines the advantages of both the solid-phase micro-extraction (SPME) and membrane separation. For extraction the SPMEM strips were incubated in the blood sample for 1 h, and were then

taken out, washed and dried with filter-paper. In the next step, the adhering compounds were extracted with ethanol and analyzed using GC-MS. The authors were able to show that the proposed method is suitable for the detection of DDVP, but they did not present any validation data.

In addition to this novel SPMEM method, two SPME methods for the detection of the OP*s* malathion[53] and methyl-parathion[54] have also been described. As already discussed above, Musshoff *et al.*[42] used this technique for the screening of 22 OP*s*. Tsoukali *et al.*[54] examined post-mortem biological samples (i.e. tissues and blood) from a woman fatally poisoned by intravenous injection of methyl-parathion. Methyl-parathion was extracted on polyacrylate fibers and analyzed using GC-NPD. The recoveries were found to be low, 46% in whole blood and 53–54% in tissues. Namera *et al.*[53] also applied the SPME technique when extracting malathion from blood. The SPME method implied exposing the extraction fiber in the headspace of a vial heated at 90°C for 5 min. The low background noise levels in the selected-ion monitoring (SIM) (GC-MS) chromatograms were due to the high absorption potential of the SPME fiber for the vaporized drug. The authors reported recoveries of around 86%. In contrast, Thompson *et al.*[55] used conventional liquid–liquid extraction for the determination of malathion in a case of fatal OP poisoning. Whole blood and homogenized tissues samples were extracted three times with 1 mL aliquots of methyl butyl ether. The analyses were performed using a GC-MS system. The LOD of 1 $\mu g\ g^{-1}$ is similar to the value obtained using the SPME technique.

The OP parathion and its active metabolite paraoxon were simultaneously determined in plasma and tissues by Abbas and Hayton.[56] Their method involved a simple liquid–liquid extraction with isooctane with subsequent GC-electron capture detection and yielded recoveries from 79–110% for tissues and 91–100% for plasma. Fenitrothion, another OP, was detected in blood samples and tissues by Yoshida *et al.*[57] using a GC-FPD method. Kojima *et al.*[58] reported a case of attempted suicide by ingestion of a fenitrothion emulsion: fenitrothion and its metabolites were extracted from body fluids by an Extrelut column extraction method and subsequently detected by GC with either FID or FPD. Data were confirmed using GC-MS. No validation data were given.

In addition to the often used GC coupled with different detection systems for the determination of OP*s*, the use of HPLC has also been described. For example, Meyer *et al.*[59] presented an analytical method for the detection of fenthion in post-mortem samples by HPLC with diode-array detection (DAD) preceded by SPE on C_{18} columns. Peak identification is accomplished via comparison with the UV spectrum of the standard substance. Comparison of this SPE method with traditional liquid–liquid extraction shows similar recoveries for both procedures ($71 \pm 12.5\%$). The LOD is lower using GC-MS ($0.10\ \mu g\ mL^{-1}$) than HPLC–DAD ($0.25\ \mu g\ mL^{-1}$). A completely validated LC-MS method for the determination of oxymeton-methyl (ODM) and its main metabolite demeton-*S*-methylsulfon (DSMS) in human blood and various tissue samples was established by Beike *et al.*[60] After SPE using C_{18} cartridges, the extracts were analyzed by HPLC-MS (ESI ion trap, positive mode). The LOD was with 1 $ng\ g^{-1}$ blood for the parent substance ODM and 2 $ng\ g^{-1}$ blood for the metabolite DSMS, comparatively low. The recoveries were in the 82–88% range for ODM and 79–84% for DSMS.

In summary, for the determination of single OP compounds in blood or organic tissues, different GC methods in combination with various detection methods (MS, NPD, FPD) are used. For sample preparation, some authors prefer the traditional liquid–liquid extraction, others use SPE on conventional C_{18} materials. In addition to these methods, novel techniques

such as SPMEM or SPME have also been described. The latter offer the advantage of extremely reduced background noise in the acquired chromatograms. In addition to GC, analytical methods involving HPLC and LC-MS have also been described. Judging by the validation data, the LC-MS approach outperforms the other methods, which is why it is to be expected that LC-MS will gain popularity in the field of OP analysis.

9.2.5.3 Alkylphosphate metabolites

OP*s* are unstable in aqueous solution and even more so in blood owing to the presence of esterase. After ingestion, OP*s* decompose in the human body to yield alkyl phosphates. Typical degradation products are dimethyl phosphate (DMP), dimethyl phosphorothionate (DMTP), dimethyl phosphorodithioate (DMDTP), diethyl phosphate (DEP), diethyl phosphorothionate (DETP) and diethylphosphorodithioate (DEDTP), which are formed by the hydrolysis of the ester bond in the OP molecule. In Figure 9.5 the chemical structures of the respective alkylphosphates are shown.[61]

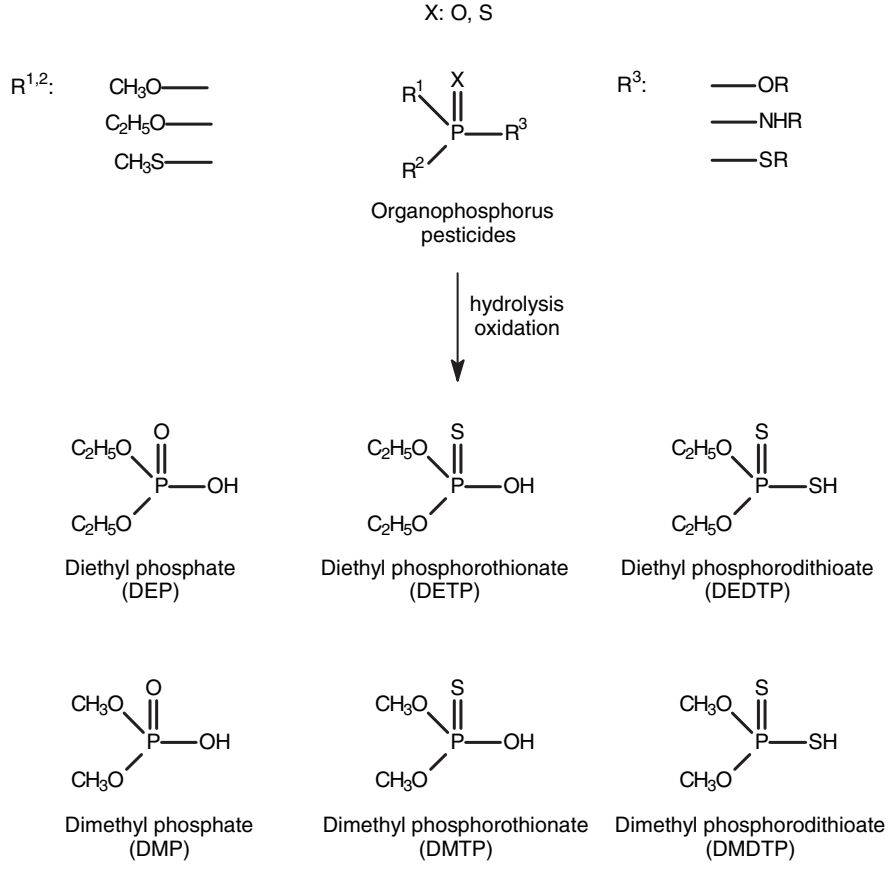


Figure 9.5 Dialkylphosphate metabolites

The most commonly used analytical methods for the quantification of the six alkyl phosphates are based on their purification from acidified samples via liquid–liquid extraction. This is followed by their conversion to volatile derivatives which can be detected using GC-MS or GC-FPD. In Table 9.4 some analytical procedures for the determination of the above substances in biological samples are listed.

In contrast to the determination of the unmetabolized OP*s*, the determination of their alkyl phosphate metabolites is more complex, as it includes several sample preparation steps for which mostly liquid–liquid extraction is used. Tarbah *et al.*[62] presented a GC-MS method following toluene extraction of the phosphamidon metabolite DMP from blood, serum, urine and gastric fluid after intoxication with the mother substance. The average recovery of DMP in plasma samples was 60% with an LOD below 0.06 mg L^{-1}. Whyatt and Barr[63] used a GC-MS-MS method for the determination of the six alkyl phosphate metabolites in postpartum meconium as a potential biomarker of prenatal OP exposure. Following lyophilization, the meconium was extracted with methanol and after the subsequent derivatization it was analyzed using GC-MS-MS. The recoveries varied between 18 and 66% depending on the compound. The LODs with this method are in a similar range to those for other methods. Hardt and Angerer[64] also used liquid–liquid extraction to enrich the six metabolites from acidified urine into a mixture of diethyl ether and acetonitrile. After derivatization, the extracts were analyzed using GC-MS, giving higher LODs than reported by Tarbah *et al.*[62] and Whyatt and Barr[63] (see also Table 9.4).

Barr *et al.*[65] and Bravo *et al.*[66] used a different technique for sample preparation and determination of alkyl phosphates in urine: the urine was spiked with internal standard and then concentrated to dryness via azeotrope codistillation with acetonitrile. The residue was dissolved in acetonitrile, derivatized and then analyzed using GC-MS. Both studies reported satisfactory validation data with coefficients of variation of $<20\%$.

Moate *et al.*[67] and Kupfermann *et al.*[68] presented analytical methods involving SPE for the extraction of alkyl phosphates from urine. Moate *et al.*[67] used SPE for sample cleanup followed by azeotrope distillation and then derivatization of the analytes. Determination of the analytes was achieved using GC-FPD. In contrast, Kupfermann *et al.*[68] purified the derivatized extract of urine samples from a case of OP poisoning on silica SPE columns followed by quantitative analysis by GC-MS. The LODs ranged from 3 to 6 ng mL^{-1}.

For the detection of the alkyl phosphate metabolites using GC-MS, derivatization of the compounds is necessary. Most often the derivatization agents are diazoalkanes,[69,70] triazenes,[71] anilinium hydroxide[72,73] and pentafluorobenzyl bromide.[64,74–78] The last compound has the advantage of yielding a single reaction product for both DMTP and DETP, whereas the others form two isomers for each metabolite. Diazomethane derivatization is popular because of its rapid and efficient reaction with dialkyl phosphates. However, it is not a suitable reagent to measure DMP accurately: on derivatization with diazomethane, DMP and inorganic phosphate, which is endogenous in human urine, both form the same product, i.e. trimethyl phosphate.[73]

9.2.5.4 Other metabolites

In addition to the above-described alkyl phosphates, a few less common metabolites can be mentioned, e.g. 3,5,6-trichloro-2-pyridinol (TCP). TCP is a major product of esterase cleavage of the OP*s* chlorpyrifos and chlopyrifos-methyl.[47,79,80] Urinary TCP, like other

Table 9.4 Analytical procedures for the determination of alkyl phosphates in biological samples

Analyte	Matrix	Sample preparation	Apparatus	LOD	Recovery (%)	RSD (%)	Reference
DMP, DEP, DMTP, DETP, DEDTP, DMDTP	Urine	Extraction with solvents, evaporations, SPE	GC-MS	3–6 ng L^{-1}	—	3.8–11.2	68
DMP	Blood, urine, gastric fluid	Extraction with toluene	GC-MS	<60 μg L^{-1}	60	1.9–9.3	62
DMP, DEP, DMTP, DETP, DEDTP, DMDTP	Urine	Azeotropic codistillation with acetonitrile	GC-MS	0.58 μg L^{-1} (DMP), 0.18 μg L^{-1} (DMTP), 0.08 μg L^{-1} (DMDTP), 0.2 μg L^{-1} (DEP), 0.09 μg L^{-1} (DETP), 0.05 μg L^{-1} (DEDTP)	—	—	65
DMP, DEP, DMTP, DETP, DEDTP, DMDTP	Meconium	Extraction with methanol, derivatization	GC-MS-MS	0.51 μg L^{-1} (DMP), 0.18 μg L^{-1} (DMTP), 0.08 μg L^{-1} (DMDTP), 0.2 μg L^{-1} (DEP), 0.09 μg L^{-1} (DETP), 0.05 μg L^{-1} (DEDTP)	18–66	—	63
DMP, DEP, DMTP, DETP, DEDTP, DMDTP	Urine	Azeotropic codistillation with acetonitrile, derivatization	GC-MS-MS	1.0 mg L^{-1}(DMP), 1.6 mg L^{-1}(DMTP), 0.78 mg L^{-1}(DMDTP), 0.72 mg L^{-1}(DEP), 0.38 mg L^{-1}(DETP), 0.25 mg L^{-1}(DEDTP)	17–65	11.2–19.7	66
DMTP, DETP, DEDTP, DMDTP	Urine	Anion-exchange disk extraction	GC-FPD	9 μg L^{-1} (DMTP), 12 μg L^{-1} (DMDTP), 12 μg L^{-1} (DETP), 13 μg L^{-1} (DEDTP)	17.9–77.2	3.2–13.2	99

DMP, DEP, DMTP, DETP, DEDTP, DMDTP	Urine	Freeze-drying and derivatization	GC-FPD	50 μg L^{-1} (DMP), 10 μg L^{-1} (DMTP), 10 μg L^{-1} (DMDTP), 10 μg L^{-1} (DEP), 5 μg L^{-1} (DETP), 5 μg L^{-1} (DEDTP)	—	6.1–23.0	100
DMP, DEP, DMTP, DETP, DEDTP, DMDTP	Urine	Extraction with acetonitrile	GC-FPD	2 μg L^{-1} (DMTP, DMDTP, DETP, DEDTP), 3 μg L^{-1} (DMP, DEP)	85.8–101.0	1.9–11.4	74, 101
DMP, DEP, DMTP, DETP, DEDTP, DMDTP	Urine	Extraction with diethyl ether and acetonitrile	GC-MS	1 μg L^{-1} (DMTP, DMDTP, DETP, DEDTP, DEP), 5 μg L^{-1} (DMP)	68–114	9.7–15.5	64
DMP, DEP, DMTP, DETP, DMDTP	Urine	SPE	GC-FPD	10 μg L^{-1} (DMP, DEP) 2 μg L^{-1} (DMTP, DMDTP, DETP)	57–118	4.4–46.4	67

OP metabolites, may be used as an indicator of exposure to chlorpyrifos and chlorpyrifos-methyl, although the available data are still insufficient to define biological exposure limits. For the detection of TCP in urine, predominantly reversed-phase HPLC with UV detection and GC are used. In Table 9.5, exemplary analytical methods are listed. For example, Bartels and Kastl[81] presented a sensitive GC-negative ion chemical ionization mass spectrometric (GC-NCI-MS) method for measuring levels of TCP in human urine. The metabolite was isolated from urine by acid hydrolysis of urine aliquots followed by diethyl ether extraction. The LOD was very low (0.5 $\mu g\,mL^{-1}$) and the recoveries varied between 80.6 and 89.9%. Subsequently, Hill *et al.*[82] presented a method for measuring 12 analytes, pesticides or their metabolites, in urine. The sample preparation involved enzyme hydrolysis and solvent extraction through the use of laboratory robotics, followed by phase-transfer catalysis derivatization and silica clean-up. Samples were analyzed by GC-MS and the LODs were 1 $\mu g\,mL^{-1}$ for most analytes. Aprea *et al.*[83] also used GC with different detection methods (MS or ECD) for the determination of urinary TCP in the general Italian population. The samples were hydrolyzed using hot acid, extracted with hexane and then derivatized. In contrast, Chang *et al.*[84] used a much simplified sample preparation scheme with subsequent HPLC analysis for the determination of TCP in urine. The detection limit was not as low as that obtained with GC-MS[81] and GC-ECD.[47] It was, however, about 5–20 times more sensitive than the HPLC study reported by Sultatos *et al.*[85], who reported an HPLC method with a sample preparation involving an ethyl acetate extraction and evaporation step.

Another product of esterase cleavage is *p*-nitrophenol (PNP), which is generated in the human body by the degradation of parathion, parathion-methyl and parathion-ethyl.[86] Some methods for the determination of PNP in urine are summarized in Table 9.5. PNP can, for example,[87] be determined in the urine of occupationally exposed subjects by means of GC-ECD. Sample preparation involves acid hydrolysis, extraction with diethyl ether, derivatization with diazoethane and purification on silica gel columns. The LOD was 20 $\mu g\,L^{-1}$ with a recovery of 85–98%. This method has also been used in a study on selected pesticide residues and metabolites in urine from a survey of the US general population.[88] In the context of another survey study, a more complicated technique to detect PNP in urine has recently been used by Hill *et al.*[89] involving GC-MS-MS with positive chemical ionization after derivatization with 1-chloro-3-iodopropane. The authors also used this method for the determination of the metabolite TCP and 10 other analytes in urine.[82] Sample preparation involved hydrolysis with β-glucuronidase, several extraction steps using different solvents and purification by SPE (silica gel column). The LOD was 1 $\mu g\,L^{-1}$ with an inter-assay RSD of 24%.[90] Barr *et al.*[86] developed a rapid, high-throughput, selective method for quantifying PNP using LC-MS-MS. Sample preparation is less complicated than in the other methods: after hydrolysis with β-glucuronidase/sulfatase the hydrolyzate was acidified, extracted with dichloromethane and then analyzed. The method LOD was calculated to be 25 $\mu g\,L^{-1}$. Although this LOD is higher than that of previously published methods (see above), the method was suitable to categorize samples into the various priority levels required for public health intervention.

The ester cleavage of fenitrothion leads to the formation of the metabolite 3-methyl-4-nitrophenol (MNP). Shafik *et al.*[87] used GC-ECD for the detection of MNP in occupationally exposed subjects. Sample preparation involved extraction with diethyl ether, derivatization with diazoethane and purification on silica gel columns. The LOD was determined as 50 $\mu g\,L^{-1}$ with a recovery of 88–98%.

Table 9.5 Analytical procedures for the determination of different metabolites of organophosphorus pesticides in urine

Analyte	Hydolysis/sample preparation	Apparatus	LOD	Recovery (%)	RSD (%)	Reference
TCP	Hot HCl and extraction with diethyl ether	GC-NCI-MS	0.5 μg L^{-1}	80.6–89.9	0.4–2.6	81
	Hot HCl and extraction with toluene	GC-MS, GC-ECD	MS: 1.5 μg L^{-1} ECD: 1.2 μg L^{-1}	— ECD: 91.7	MS: 8.2 ECD: 8.7	83
	Hot HCl and extraction with toluene	GC-ECD	5.0 μg L^{-1}	71.8	7	102
	Hot H_2SO_4 and SPE C_{18}	GC-ECD	10 μg L^{-1}	—	—	47
	β-Glucoronidase and extraction with 1-chlorobutane–diethyl ether and 1-chloro-3-iodopropane	GC-MS-MS	1 μg L^{-1}	—	24	82
	Hot acid hydrolysis and perchloric acid	HPLC-UV	2.2 ng per 20-μL injection	102 ± 15	<10	84
TCP in plasma and tissues	Extraction with ethyl acetate	HPLC-UV (290 nm)	—	85 ± 8	—	85

(*continued*)

Table 9.5 (*Continued*)

Analyte	Hydolysis/sample preparation	Apparatus	LOD	Recovery (%)	RSD (%)	Reference
PNP	Hot acid hydrolysis and extraction with diethyl ether	GC-ECD	20 μg L^{-1}	85–98		87
	β-Glucoronidase and extraction with 1-chlorobutane–diethyl ether and 1-chloro-3-iodopropane	GC-MS-MS	1 μg L^{-1}	—	—	89
	β-Glucoronidase/ sulfatase, addition of HCl and extraction with dichloromethane	LC-MS-MS	25 μg L^{-1}	85 ± 8	—	86
MNP	Hot acid hydrolysis and extraction with diethyl ether	GC-ECD	50 μg L^{-1}	88–98	—	87
MCA and DCA	Extraction with diethyl ether, SPE	GC-FPD	MCA: 5 μg L^{-1} DCA: 20 μg L^{-1}	MCA: 98–104 DCA: 98–101	—	91
	Alkaline hydrolysis	GC-FPD	—	—	—	103

The respective mono- and dicarboxylic acid [malathion α-monocarboxylic acid (MCA) and malathion dicarboxylic acid (DCA)] are the main metabolites of the OP malathion generated by the hydrolysis of one or both of the side-chain diethylsuccinic esters.[35,91] Bradway *et al.*[91] presented a GC-FPD method for the determination of both substances in spiked urine and also in urine from rats exposed to malathion at five levels (see also Table 9.5). The clean-up procedure was modified based on a method developed by Shafik *et al.*[92] and involves extraction of acidified urine with diethyl ether, derivatization with diazomethane and purification on a silica gel column. Another method used by some authors involves the conversion of MCA and DCA into the respective alkyl phosphate metabolites DMTP and DMDTP (see Section 9.2.5.3) by alkaline hydrolysis. These compounds can then be derivatized with pentafluorobenzyl bromide and analyzed using GC-FPD.

9.3 CONCLUSION

In this chapter a short introduction to organophosphorus pesticides (OP*s*) and their mechanism of action has been given. Symptoms of intoxication in humans following exposure have been discussed along with the signs of progressing intoxication, long-term risks of OP poisoning and possible therapy regimes. Furthermore, a wide range of studies concerned with the analytical aspects of biological monitoring of OP exposure were reviewed.

Pesticides can be subdivided into three groups based on their mechanism of action. In addition to pyrethroid and organochlorine compounds, the OP*s* represent a large group within this substance class. OP*s* act through inhibition of the enzyme acetylcholinesterase in the CNS that is essential for the hydrolysis of the neurotransmitter acetylcholine. Symptoms of intoxication appear a few minutes after inhalation or swallowing and 2–3 h after cutaneous resorption of the respective OP. The more severe symptoms of OP intoxication include coma, tremor, muscle cramps and others. In cases of intoxication, the first measure is to stabilize respiration and circulation. In the next step, treatment with oximes provides the opportunity to reactivate the acetylcholinesterase, thereby alleviating the cholinergic symptoms.

For the detection of OP*s*, e.g. in the context of a medical diagnosis, many different analytical methods have been reported. One possibility is to measure the cholinesterase activity as an acute intoxication characterized by a 20% decrease in acetylcholinesterase activity. Furthermore, analytical methods involving OP*s* do not exclusively focus on the determination of the OP*s* themselves; the latter are rather an integral part of several toxicological screening procedures.

In cases of poisoning, blood and/or urine or tissue samples can be tested for unmetabolized OP*s*. A review of available analytical methods and their main characteristics is given in Table 9.3. For the screening methods and for the quantification of single compounds, often GC techniques in combination with various detection methods are used. Furthermore, several HPLC methods have been described. For sample preparation, some authors prefer the traditional liquid–liquid solvent extraction, whereas others use SPE on conventional materials. In addition to these, novel techniques such as SPME or SPMEM are also used.

In addition to the determination of the unmetabolized compounds to confirm exposure, the quantification of their metabolites, e.g. alkyl phosphate metabolites or other characteristic metabolites, is a possibility. The most commonly used analytical methods for the

quantification of the six alkyl phosphates are based on their purification from acidified samples via liquid–liquid extraction. This is followed by their conversion to volatile derivatives which can be detected using GC-MS or GC-FPD (see Table 9.4). A review of analytical methods for the determination of other metabolites, e.g. 3,5,6-trichloro-2-pyridinol (TCP), the major product of ester cleavage of chlorpyrifos, is given in Table 9.5. These metabolites can be quantified with GC but also using HPLC or HPLC-MS methods.

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10

Chromatographic Analysis of Nerve Agents

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10.1 INTRODUCTION

The word 'paralytic' is medically defined as 'relating to paralysis' or 'a person affected with paralysis'.[1] The analyses of paralytics in the clinical laboratory include therapeutic drug monitoring (TDM), diagnosis of overdose and treatment of medical emergencies following exposure. Various chromatographic techniques are employed by the clinical laboratory for the routine analysis of paralytics whereas other techniques such as tandem mass spectrometry are reserved for clinical research. The different chromatographic methods utilized are dependent on the agent and include gas chromatography (GC), high-performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC-MS and LC-MS-MS). This chapter focuses on the chromatography and detection of therapeutic and natural paralytic agents including neuromuscular blocking agents and saxitoxin. While botulinum neurotoxins have recently been analyzed for clinical purposes, chromatographic techniques are exclusively used to separate and detect sequence variations in genes and, consequently, relevance to this chapter is limited and will not be discussed further.[2]

10.2 NEUROMUSCULAR BLOCKERS

10.2.1 Background and uses

The use of neuromuscular blocking agents (NMBAs) dates back as early as the 1500s, when South American Indians created deadly arrows, by dipping them into various curare poisons,

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to paralyze their prey. Sir Walter Raleigh and other explorers brought many forms of curare back to Europe upon returning from the Americas. It was not until 1932 that highly purified curare was first introduced to patients with tetanus and spastic disorders. It is still utilized today for this clinical purpose. Ten years later, Griffith and Johnson first reported curare for purposes of muscle relaxation during anesthesia.[3]

Today, there are five main therapeutic uses of neuromuscular blockers. The primary use of NMBAs is as an adjuvant to promote muscle relaxation during surgical anesthesia to reduce the amount of anesthesia needed. Additionally, neuromuscular blockades can effectively prevent asynchronous breathing and barotraumas, particularly in neonates and small infants.[4] Orthopedists employ NMBAs to facilitate correction of dislocations and alignments of fractures. NMBAs are also given to prevent trauma in electroconvulsive shock therapy and to treat severe cases of tetanus. Finally, neuromuscular blockers can be used in assisting extended mechanical ventilation (i.e. endotracheal intubation). Several US surveys, spanning more than a decade, assessing the use of sedating drugs and NMBAs in patients requiring mechanical ventilation demonstrated, however, that agents such as opiates and benzodiazepines were used more frequently (36 vs 20%; 68 vs 13%)[5–7] and in different situations than NMBAs. One survey concluded that the use of NMBAs was associated with longer duration of mechanical ventilation, weaning time and stay in the intensive care unit (ICU) and higher mortality.[6]

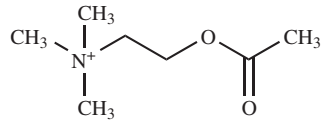
10.2.2 Classification, mechanism and duration of action

Mechanistically, neuromuscular blockers combine with nicotinic receptors on the postsynaptic membrane to block competitively acetylcholine (ACh) binding. This prevents conformational changes of, and sodium passage through, the ion channels in the membrane. Once applied to the neuromuscular end-plate, NMBAs desensitize the muscle cells to motor-nerve impulses and ACh.[3] Chemical structures of acetylcholine and representative NMBAs are depicted in Figure 10.1.

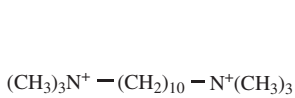
Neuromuscular blocking agents are divided into two classes according to their actions: non-depolarizing, for which the prototype is curare, and depolarizing agents, such as succinylcholine. Curare, also known as d-tubocurarine, is a plant alkaloid, which acts in the opposite manner to nicotine. The systematic name of the active principle is 7′,12′-dihydroxy-6,6′-dimethoxy-2,2′,2′-trimethyltubocuraranium hydrochloride. This structure and variants of this structure comprise the non-depolarizing neuromuscular blocking agents. Non-depolarizing neuromuscular blocking agents create a competitive blockade of acetylcholine receptors, thereby preventing depolarization of motor end-plate. Non-depolarizing agents do not cause fasiculations and their effects can be reversed by anticholinesterase agents.

Succinylcholine (suxamethonium) and decamethonium (C-10) are depolarizing neuromuscular blockers that act as acetylcholine analogs by binding directly to nicotinic receptors causing persistent depolarization of the motor end-plate. Because these drugs are slowly hydrolyzed, repolarization of the muscle is delayed. Depolarization by these drugs is divided into Phase I and II responses. For a Phase I response, brief initial twitches known as ‘fasiculations’ are followed by flaccid paralysis. Flaccid paralysis is induced by a refractory state of the nerve awaiting a rapid increase of membrane potential resulting in an action potential. Acetylcholinesterase (AChE) potentiates the Phase I blockade and competitive

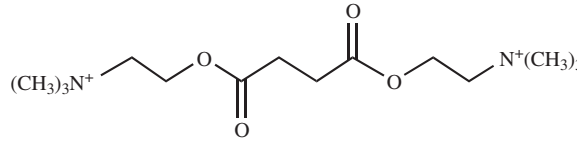
ACETYLCHOLINE



DEPOLARIZING AGENTS

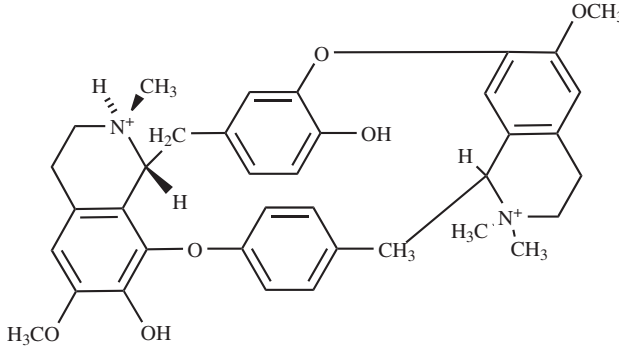


Decamethonium

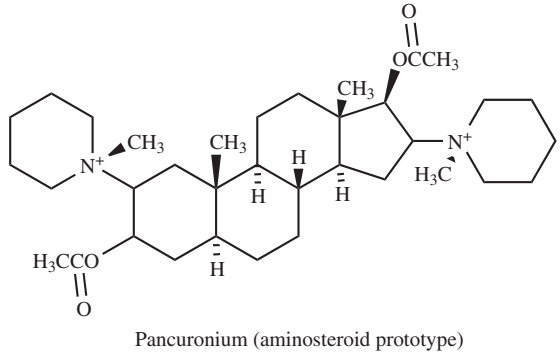


Succinylcholine (benzylisoquinolines)

NON-DEPOLARIZING AGENTS



d-Tubocurarine (curare)



Pancuronium (aminosteroid prototype)

Figure 10.1 Structures of acetylcholine and representative neuromuscular blocking agents

blockers antagonize it. A Phase II response maintains a flaccid paralysis by desensitizing nicotinic receptors with a long blockade or a high concentration of NMBA. Desensitized receptors result in a smaller effect for a given degree of receptor occupancy, essentially competitive inhibition. Therefore, acetylcholinesterases antagonize the Phase II blockade

whereas competitive blockers potentiate it.[3] The muscle fasiculations of depolarizing agents are followed by relaxation. Paralysis caused by depolarizing agents is terminated by metabolism of the agent by pseudocholinesterase. Succinylcholine is primarily used for short-acting paralysis such as intubation. Decamethonium has a higher risk of fatality and is no longer used therapeutically in the USA.

NMBAs are further differentiated by their duration of action during anesthesia. Succinylcholine and mivacurium are common ultra-short-acting competitive NMBAs (5–10 min). An intermediate duration of action (30–45 min) is maintained with the use of atracurium, cisatracurium, rocuronium and vecuronium. A long-lasting duration of action (90–100 min) is observed with *d*-tubocurarine, doxacurium, metocurine, pancuronium and pipecuronium.

Less commonly, NMBAs are classified based on their chemical nature to include natural alkaloids and their congeners (e.g. *d*-tubocurarine), aminosteroids (e.g. cisatracurium, vecuronium) and benzylisoquinolines (e.g. succinylcholine). This classification scheme also relates to mechanism of actions and associated effects.

10.2.3 Effects and toxicity

Neuromuscular blockers are ionized (i.e. quaternary nitrogen), prohibiting oral ingestion and CNS effects since they do not cross the blood–brain barrier. As such, NMBAs are injected intravenously, causing rapid onset of their effects. Following an NMBA injection, skeletal muscles experience motor weakness followed by flaccid paralysis. Muscle paralysis occurs first for fine muscles located in eyes and fingers, followed by appendages, neck, trunk and intercostals muscles, and finally the diaphragm and muscles of respiration. Recovery occurs in reverse order. Death is attributed to paralysis of respiratory muscles. In addition to skeletal muscle paralysis, competitive NMBAs can block nicotinic receptors at the autonomic ganglia and adrenal medulla, resulting in hypotension and tachycardia, decreased gastrointestinal muscle tone and motility. Finally, competitive NMBAs block histamine release, potentially leading to hypotension, bronchospasm and excessive bronchial and salivary secretions. Toxicity of NMBAs is summarized in Table 10.1. The blockade of competitive neuromuscular agents can be overcome with the use of cholinesterase inhibitors (e.g. physostigmine), which increase ACh survival in the neuromuscular junction, thereby increasing the probability that the receptor will become occupied by ACh and not an NMBA.[3]

10.2.4 Analysis

Neuromuscular blockers can be analyzed using HPLC with fluorescence, ultraviolet (UV), or electrochemical detection (ECD). As an alternative to HPLC, GC with nitrogen–phosphorus detection (NPD) and LC coupled with electrospray ionization tandem mass spectrometry (ESI-MS-MS) have also been investigated as viable techniques to measure neuromuscular blockers (Table 10.2).

Regardless of the chromatographic technique, NMBAs are purified from biological tissues and fluids by both liquid–liquid extractions (LLE) and solid-phase extractions (SPE). Because many NMBAs have a short half-life (e.g. suxamethonium, $t_{½} = 0.7$ min),

Table 10.1 Toxicity of neuromuscular blockers

Mechanism of toxicity	Non-depolarizing NMBAs	Depolarizing NMBAs
Skeletal muscle blockade	Prolonged apnea (>20 s)	Prolonged apnea
Ganglionic blockade		
	Hypotension	Hypotension
	Tachycardia	Tachycardia
	Decreased GI motility/tone	
Histamine release	Hypotension	
	Bronchospasm	
	Increased secretions	
Calcium release from sarcoplasmic reticulum		Malignant hyperthermia • increased muscle tone (rigidity) • increased cellular metabolic rate (hyperthermia)
Loss of potassium in muscle cells		Hyperkalemia
Abnormal cholinesterase activity in individual		Prolonged paralysis and delayed recovery

urine may need to be collected and analyzed in addition to blood.[8] Furthermore, metabolites of a drug may be the only analyte identified after administration of an NMBA since the parent drug may be metabolized to undetectable amounts in a matter of minutes (e.g. atracurium and mivacurium). LLEs require ion-pair formation between the NMBA and a salt such as potassium iodide. SPE methods are performed with silica-based hydrocarbon sorbents, typically C_{18} or C_8. HPLC methods to detect NMBAs are usually optimized for one or two agents. A majority of HPLC techniques rely on either UV or fluorescence detection. As such, HPLC coupled with UV detection is limited to NMBAs that absorb UV light, namely mivacurium, cisatracurium, atracurium and its degradation products laudanosine and quaternary monoacrylate. Similarly, HPLC with fluorescent detection requires agents that fluoresce or that are labeled with a fluorescent marker, and electrochemical detectors require analytes that are either oxidizible or reducible. For example, Reich *et al.*[4] successfully detected cisatracurium and vecuronium by fluorescent and electrochemical detection, respectively, in 19 neonates and small infants infused with these drugs during congenital heart surgery.

Pitts *et al.*[9] successfully determined succinylcholine in plasma by HPLC with ECD. Owing to rapid butyrylcholinesterase (BuChE) hydrolysis of succinylcholine to succinylmonocholine and choline in blood, the parent drug is difficult to determine. Since succinylmonocholine is further hydrolyzed to choline and inactive succinic acid at physiological pH, these investigators measured succinylcholine concentrations indirectly by determining the concentration of its breakdown product, choline. Complete hydrolysis of succinylcholine to choline and succinic acid is a two-step reaction, yielding choline at each step. Under the assayed conditions, none of the measured choline was derived from succinylmonocholine. In this method, choline was measured in two blood or plasma samples, in one of which succinylcholine hydrolysis was inhibited immediately (e.g. endogenous choline or hydrolyzed aliquot) by the addition of physostigmine (10^{-5} M),

Table 10.2 Chromatographic Detection of Neuromuscular Blockers

Author (Year)	Neuromuscular Blocker(LOQ)	Technique & Specimen Type	Method Summary
Pitts (2000)[9]	Succinylcholine (SUC; 16 ng ml^{-1})	HPLC-ECD Plasma (2 mL)	*Pretreatment:* • Total choline: addition of physostigmine (10^{-5} M, 0.01%) • Endogenous choline: addition of BuChE (200 mU at 37°C) in 0.5 mL of 0.05 M PB (pH 7.4) • Reaction stopped: 1 mL ice cold 0.1 M perchloric acid. • Centrifuge: 5000*xg* at 4°C for 5 min to obtain plasma • Storage: −70°C *Sample Preparation:* • Choline Standards: prepared in 52 mM acetic acid (pH 5.5) with 0.01% antimicrobial agent, Kathon (BAS- Bioanalytical Systems, Lafayette, IN) *Reverse Phase Column:* • Physostigmine trapping column: pre-column 20 µL injection loop (MF-6262, BAS) • ACh analytical column (BAS)- 96 mm × 5 mm i.d. • Choline retained 11.1 min then passed through 300 mm long immobilized enzyme column containing AChE (60U) and choline oxidase (60U) from BAS analytical kit • Platinum working electrode: measured peroxide reaction product • Electrode potential: 0.5 mV relative to Ag/AgCl reference electrode • Resulting current measured with LC-4B amperometric detector (BAS) • Output: 5–20 nA full scale depending on concentration and electrode sensitivity *Mobile Phase:* • Flow rate: 0.7 mL min^{-1} • 0.05 M Tris/$NaClO_4$ (pH 8.5) with 0.5% antimicrobial agent, Kathon (BAS)

(*continued*)

Table 10.2 (*Continued*)

Author (Year)	Neuromuscular Blocker(LOQ)	Technique & Specimen Type	Method Summary
			Detection: • Electrochemical Signal measured: analyte peak height relative to concentration *Run Time:* Not Reported
Reich (2004)[4]	Cisatracurium (CST; 20 ng ml^{-1})	HPLC-FL Plasma	*Pretreatment:* • Acidified and frozen *Standard Preparation:* • SPE on C18 Bond Elut column (Varian, Palo Alto, CA) *Reverse Phase Column:* Regis C18 Spherisorb Little Champ (Regis Technologies Inc., Morton Grove, IL) 5 cm × 4.6 mm, 5 μm *Mobile Phase:* • 0.03 M NPB (pH 3.0) with 0.14% $(CH3)_4NH_4OH$ in ACN (60:40, *v/v*) *Detection:* • Fluorescence • Wavelength not reported *Run Time : Not Reported:*
Reich (2004)[4]	1. Vecuronium (VE; 15 ng ml^{-1}) 2. 3-OH Vecuronium (3OH-VE; 15 ng ml^{-1})	HPLC-ECD Plasma	*Pretreatment:* Acidified and frozen *Sample Preparation:* • SPE on C1 Bond Elut column (Varian, Palo Alto, CA) • Precondition: MeOH & H_2O • Wash: H_2O, ACN, MeOH • Elute: 0.01 M $NaClO_4$ in MeOH • Evaporate in solvent Dissolve: 0.1 mL mobile phase

Probst (1997)[11]	1. Rocuronium (ROC; 10 ng ml^{-1}) 2. 17-OH-ROC (17OH-ROC; 50 ng ml^{-1})	GC-NPD Plasma	*Reverse Phase Column:* Waters Nova-Pak CN (Waters Corp., Milford, MA) 3.9 (units NR) × 150 mm, 4 µm *Mobile Phase:* • 0.033 M NPB (pH 5.5) in ACN (60:40, *v/v*) *Detection* • Electrochemical *Run Time:* Not Reported: *Pretreatment:* • Heparinized blood centrifuged 5 min (2500 U/min-1) • To prevent hydrolysis of ROC, decanted plasma acidified by diluting equally with 0.8 M PB (pH 5.4) • Stability study to confirm samples stable for 1 year. 600 µL of pretreated sample (−70°C) at different concentrations were evaluated. *Sample Preparation:* • IS: 3OH-VE; LLE: 500 µL sample (250 µL specimen/250 µL PB) treated with saturated KI solution (500 µL) and $MeCL_2$ (5 mL); Rotary mix: 30 min and decant $MeCL_2$ (Breda Scientific); Evaporate: 60°C in SpeedVac Plus SC 110A (Savant; Bierbeek, Belgium) CAN Dissolve: 200 µL Derivatize: MTBSTFA at 70°C overnight Evaporate: 60°C in SpeedVac; Dissolve: 100 µL Acetone *Alternate Sample Preparation Method:* • IS: 3OH-VE; SPE: 500 µL sample (250 µL specimen/250 µL PB) & saturated KI solution (500 µL) added to luer locked Extrelut cartridge (Merck; Darmstadt, Germany); Add: $MeCL_2$ (5 mL) and equilibrate for 5 min; Evaporate: 60°C in SpeedVac; Dissolve: 200 µL CAN; Derivatize: MTBSTFA at 70°C overnight; Dissolve: 100 µL Acetone *GC Column:* DB1 (J &W Scientific; Koln, Germany) • 10m × 0.32 mm i.d. × 0.25 µm

(*continued*)

Table 10.2 (*Continued*)

Author (Year)	Neuromuscular Blocker(LOQ)	Technique & Specimen Type	Method Summary
			GC System: • injector: 300°C, split (1:25); injection volume: 2 µL • oven preramp: 5 min at 120°C • oven ramp: 30°C min-1 to 300°C hold 6.5 min • carrier gas (helium, 5.0): 3 ml min-1; make up gas (nitrogen): 25 ml min^{-1}; hydrogen: 3.5 ml min^{-1}; synthetic air: 175 ml min^{-1} *Detector:* Nitrogen specific detector- TSD (Varian, Darmstadt, Germany) TSD bead probe modified by applying rubidium dotted glass bead Detector temp: 320°C Detector current: 2.9-3.0 A *Run Time:* 13 min:
Gutteck-Amsler (2000)[12]	a) Pancuronium (IS)-PE-10 ng ml^{-1} b) ROC- 5 ng ml^{-1} c) VE-15 ng ml^{-1} d) 3OH-VE-15 ng ml^{-1}	LC-ESI-MS Plasma	*Pretreatment:* preservative- 1 mol L^{-1} sodium hydrogen phosphate solution *Sample Preparation:* • Add: IS (50 µL) & 6 mol/L KI (1 mL) • LLE: toluene (5 mL); shaken for 20 min • Centrifuge, separate, & evaporate organic layer Dissolve: 0.1 mL mobile phase *Reverse Phase Column:* Nucleosil C18 12.5 cm × 4mm, 5µm Macherey-Nagel *Guard Column* • Nucleosil C 18; 8 × 4 mm, 5 µm Macherey-Nagel *Mobile Phase:* 50 µM formate buffer (pH 3) in MeOH (73:27, *v/v*)

			Detection LCQ ion trap MS (Thermoquest, San Jose, CA) Positive ESI Voltage: 3.8 kV Capillary: 230°C Product ions (*m/z*)-a) PA: 286.4 → 236.7; b) ROC: 529.4 → 487.7; c) VE: 279.2 → 249.4; d) 3OH-VE: 258.5 → 100.2 *Retention Time:* a) PA: 8.2 min; b) RO: 6.6 min; c) VE: 15.4 min; d) OHV: 9.7 min
Cirimele (2003)[14]	a) d-TBC b) PA c) ALC d) ROC e) OHV f) VE g) ATC h) MVC i) MBZ	LC-ESI-MS Blood Urine	*Sample Preparation:* • Extract: Blood (1 mL) mixed with saturated KI (1 mL) and 0.8 M PB (NaH_2PO_4, 1 mL, pH 5.4) and $MeCl_2$ (5 mL) • Shake: 15 min at 100 cycles min-1 • Centrifuge 10 min (4000 rpm) Decant: Organic phase and evaporate • Reconstitute: 30 µL mobile phase *Reverse Phase Column:* • Nucleosil C18 • 150 cm × 1.5 mm i.d., 5 µm • Protected by 0.5 µm frit • Injection volume 2 µL *Mobile Phase:* 50 mM NH_4COOH (pH 3) in MeOH using formic acid to adjust pH Linear gradient: 30 to 90% MeOH in 6 min Flow: continuous 50 µL min-1 *Detection:* • LCQ ion trap MS (PE Sciex API-100); Positive ESI; Voltage: 4.2 kV; Capillary: NR • Nebulizing gas: Nitrogen (99.95%) • Ion source orifice: 25 µm at +40V • Full scan mode: 200 to 720 *m/z* Fragment ions (*m/z*) d-TBC: **609**, 552,521; PA: **430**, 472, 617; ALC: **333**, 312, 711; ROC: **487**, 530; OHV: **515**, 258, 356; VE: **557**,

(*continued*)

Table 10.2 (*Continued*)

Author (Year)	Neuromuscular Blocker(LOQ)	Technique & Specimen Type	Method Summary
			356, 398; ATC: **464**, 516, 570; MVC: **514**, 600, 671; MBZ: **294**, 276, 208; (Quant ion bolded) *Retention Time (min):* d-TBC: 11.7; PE: 12.3; ALC: 12.4; ROC: 12.4; OHV: 12.4; VE: 12.7; ATC: 13.2; MVC: 13.4; MBZ: 15.1;
Kerskes (2004)[13]	a) ROC b) VE c) Suxamethonium (SUX, succinylcholine) d) Mivacurium (MVC) e) Gallamine (GAL) f) Pancuronium (IS) (PE-10 ng ml^{-1}) g) Atracurium (ATR) h) Laudanosine (LAU) i) Quaternary monoacrylate (QMA) LOQ-NR	LC-ESI-TMS Blood Serum Urine Bile Liver Muscle Brain	*Pretreatment:* • Incubate urine, bile, liver at 36°C, 3 h; 5 μL β-glucuronadase/aryl sulfatase (*helix pomatia*, Merck); Homogenate tissues (4:1 in $CH_8N_2O_3$); Vortex/Centrifuge; Use supernatant • All other matrices:1 mL in $CH_8N_2O_3$ (2 mL); Vortex/Centrifuge; Use supernatant *Sample Preparation:* • SPE: BondElute C18 HF (Varian, Harbor City, CA) • Precondition: MeOH & $CH_8N_2O_3$ (2 mL each) • Wash: Ammonium carbonate buffer (1.5 mL × 2) • Dry 5 min, add hexane (50 μL), & dry another 5 min • Elute: 0.1 M acetic acid in MeOH (1 mL) • Evaporate in heated, vacuumed centrifuge (Savant SpeedVac AE2000) for 25 min • Dissolve: 0.1 mL $NH_4C_2H_3O_2$ and ACN (7:3, *v/v*) Centrifuge 5 min (4000 rpm) *Reverse Phase Column:* Intertsil ODS2 (Chromopack) *Mobile Phase:* 50 μM $NH_4C_2H_3O_2$ (pH 5) in water; Flow: continuous; 0.4 mL min-1;

(*continued*)

Percent Gradient

Mins:	*Buffer/ACN:*
0→5	95/5
5→15	70/30
15→25	70/30
25→30	65/35
30→33	30/70
33→33.5	30/70
33.5→35	95/5

Detection:

- LCQ ion trap MS (Thermoquest, San Jose, CA); Positive ESI; Voltage: 3-46 kV; Capillary: 125–250°C; Sheath Gas: 45–85 psi; Collision Energy: 32–37%

Product ions (*m/z*)- PE: 286.4 → 236.6; 472.3; 430.2; 206.7; 100.3; ROC: 529.4 → 487.4 → 427.4; 376.3; 358.2; 418.2; 487.3; 400.3; 445.4 (MS/MS/MS); VE: 557.5 → 497.4; 398.2; 338.23; 416.2; 458.2; 356.3; SUX: 145.2 → 115.6; MVC: 514.4;

342.3 → 671.3; 357.1; 342.2; 600.3; 428.2; 325.2; GAL: 284.7; ATC: 464.3; 358.2; 570.2 → 327.0; 307.2; 370.2; 358.1; 296.1; LAU: 358.2 → 206.2; 327.0; QMA: 570.2 → 370.2; 327.0; 412.1; 256.1

- *Retention Time (min):* PE: 8.6; ROC: 7.9; VE: 9.5; SUX: 1.6; MVC: 18.5, 20.5, 22.2; GAL: 4.5; ATC: 17.1, 17.8, 18.4; LAU: 7.9; QMA: 23.9, 25.6

Abbreviations: LOQ: limit of quantitation; BuChE: butylcholinesterase; ACh: Acetylcholine; AChE: acetylcholinesterase; Ag/AgCl: silver/silver chloride; $NaClO_4$: sodium perchlorate; $(CH_3)_4NH_4OH$: tetramethyl ammonium hydroxide; CN: cyanide; SPE- solid phase extraction; $CH_8N_2O_3$: ammonium carbonate; $NH_4C_2H_3O_2$: ammonium acetate buffer; PB: phosphate buffer; LLE- liquid-liquid extraction; HF: hydrofluoric acid; $MeCl_2$: methlene chloride; MeOH: methanol; ACN: acetonitrile; H_2O: water; MTBSTFA: *N*-Methyl-*N*- (tert-butyldimethylsilyl)-trifluoroacetamide; IS: internal standard; KI: potassium iodide; NPB: sodium phosphate buffer; NaH_2PO_4: sodium phosphate, monobasic; NH_4COOH: formate buffer; HPLC-ECD: high performance liquid chromatography-electrochemical detection; HPLC-FL: high performance liquid chromatography-fluorescence detection; GC-NPD: gas chromatography-nitrogen phosphorus detection; LC-ESI-TMS: liquid chromatography electrospray ionization-tandem mass spectrometry; LC-ESI-MS: liquid chromatography electrospray ionization-mass spectrometry; NR: not reported

and the other which was completely hydrolyzed (e.g. total choline or inhibited aliquot) in 20 min by addition of BuChE (200 mU at 37 °C). These investigators used a commercially available acetylcholine/choline analytical kit [Bioanalytical Systems (BAS), West Layfayette, IN, USA] that measures these analytes by HPLC-ECD following reversed-phase column separation and pass through an immobilized enzyme reactor containing AChE and choline oxidase. In this process, only the oxidation of choline relates to the succinylcholine concentration. A 95% recovery of choline was achieved and the difference in the two choline measures represented the succinylcholine concentration. Choline calibration curves were linear from 16.3 to 20 900 ng mL^{-1} (156 pmol mL^{-1} to 200 nmol mL^{-1}) with correlation coefficients $\geq$0.999. The within-day ($n = 6$) and between-day precisions ($n = 10$) of HPLC choline detection were assessed by repeatedly injecting choline calibration standards multiple times within a day and over a 3-week period. Mean coefficients of variation (CVs) with standard deviations for this procedure were 3.7 $\pm$ 1.2 and 3.8 $\pm$ 1.6%, respectively. The chosen chromatographic conditions (Table 10.2) did not yield any succinylcholine or interfering peaks.

GC methods are less common than LC for the analysis of NMBAs. Their mono- or bisquaternary ammonium structure, however, makes them ideal for nitrogen–phosphorus detection (GC-NPD). Most GC techniques for NMBAs follow similar parameters exemplified in a paper published by Furuta *et al.* in 1988.[10] They reported the use of GC-NPD to detect pancuronium (PA), pipecuronium (PIP), vecuronium (VE) and their 3-desacetyl metabolites (3-Des-) with detection limits of 2 ng mL^{-1} for the parent drugs and 4 ng mL^{-1} for their respective metabolites. Plasma samples from 106 surgical patients were acidified, derivatized at the 3-hydroxy steroidal position with an *O-tert*-butyldimethylsilyl derivatizing agent, washed with diethyl ether and finally extracted with an aliquot of dichloromethane. Lower limits of detection and upper limits of linearity were as follows: PA, VE, PIP, 2–5000; 3-Des-VE, 4–5000; 3-Des-PA, 8–500; and 3-Des-PIP, 25–1000 ng mL^{-1}. Adequate precision was demonstrated with coefficients of variation ranging from 2 to 20%. Reproducibility for clinically relevant concentrations of these drugs in human serum was 5–16% PA, 2–4% VE and 6–16% PIP. No interferences were observed in the analyses of over 100 clinical specimens.

In 1997, Probst *et al.*[11] modified Furata *et al.*'s method to quantify rocuronium and its 17-desacetyl metabolite in heparinized plasma. This group performed parallel studies of the previously described LLE extraction and an on-column ion pairing using Extrelut solid-phase extraction cartridges. The intra-assay precision ($n = 5$) at two concentrations (400 and 1052 ng mL^{-1}) for both parent drug and metabolite showed mean concentrations within 4–17% (CV = 9.7–10.5%) of the target concentration of rocuronium and within 7–8% (CV = 13.2–15.3%) of the target concentration of its metabolite. The inter-assay precision at three concentrations (400 ng mL^{-1}, $n = 15$; 800 ng mL^{-1}, $n = 13$; and 1480 ng mL^{-1}, $n = 15$) demonstrated similar results with mean concentrations within 0.5–11.7% (CV = 8.2–17.7%) and within 7–21% (CV = 16.4–21.3%), respectively. The mean accuracy ranged from 88 to 103% for rocuronium (ROC) from 108 to 121% for 17-desacetylrocuronium (17OH-ROC). The linearity of the ROC calibration curve was 50–6400 ng mL^{-1} and the linearity of 17OH-ROC was 80–6400 ng mL^{-1}. The lower limit of linearity was set as the limit of quantification determined as a reliable repeated signal-to-noise ratio between 5 and 10 of the peak area ratio of analyte to internal standard. Analytical interferences were not noted. These analytical modifications simplified the extraction procedure (e.g. separation of aqueous and organic phases not required) and improved sample preparation time (5–10 vs

30 min) in large-scale determinations as compared with LLE. In addition, further attempts to improve sensitivity such as use of alternative SPE cartridges (RP-8, RP-18, cyanopropyl or cation-exchange) or organic solvents (1,1,1-trichloroethane, trichloromethane, diethyl ketone, and ethyl methyl ketone) with and without potassium iodide were not successful. ROC and 17OH-ROC recoveries were between 30% and 48% for heparinized plasma and 52% and 73% for phosphate-buffered heparinized plasma, respectively. Despite these ROC recoveries, concentrations less than 100 ng mL^{-1} were successfully detected in patients' samples 4 h after a 22 mg h^{-1} ROC infusion. 17OH-ROC was not detected in any of the patients' samples.

Since 2000, a myriad of LC-MS techniques have been reported to detect an array of NMBAs. These methods are valuable because they are generally more sensitive and more practical because they can measure a number of NMBAs simultaneously. Likewise, pharmacokinetic studies of neuromuscular blockers require low detection limits ($<10\,\mu g\,L^{-1}$) and acceptable reproducibility (CV <10%). Hence more sensitive chromatographic techniques such as tandem mass spectrometry with electrospray ionization combined with liquid chromatographic separation (LC-ESI-MS-MS) are better suited for these types of clinical applications.[12] Brief descriptions of representative LC-MS methods follow.

In 2002, Kerskes *et al.*[13] reported an LC-ESI-MS method detecting seven neuromuscular blocking agents and two additional metabolites. ESI into an ion trap was the optimal interface for the charged structures of the NMBAs. A 35-min HPLC gradient using ammonium acetate buffer in acetonitrile effectively eluted all nine analytes on an Intertsil ODS2 Chromopack column within 26 min. Autotuning to optimize the tube lens offsets, octapole offsets and capillary voltage were carried out for each NMBA. Further tuning was done manually to determine the best sheath gas flow-rate and capillary temperature for each NMBA. For an unknown compound, the samples were screened using a method containing a suxamethonium (SUX) tune file from 0 to 6 min and an atracuronium (ATC) tune file from 6 to 35 min. For cases of known analyte, the specimens were analyzed with the specific tuning parameters for the given NMBA. The optimal collision energy for MS-MS measurements ranged from 32 to 37%. Performance studies for this procedure were not discussed by the authors.

NMBAs were analyzed by the MS configuration that gave the most intense ions in which the relative abundance was more than 10%. In the MS mode, the molecular ions were observed for gallamine ($m/z = 284.7$), pancuronium ($m/z = 286.4$), vecuronium ($m/z = 557.5$) and rocuronium ($m/z = 529.3$). In the MS-MS mode, the product ions of the last three molecular ions were unambiguous and easily identified. Gallimine did not have an appreciable signal in the MS-MS or MS-MS-MS modes. ROC and SUX were the only NMBAs requiring triple quadrapole identification, as only one peak (ROC, $m/z = 487.4$; SUX, $m/z = 115.6$) was observed in the MS-MS mode, hence a unique fragmentation pattern was not evident without further fragmentation. In addition to a small peak, mivacurium has a base peak molecular ion ($m/z = 514.4$) that can be fragmented into six product ions. Molecular ions were observed for atracurium ($m/z = 570.2$) and its metabolites, quaternary monoacrylate ($m/z = 570.2$) and laudanosine (protonated at eluent pH; m/z M + 1 = 358.2) and these precursor ions are additionally fragmented to four, four and two product ions, respectively. Mivacurium and atracurium both had several isomeric peaks between 16 and 22 min. Although there was no baseline separation of these two analytes, they could be uniquely identified based on their different mass spectra.

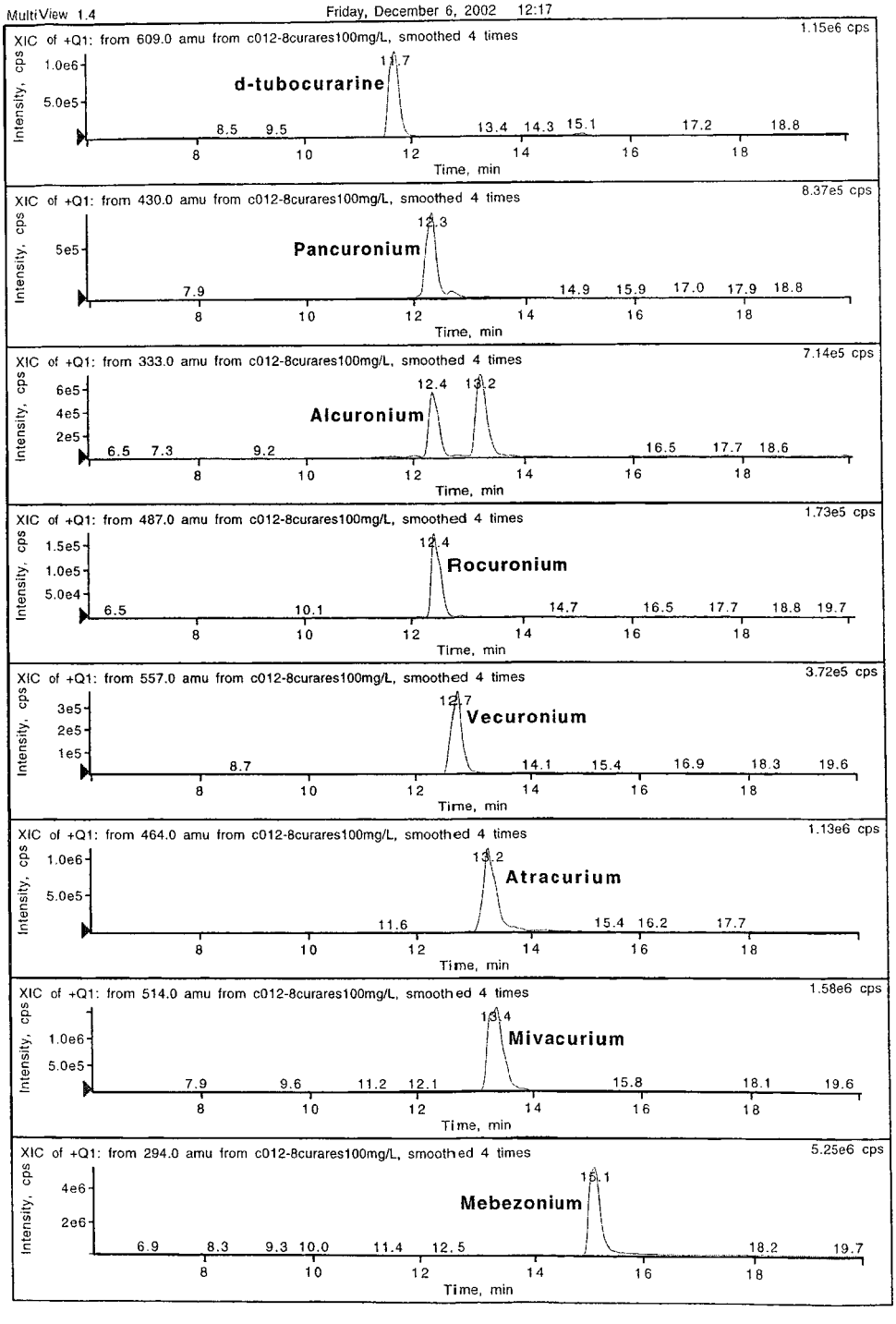


Figure 10.2 Typical chromatographic profiles for the eight quaternary muscle relaxants. Reprinted from *Journal of Chromatography B*, **789**, V. Cirimele, M. Villain, E. Pépin, B. Ludes and P. Kintz, Screening procedure for eight quaternary nitrogen muscle relaxants in blood by high-performance liquid chromatography–electrospray ionization mass spectrometry, 107–113, with permission from Elsevier

This multi-analyte LC-MS method for NMBAs investigated both clinical and post-mortem applications. First, suxamethonium and rocuronium were detected in serum and urine administered to a woman during a Caesarian section operation. Suxamethonium was detected in urine 35 min post-administration, but it was not present in the serum. About 25 min following the administration of rocuronium, it was detectable in both matrices. Another case investigated by this method involved a possible assisted suicide by pancuronium (Pavulon) injection. Biological specimens investigated by this method included blood, urine, bile, brain, muscle and liver collected during autopsy. Pancuronium was detected in all matrices with the highest concentration found in the liver (2.4 mg kg^{-1}) and the lowest in the brain (0.1 mg kg^{-1}). Two additional peaks were consistently present and thought to correspond to 3- and 17-hydroxypancuronium.

Another clinical and forensic method that identified and quantified eight quaternary nitrogen NMBAs in blood was reported by Cirmele *et al.* in 2003.[14] The muscle relaxants detected included *d*-tubocurarine (*d*-TBC), alcuronium (ALC), PA, VE, atracuronium (ATR), ROC and mebezonium (MBZ), with mivacurium (MVC) as the internal standard. The procedure used ion-pair extraction with methylene chloride (pH 5.4), reversed-phase HPLC separation with a binary mobile phase of 50 mM ammonium formate buffer (pH 3.0) in methanol and ESI-MS detection. Linearity of the procedure was established to be 0.1–10 mg L^{-1} with a correlation coefficient $r^2 > 0.929$. All analytes demonstrated a limit of detection of 0.1 mg L^{-1} at a signal-to-noise >5.0. With eight replicates each, accuracy was demonstrated at a 1.0 mg L^{-1} concentration with a CV between 6.9 and 17.8% and the relative extraction recovery was between 46.0 and 91.1% at 1.0 mg L^{-1}. Typical chromatographic profiles for the eight quaternary muscle relaxants are depicted in Figure 10.2. This screening procedure performed satisfactorily in the investigation of two fatalities. One successfully detected vecuronium (1.2 and 0.6 mg L^{-1}) and 3-hydroxyvecuroinium (4.4 and 0.7 mg L^{-1}) in blood and urine, respectively. Blood obtained at autopsy in a second case revealed the presence of MBZ at 6.5 mg L^{-1}. The full-scan chromatogram and specific mass spectrum of mebezonium in post-mortem blood are depicted in Figure 10.3. While the first case showed slightly elevated therapeutic concentrations of vecuronium in blood (0.4–1.0 mg L^{-1}), the absence of medical control or assistance may have contributed to the death. The post-mortem mebezonium concentration in the second case was consistent with three other reported deaths.

A once exclusive instrumental technique for research, LC-MS is becoming ever more utilized in special applications. Since hospitals are requiring more robust, sensitive and specific assays for routine TDM analysis, it is inevitable, as demonstrated with the aforementioned NMBAs, that LC-MS methods will eventually become routine procedures in clinical settings.

10.3 PARALYTIC SHELLFISH POISONING: SAXITOXIN

10.3.1 Background

Shellfish poisoning occurs when shellfish feed on planktonic algae (e.g. dinoflagellates, especially *Gonyaulax* and *Alexandrium* species) that accumulate and metabolize toxins, which are subsequently ingested by humans. A particularly serious form of shellfish poisoning is paralytic shellfish poisoning (PSP) owing to its extreme toxicity caused by

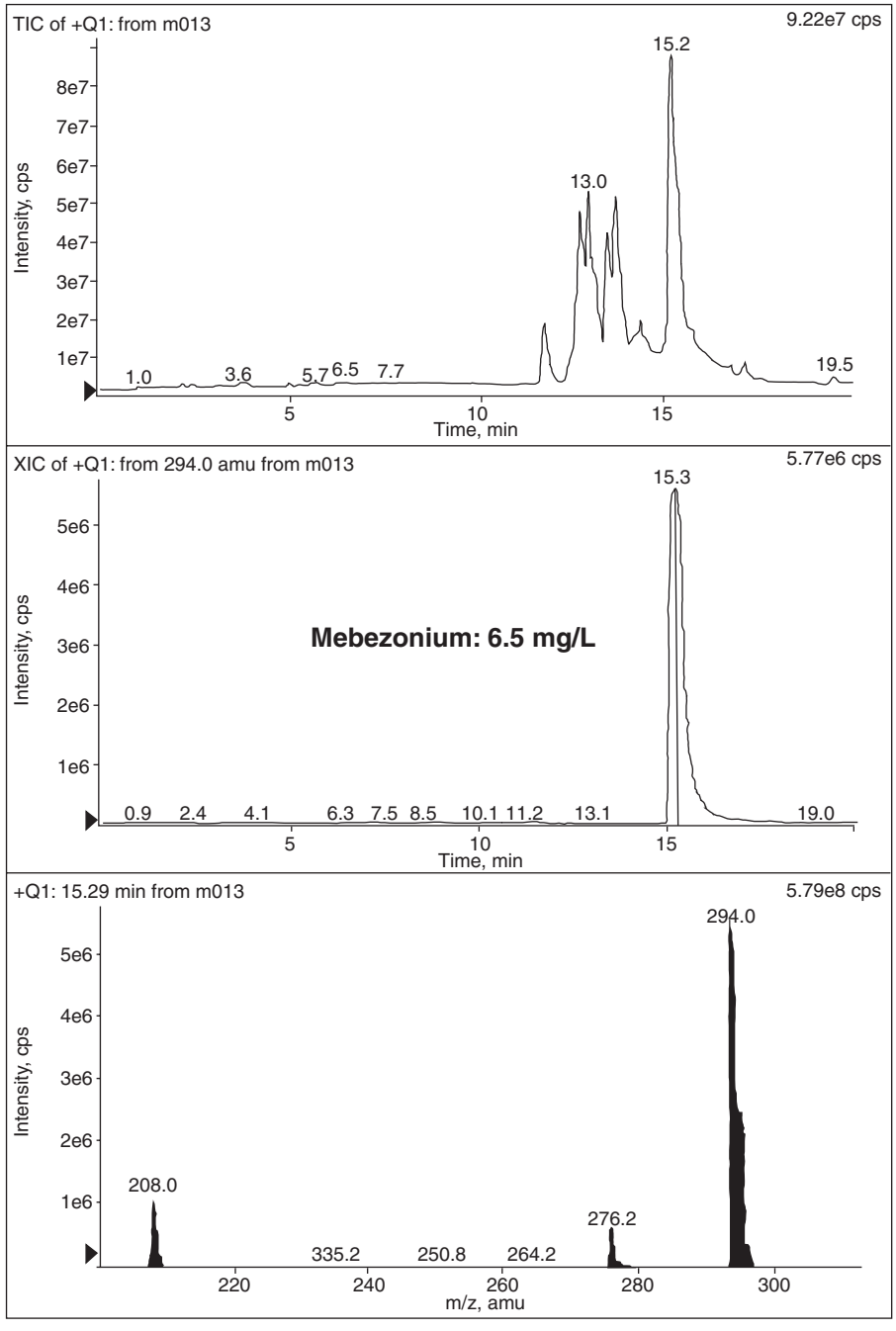


Figure 10.3 Full-scran chromatogram and specific mass spectrum of mebezonium in post-mortem blood. Reprinted from *Journal of Chromatography B*, **789**, V. Cirimele, M. Villain, E. Pépin, B. Ludes and P. Kintz, Screening procedure for eight quaternary nitrogen muscle relaxants in blood by high-performance liquid chromatography–electrospray ionization mass spectrometry, 107–113, with permission from Elsevier

over 21 structurally related toxins derived from saxitoxin (STX), a neurotoxin to mammals. STX toxins block the sodium channels in the excitable membranes of nerve cells and associated muscles; this depolarization blockade inhibits the action potentials and nerve transmission impulses, ultimately resulting in respiratory failure. Saxitoxin analogs fall into three subgroups based on their structure, with the least potent being carbamate toxin followed by sulfamate toxins and decarbamoyl gonyautoxins.

10.3.2 Toxicity

Saxitoxin manifests toxicoses, which may ultimately be fatal. Deadly outbreaks, such as that experienced in 1987 in Guatemala, have reported as many as 187 cases and 26 deaths occurring after the ingestion of a single exposure to a clam soup. The high mortality rate associated with PSP has led many countries to mandate long-term closure of water areas in which molluscan shellfish are harvested during particularly unsafe seasons (e.g. summer months) when planktonic blooms make PSP a natural phenomenon.[15] Today, PSP has relatively few outbreaks in the USA owing to strong control programs that prevent human exposure to toxic shellfish such as mussels, clams, cockles and scallops. During a 5-year period (1988–1992), there were five outbreaks of PSP (65 cases, two deaths) reported by the Center for Disease Control and Prevention (CDC) through its surveillance program for foodborne-disease outbreaks established in 1973.[16]

PSP has a rapid onset of symptoms occurring within 0.5–2 h after ingestion of the shellfish, depending on the amount of toxin consumed. Symptoms include

- tingling
- burning
- numbness
- drowsiness
- incoherent speech
- respiratory paralysis.

Without clinical support, death may occur most commonly by respiratory paralysis or occasionally by cardiovascular collapse, despite respiratory support, caused by the weak hypotensive action of the toxin. With less serious cases (e.g. lower concentration of saxitoxin in shellfish or smaller amount of shellfish consumed), complete recovery with no lasting side-effects is expected if respiratory support transpires within 12 h of exposure. In humans, 120–180 μg of STX can produce moderate symptoms such as tingling and numbness, 400–1060 μg of STX can cause deaths, and concentrations >2000 μg constitute a fatal dose.[17]

Although saxitoxin is best known as the agent producing PSP after ingestion of contaminated marine life, this neurotoxin is also deadly if inhaled; an individual may die within minutes after inhaling a lethal dose. When inhaled, saxitoxin acts by blocking nerve conduction directly and its lethality results from paralyzing muscles of respiration. Because of its threat as a chemical warfare agent and reported use as a biological weapon, saxitoxin has been placed on the Schedule of Chemical Warfare Agents. In comparison with other

potential chemical weapons, saxitoxin is of the same magnitude of lethality as ricin and 50 times more potent than sarin gas based on LD_{50} measurements in mice.[18]

10.3.3 Analysis

Analysis of PSP toxins can be achieved by bioassays and chemical assays. Historically, *in vivo* and *in vitro* bioassays were introduced first, subsequently followed by biochemical assays such as receptor binding assays and immunoassays. Chemical assays employing various analytical techniques including fluorimetry and liquid chromatography (e.g. HPLC, LC-MS) are currently used for clinical applications. Although bioassays are less sensitive and specific than newer techniques, they are still widely utilized owing to their low cost, simplicity and regulatory acceptance by governmental agencies such as the US Food and Drug Administration (FDA).

The mouse biosassay first introduced for detection of PSP toxins in 1937 by Sommer and Meyer was adopted by the Association of Official Analytical Chemists in 1984 as a non-selective bioassay with animal symptoms and time to death utilized as the measure of PSP toxicity. Its LOD of 40 μg per 100 g of tissue is adequate to determine if a tolerance level of 80 μg for humans has been reached during a PSP episode and whether actions should be taken to maintain public safety.[19]

In the early 1990s, chemical assays began to replace bioassays as both a screening and confirmatory test to detect toxins within human fluids and tissues. *In vitro* receptor binding assays, such as the hippocampal slice assay[20] and the sodium channel blocking assays and saxitoxin-specific ligand binding assays, are utilized to detect saxitoxin in addition to immunoassay techniques such as enzyme-linked immunosorbent assay (ELISA).[21,22] These chemical assays can detect saxitoxin at nanogram to picogram levels with equivalency calculated at 2 μg STX eq. per 100 g of shellfish.[23–25]

HPLC is the most common chromatographic assay, predominantly utilizing fluorescence or mass spectrometric detection to measure presence of individual PSP toxins with a detection limit for saxitoxin of 20 fg per 100 g of tissue (0.2 ppm).[26] For clinical purposes, serum and urine are the preferred matrices for analysis of saxitoxin. For post-mortem analysis other organ tissues have been analyzed (e.g. heart, brain, liver, gastric, spleen).[27]

Two commonly used HPLC techniques employ reversed-phase liquid chromatography with fluorescence detection (HPLC-FLD). Sullivan *et al.*[28] used a polymer reversed-phase cyano column and gradient elution to separate the 10 most common PSP toxins in a 20-min run, and Oshima[29] used a C_8 bonded silica gel column and isocratic elution to separate all 21 PSP toxin fractions in three separate chromatographic runs for sulfamate toxin, gonyautoxins and saxitoxins. Most HPLC methods reported in the literature are modifications of these procedures. Better resolution and chromatographic peaks by these methods are achieved through oxidation of the saxitoxin analogs. The oxidation can be performed before chromatographic separation using peroxide and a base such as sodium hydroxide, as depicted in Figure 10.4.[28] Alternatively, the use of periodic acid in sodium phosphate buffer in a heated post-column reaction coil to oxidize PSP toxins to fluorescent derivatives has been reported.[29,30] The latter method can give false identification of imposter peaks. To avoid misidentification of toxins, samples should be reanalyzed following purification by SPE (C_{18} Sep Pak, Waters, Milford, MA, USA) followed by ultrafiltration (5000 Da;

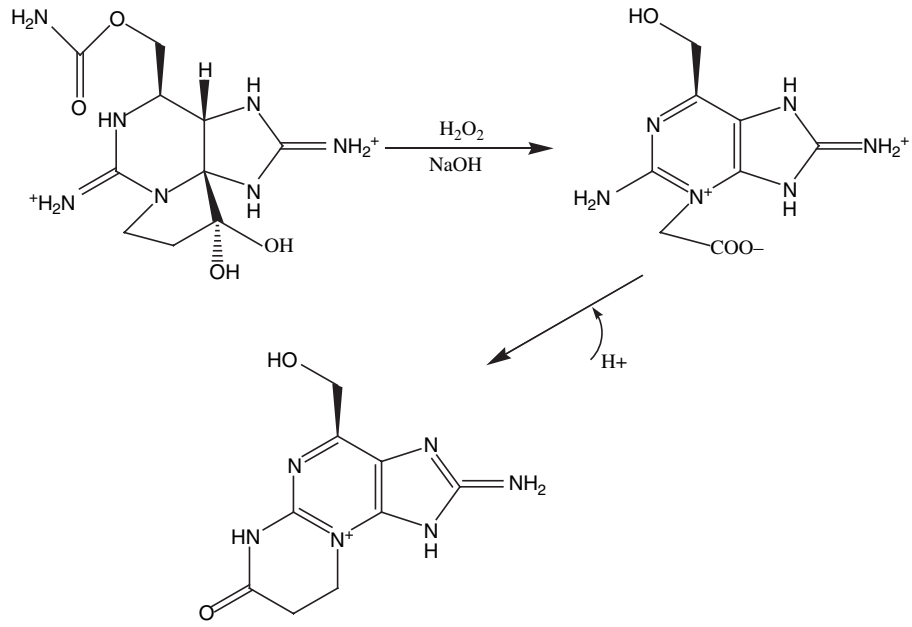


Figure 10.4 Fluorescent oxidation of saxitoxin

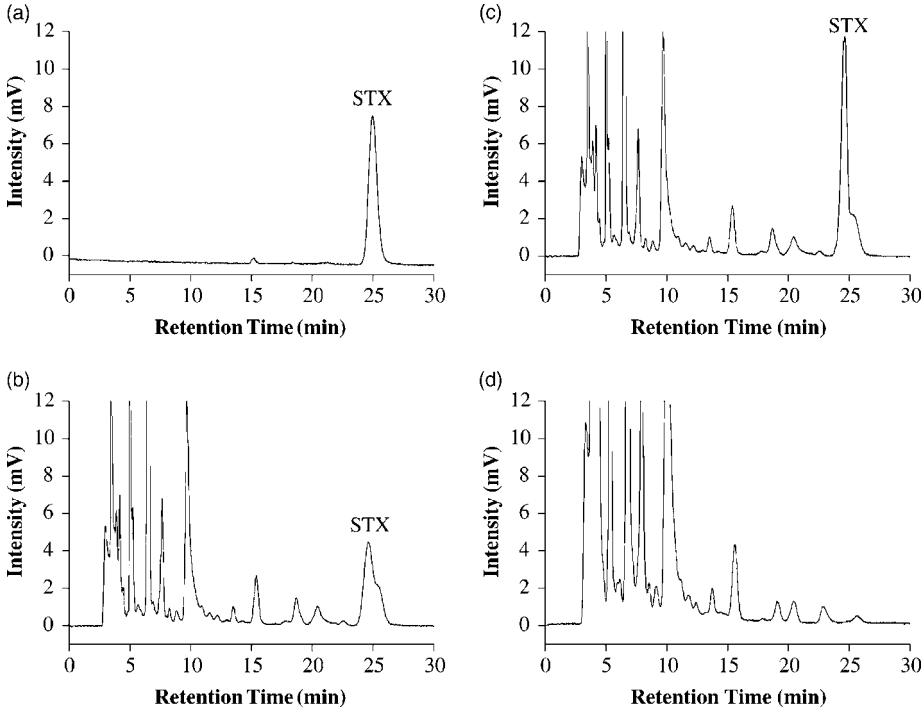


Figure 10.5 HPLC–FLD chromatogram of calibrated standard STX injection (a) compared with a toxic extract of *Octopus* (*Abdopus*) sp. 5 showing peale retention (b), spiking of the extract with an authentic STX standard (c) and with no post-column oxidation (d). Reprinted from *Toxicon*, **44**, A. Robertson, D. Stirling, C. Robillot, L. Llewellyn and A. Negri, First report of saxitoxin in octupi, 765–771, 2004, with permission from Elsevier

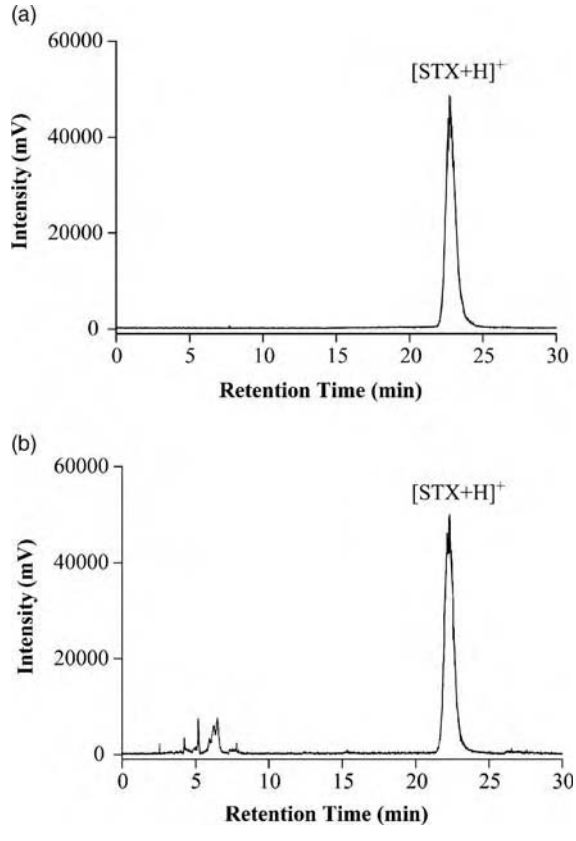


Figure 10.6 LC–MS chromatograms of an authenic STX standard (a) compared with extract of *Octopus* (*Abdopus*) sp. 5 (b) Reprinted from *Toxicon*, **44**, A. Robertson, D. Stirling, C. Robillot, L. Llewellyn and A. Negri, First report of saxitoxin in octupi, 765–771, 2004, with permission from Elsevier

Millipore, Bedford, MA, USA) and the oxidizing reagent should be replaced with distilled water to minimize oxidation. The fluorescence intensities of the peaks are compared to determine which peaks are true saxitoxins. Similarly, suspect toxin peaks such as tetrodotoxin, another marine toxin found in shellfish and octopi, can be identified using LC-MS performed in the single ion monitoring (SIM) mode.[31] Robertson *et al.*[31] presented representative chromatograms obtained using both HPLC-FLD and LC-MS, as depicted in Figures 10.5–10.7. Today, analysis of shellfish with simple toxin profiles will most likely employ the first method and, if the source of the PSP is unknown, then analysis by the second method is most appropriate. Representative LC techniques for the detection of saxitoxin are detailed in Table 10.3.

Advantages of using HPLC assays to measure saxitoxin include automated and continuous operation, increased sensitivity and increased precision. HPLC analysis of saxitoxin is limited in that it requires a skilled technician to operate the instrumentation, the analytical instrumentation is expensive, it requires extensive calibration and it is still not legally accepted for regulatory purposes. Advantages and limitations of LC-MS are similar to those

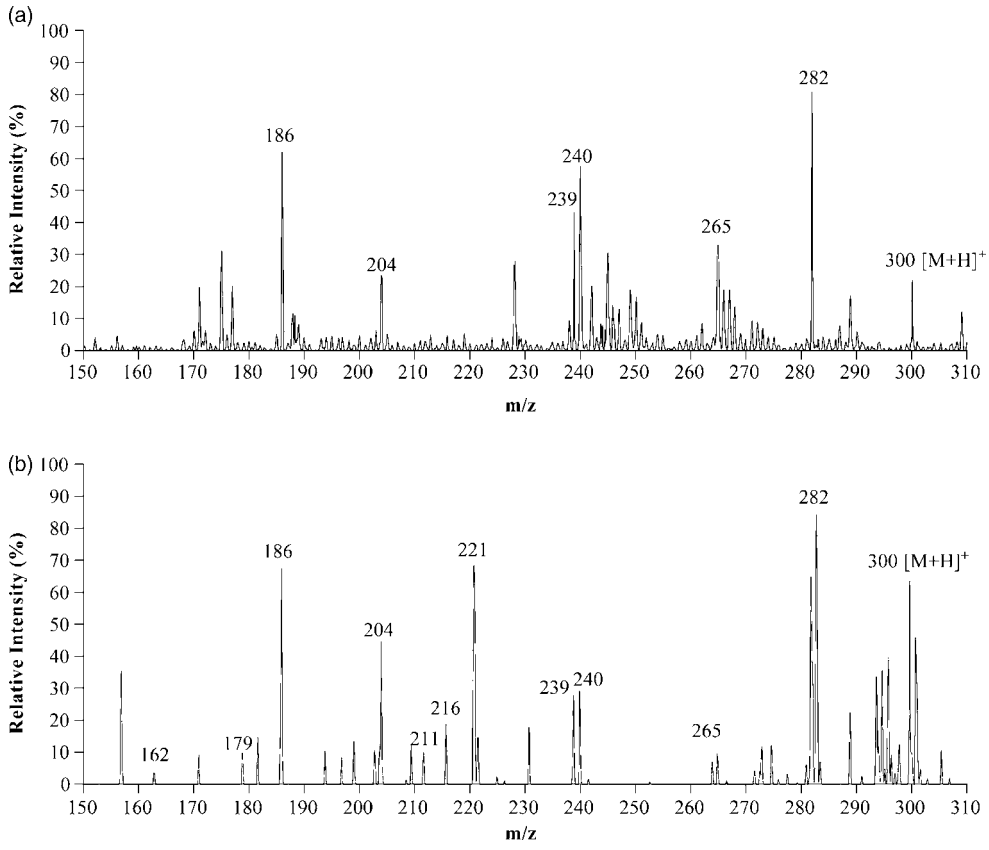


Figure 10.7 Fragment ion mass spectra for authenic STX (a) and an extract of *Octopus* (*Abdopus*) sp. 5 (b) Reprinted from *Toxicon*, **44**, A. Robertson, D. Stirling, C. Robillot, L. Llewellyn and A. Negri, First report of saxitoxin in octupi, 765–771, 2004, with permission from Elsevier

of HPLC; however, LC-MS still remains largely a research tool for the detection of saxitoxin owing its prohibitive cost and skill level.

10.4 SUMMARY

Chromatographic analysis of nerve agents has specialized purposes in many fields of analytical science, including clinical chemistry, food science, therapeutic drug monitoring and death investigation. As general analytical technologies have advanced, methodologies to improve the detection of nerve agents such as neuromuscular blocking agents and natural toxins such as saxitoxin have been introduced. Although chromatographic analyses of nerve agents are not heavily represented in the literature, techniques have been adequately presented and reproduced or modified by subsequent researchers. This chapter reviews both summarized and specific chromatographic techniques to analyze nerve agents presented in the past 20 years, in an effort to organize these technologies for the reader to evaluate carefully and understand.

Table 10.3 Liquid Chromatographic Detection of Saxitoxin

Author (Year)	STX Analogues (LOD equivalent)	Method Summary
Oshima (1995)	*Carbamate toxins* • STX (4 μg STX/100 g) • NEO (6 μg STX/100 g) • GTX 2 and 3 together (<1 μg STX/100 g) GTX 1 and 4 together (2 & 3 μg STX/100 g, respectively	*Sample Preparation:* • Extract STX with 0.5 M acetic acid and 50% methanolic chloroform (2:1, v/v) in • Sonicate for 5 min • Stir for 30 min at RT and centrifuge • Reextract organic layer with 2 mL 0.5 M acetic acid • Aqueous phases combined and concentrated in a rotary evaporator and reconstituted in 0.5 M acetic acid • SPE (C18 Sep Psk, Waters Corp., Milford, MA) followed by ultrafiltration (5000 dalton; Millipore Co., MA) • Immediate analysis or store at −20°C • *Reverse Phase Column:* • Prodigy 5μ C8 • $150 \times 4.6\,\text{mm}^{2}$ i.d. • 5 μm particles • 0.7 to 0.8 ml min^{-1} *Mobile Phase:* • 1 mM tetrabutylammonium phosphate (pH 5.8) for C toxin group • 2 mM 1-heptanesulfonic acid in 10 mM APB (pH 7.1) for gonyautoxin (GTXs) • 2 mM 1-heptanesulfonic acid in 30 mM APB (pH 7.1) & 3% CAN for saxitoxin (STXs) *Post-column oxidation:* 7 mM periodic acid in 10 mM PPB (pH 9.0) in a Teflon tubing coil (0.5 mm × 10 m) at 65°C. 0.5 M acetic acid to halt rxn. *Detection:* • Fluorescence • $\lambda_{ex} = 330$ nm • $\lambda_{em} = 390$ nm *Run Time* <20 min

Asp (2004)	*Carbamate toxins* 1. STX (4 µg STX/100 g) 2. NEO (6 µg STX/100 g) 3. GTX 2 and 3 together (<1 µg STX/100 g) GTX 1 and 4 together (2 & 3 µg STX/100 g, respectively	*Sample Preparation:* • 1. Extract STX with 0.1 N HCL at 90°C • 2. Protein Ppt. wit 5% tungstic acid at (9:1) with extract • 3. SPE Extraction (divinylbenzene/N-vinylpyrrolidone copolymer, Waters Corp., Milford, MA) • 4. Microcentrifugation (0.2 μm filter) • *Reverse Phase Column* • Chrompack C8 • 150 × 4.6 mm ^{2} i.d. • 5 µm particles • *Mobile Phase:* • 2 mM sodium 1-heptanesulfonate in 30 mM APB (pH 7.1) in ACN (96:4) • 2 mM sodium 1-heptanesulfonate in 10 mM APB (pH 7.1) in ACN (96:4) • *Post-column oxidation* • 7 mM periodic acid in 50 mM NPB (pH 9.0) in a reaction coil (0.5 mm × 10 m) at 85°C. 0.5 M acetic acid to halt rxn. • *Detection*: • Fluorescence • $\lambda_{ex} = 330$ nm • $\lambda_{em} = 390$ nm *Run Time* <20 min
Roberts on (2004)	1. STX	*Sample Preparation*: • Extract STX with 80% ethanol (pH2, HCl) at 5 mL/g tissue • Homogenize on ice • Sonicate for 10 min in ice bath. Repeat twice • Ultracentrifuge for 20 min at 4°C. • Repeat steps 1 thru 4 twice, pooling all supernatants • Ultrafiltration (0.2 µm nylon; Millipore Co., MA) and lyophilizing. • Dried extracts reconstituted in 0.05 M acetic acid (1 mL) and passed through 10,000 MWCO centrifugal ultra-filter (Microcon, Millipore)

(*continued*)

Table 10.3 (*Continued*)

	Column: • TSK-Gel Amide 80 column Toso Haas • 250 × 4.6 mm 2 i.d. • 5 μm particles *Mobile Phase:* • 40°C isocratic elution • 2 mM ammonium formate, 3.6 mM formic acid in 70% ACN:water (at 0.2 ml min^{-1}) *Special Treatment*: Ion spray interface introduction (9:1) waste to MS split ESI interface *Detection:* *MS/MS* • Positive ion • HV capillary+4 kV • Skimmer: 40 V • Precursor ions: *m/z* 300 Da • Isolation width: *m/z* 6 • Daughter ions scanned: • *m/z* 100–400 Da • *Product ions (m/z)* STX: 300 → 282, 265, 240, 239, 204, 186 *Run Time* <25 min

Abbreviations: LOD- limit of detection; STX- saxitoxin; NEO- neosaxitoxin; GTX- gonyautoxin; RT- room temperature; Ppt- protein precipitation; SPE- solid phase extraction; APB- ammonium phosphate buffer; CAN- acetonitrile; NPB- sodium phosphate buffer; PPB- potassium phosphate buffer; rxn- reaction

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11

History and Pharmacology of γ-Hydroxybutyric Acid

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11.1 INTRODUCTION

γ-Hydroxybutyric acid (GHB) is an endogenous compound in the mammalian central nervous system and peripheral tissues and a minor metabolite or precursor of γ-aminobutyric acid (GABA), an inhibitory neurotransmitter. Laborit and his associates first synthesized GHB in 1960 as an experimental GABA analog for possible use in the treatment of seizure disorder (Laborit *et al.*, 1960). They hypothesized that if GHB could readily cross the blood–brain barrier then perhaps it could facilitate the synthesis of GABA in the brain. Although GHB did not produce elevated GABA synthesis, the research revealed that GHB had some properties that rendered it useful as an anesthetic adjuvant. The earliest pharmacological use of GHB in humans was in this application. Blumenfeld *et al.* (1962) and Sprince *et al.* (1966) investigated the potential anesthetic properties of GBL and 1,4BD. Their observations that sleep induction time was the shortest with GBL and longest with 1,4BD as compared with GHB was an early clue to the metabolic and structural relationship among these compounds (Figure 11.1). Additional studies demonstrated that the pharmacologically active form of GBL was in fact GHB (Roth *et al.*, 1966). Roth and Giarman (1965) determined that a lactonase enzyme in blood and liver rapidly catalyzed the hydrolysis of GBL to GHB.

A genetic disorder called GHB aciduria occurs when there is a deficiency of succinic semialdehyde dehydrogenase. Persons with this disorder have elevated concentrations of GHB in their blood, spinal fluid and urine (Gibson *et al.*, 1998). The clinical manifestations of the increased GHB concentration can range from mild oculomotor problems and ataxia to severe psychomotor retardation, but it is most commonly characterized by mental, motor and language delay accompanied by hypotonia.

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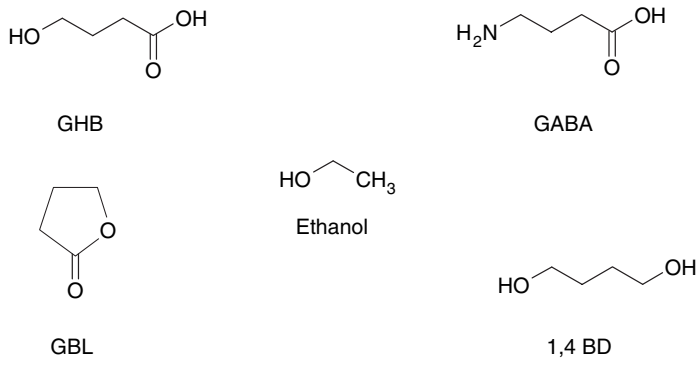

Figure 11.1 Structures of GHB, GBL, 1,4BD, GABA and ethanol

11.2 HISTORY OF ILLICIT USE OF GHB

In 1977, a study was published that would permanently change the relative obscurity of GHB, GBL and 1,4BD. Takahara *et al.* (1977) administered GHB to six healthy adult males and showed an approximately 10-fold increase in plasma growth hormone concentration that peaked at 45 min post-dose. This effect persisted for about 15 min and then the growth hormone concentration declined towards the pretreatment level. The growth hormone concentration at 120 min post-dose was still above baseline but significantly (two-thirds) below the peak concentration. Based on this report, bodybuilders assumed that they could increase growth hormone concentration by using GHB and thereby optimize their muscle-building potential. A more recent study by Van Cauter *et al.* (1997) showed that the increase in growth hormone secretion was correlated with the enhancement of slow wave sleep and growth hormone release did not occur prior to sleep onset. The growth hormone stimulating effect of a 2–3 g dose of GHB was seen during the first 2 h of sleep as an increase in amplitude and duration of the normal growth hormone secretory pulse associated with sleep onset as opposed to an increase in the number of growth hormone release pulses.

The use of GHB by bodybuilders seemed harmless in theory until emergency room reports associated with GHB toxicity started to accumulate (Centers for Disease Control, 1990). Users soon discovered that GHB had a definite mood elevating quality and introduced

Table 11.1 Common street names for GHB

Gamma OH	Liquid ecstasy
Sodium oxybate	Easy Lay
Natural Sleep 500	Salt water
Oxy-Sleep	Vita G
GHBA	Georgia Home Boy
G-caps	Grievous bodily harm
G	Great Hormones at Bedtime
Soap	Liquid X

GHB into the party drug scene, where it remains today. GHB is known by numerous street/slang names (Table 11.1). Most of these slang names utilize the letters G, H and B, such as 'Georgia Home Boy' or 'Great Hormones at Bedtime'. The slang name 'liquid ecstasy' can be confusing, as 'ecstasy' or XTC is the slang name commonly associated with methylenedioxymethamphetamine (MDMA), which is not in the same class of compounds as GHB. In November 1990, the US Food and Drug Administration (FDA) warned consumers of the danger of GHB, but the incidents of poisonings continued to rise. GHB and products containing GHB were removed from the market and GHB moved underground. Users soon discovered that GHB could easily be synthesized from readily available precursors. The industrial solvent γ-butyrolactone (GBL), when made basic with lye and heated, would yield GHB. With addition of an acid such as vinegar to adjust the pH, the solution of GHB was ready to consume. This illicit GHB is especially dangerous because its concentration is unknown and can vary greatly from batch to batch. Coupled with the fact that GHB has a very steep dose response curve, it is easy to overdose accidentally. Also, contaminants may be introduced in the clandestine manufacturing process. Toxicity of GHB is characterized by euphoria, dizziness, visual disturbances, decreased level of consciousness, nausea, vomiting, suppression of the gag reflex, bradycardia, hypotension, acute delirium, confusion, agitation, hypothermia, random clonic muscle movements (twitching), coma, respiratory depression and death. Although there have been some reports of seizures associated with GHB intoxication, there is no evidence of true seizure activity as measured by EEG in humans (Entholzner *et al.*, 1995). However, only GHB doses consistent with safe anesthesia have been evaluated in these EEG studies. Clonic muscle movements and severe parasympathomimetic activity, including profuse salvation, defecation and urination, have been documented in dogs treated with large doses (toxic and lethal) of GHB (Lund *et al.*, 1965). The clonic muscle movement was so prominent in the dogs that at anesthetic doses a barbiturate was also administered to effect convenient anesthesia. Another complicating factor is that GHB used outside a clinical setting is frequently used in combination with other drugs. This could affect the pharmacology of GHB in many ways depending upon which additional drug(s) are consumed and their dose. By far the most common drug taken in combination with GHB is ethanol (Louagie *et al.*, 1997; Li *et al.*, 1998; Centers for Disease Control and Prevention, 1999). This combination is especially dangerous because ethanol potentiates GHB's Central Nervous System (CNS) depressant effects, as demonstrated by depression of the startle response (a measure of sensory responsiveness) in rats (Marinetti and Commissaris, 1999). GHB has been implicated in fatalities both when administered alone (Marinetti *et al.*, 2000) and when used in combination with other drug(s) (Ferrara *et al.*, 1995). The use of GHB has been implicated in sexual assault. In fact, a common slang name for GHB is 'date rape drug', although it is not deserving of this title. Actual confirmed cases where GHB has been used in this capacity do exist but they are not common in comparison with other drugs more frequently chosen for this crime, namely ethanol, cannabinoids and benzodiazepines (ElSohly and Salamone, 1999). The most likely negative outcome of chronic GHB use is addiction. A GHB withdrawal syndrome has been documented with chronic GHB use (Hernandez *et al.*, 1998; Craig *et al.*, 2000; Dyer *et al.*, 2001). The clinical presentation of GHB withdrawal ranges from mild clinical anxiety, agitation, tremors and insomnia to profound disorientation, increasing paranoia with auditory and visual hallucinations, tachycardia, elevated blood pressure and extra-ocular motor impairment. Symptoms, which can be severe, generally resolve without sequelae after various withdrawal periods, although one documented death has occurred. Treatment with benzodiazepines has been successful for symptoms of a mild withdrawal syndrome.

11.3 CLINICAL USE OF GHB IN HUMANS

As discussed at the beginning of this chapter, the first clinical use of GHB was as an anesthetic adjuvant or induction agent. This application is still in use today in Europe (Kleinschmidt *et al.*, 1999). In the USA, the only medically approved use of GHB is as a treatment for cataplexy associated with the disease narcolepsy. The medically formulated GHB approved by the FDA is called Xyrem® and is a controlled substance (Federal Schedule III). This research has been ongoing since the 1970s and subjects participating in the clinical trails have experienced few adverse effects (Broughton and Mamelak, 1979). Another promising area for medically formulated GHB is in the treatment of alcohol withdrawal syndrome. This is not surprising since ethanol and GHB are similar compounds, both in structure and pharmacology (Figure 11.1). Cross-tolerance between ethanol and sub-anesthetic doses of GHB has been observed in rats, which may explain why alcoholics being treated with GHB do not experience sedation at doses that would sedate a non-alcohol-tolerant individual (Colombo *et al.*, 1995). Adverse effects have been mild except for occasional replacement of alcohol addiction with GHB addiction, resulting in some subjects self-medicating with additional GHB to enhance its effects (Addolorato *et al.*, 1997; Beghè and Carpanini, 2000). Treatment with GHB has also been investigated for opiate withdrawal syndrome (Gallimberti *et al.*, 1993), cocaine addiction (Fattore *et al.*, 2000) and fibromyalgia (Scharf *et al.*, 1998). For an in-depth review of GHB use in the treatment of withdrawal, see the April 2000, Volume 20, Issue 3 of the journal *Alcohol.*

11.4 HISTORY OF ILLICIT USE OF GBL AND 1,4BD

On 18 February 2000, GHB was placed in Federal Schedule I of the Controlled Substances Act with GBL as both a list I chemical and a controlled substance analog and 1,4BD falling under the controlled substance analog section (US Congress, 2000). Unfortunately, scheduling has not curbed the illicit use of this trio. With the placement of GHB in Federal Schedule I, there is more interest on the part of the illicit manufacturers in producing a GHB product that will stay in the lactone form and not spontaneously convert to GHB because penalties for GBL are less severe. In fact, a seizure of solid GBL has been reported in California where the liquid GBL was adsorbed on silicon dioxide powder, which was then placed in a clear capsule (DEA Southwest Laboratory, 2001). This adds additional danger because the lactone form, based on its physical characteristics and its increased solubility in lipids, has been shown in animal studies to be absorbed by the gut more efficiently than the GHB acid. The FDA has requested removal of health supplement products containing GBL, but this is a small fraction of the products that contain this compound (Food and Drug Administration, 1999a). The most common use of GBL is as an industrial solvent with domestic production of approximately 80 000 tons per year. In industry, it is widely used and would be difficult to replace based on its excellent properties as a safe, effective and biodegradable degreaser. Some manufacturers of diet aid products containing GBL have masked the presence of this ingredient by using one of the many chemical synonyms for GBL in the list of ingredients on the product label (Table 11.2).

GBL has been detected in alcoholic beverages, tobacco smoke, coffee, tomatoes, cooked meats and several foodstuffs (IARC, 1999). GBL produces the same pharmacological effects as GHB as it is rapidly converted to GHB in the body. At equimolar doses GBL produced a

Table 11.2 Chemical synonyms for GBL

Dihydro-2(3*H*) furanone	4-Hydroxy-γ-lactone	4-Deoxytetronic acid
Butyrolactone-4	Butyrylactone	1,2-Butanolide
4-Butyrolactone	Butyrl lactone	1,4-Butanolide
Butyric acid	Gamma-6480	4-Hydroxybutyric acid γ-lactone
Butyric acid lactone	Gamma BL	γ-Hydroxybutyric acid cyclic ester
Butyrolactone	BLO or BLON	NCI-C55875
α-Butyrolactone	4-Butanolide	Tetrahydro-2-furanone

more prolonged hypnotic effect in rats as compared with GHB (Lettieri and Fung, 1978). To a lesser extent, 1,4BD has followed the same path as GBL, and is gaining in popularity and has recently been associated with adverse events, including death (Kraner *et al.*, 2000; Zvosec *et al.*, 2001). As with GBL, the major use for 1,4BD in the USA is as an industrial compound. Unlike GBL, however, 1,4BD is not used to manufacture GBL or GHB illicitly. This conversion is an industrial process and cannot be accomplished in a household setting. The pharmacological effects of 1,4BD are ultimately those of GHB, which is produced metabolically from 1,4BD (Figure 11.2). With the increased attention on GHB toxicity, the

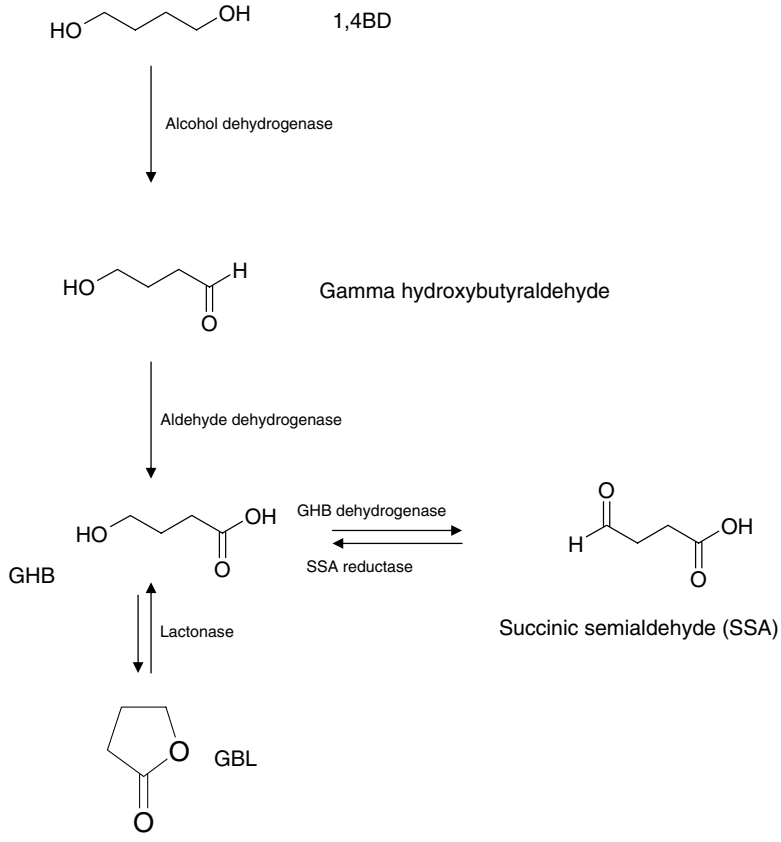

Figure 11.2 Metabolic pathway of GBL and 1,4BD

Table 11.3 Chemical synonyms for 1,4BD

Butane-1,4-diol	Sucol B
1,4-Butylene glycol	1,4-Tetramethylene glycol
1,4-Dihydroxybutane	Butylenes glycol
Diol 14B	Tetramethylene-1,4-diol

FDA has also requested that products containing 1,4BD be removed from the market (Food and Drug Administration, 1999b). As with GBL, this action could also lead manufacturers to replace the name, 1,4-butanediol, with one of its many chemical synonyms to disguise its presence in the product (Table 11.3).

In addition to GBL and 1,4BD, the recent emergence of diet aid products containing γ-valerolactone (GVL) has caused some concern as to the pharmacological effects of this compound and its safety for human consumption. GVL is an FDA-approved food additive and/or adjuvant and it is safe for human consumption at 0.0009322 mg kg^{-1} per day per person (FDA data). One currently available diet aid product, Tranquili-G, recommends a dose of GVL around 3 g, which is much greater than the amount that was approved by the FDA for safe human consumption. In rats and rabbits, administration of GVL produced the following effects; marked muscular weakness, mild anesthesia and an increase in the rate of respiration with oral LD_{50} values for GVL at 8.8 and 2.5 g kg^{-1} for rats and rabbits respectively (Deichmann *et al.*, 1945). Past research has documented that GVL is quickly metabolized by the lactonase enzyme to 4-Me-GHB in human blood and rat liver microsomes (Fishbein and Bessman, 1966), similar to the way in which GBL is metabolized by this enzyme to GHB (Figures 11.2 and 11.3). 4-Me-GHB is a structural analog of GHB but GVL is not. It has been demonstrated in the rat model that GVL has similar pharmacological properties to GHB mediated through the 4-Me-GHB metabolite (Marinetti, 2003).

11.5 DISTRIBUTION AND PHARMACOKINETICS OF GHB, GBL AND 1,4BD

Normal endogenous concentrations of GHB in CSF in humans are dependent on age and the presence of seizure disorder. Infants had higher concentrations of GHB (0.26–0.27 μg mL^{-1}) in the CSF than older children (0.11–0.13 μg mL^{-1}), who, in turn, had higher concentrations than adults (0.02–0.03 μg mL^{-1}). Children with myoclonic-type seizures had the highest concentrations of GHB in the CSF (0.78–0.97 μg mL^{-1}), whereas children with other types

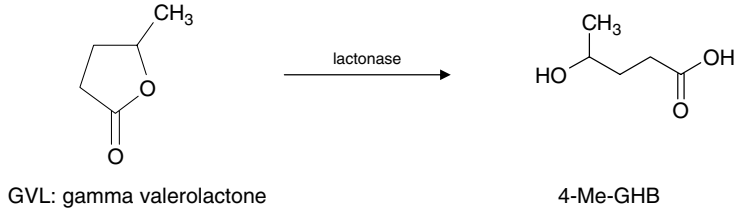

Figure 11.3 Production of 4-Me-GHB from GVL *in vivo*

of seizures had the next highest concentrations (0.37–0.48 $\mu g\ mL^{-1}$) (Snead *et al.*, 1981). Along with the analysis of the CSF for GHB, the serum of all the subjects was also analyzed. Of the 130 subjects, none had any measurable amount of GHB in the serum, with a limit of sensitivity of the assay of 0.002 $\mu g\ mL^{-1}$.

In another study, GHB concentrations were determined in various tissues and blood of rats (Nelson *et al.*, 1981). The results showed that brown fat, kidney, muscle and heart showed concentrations $>1\ \mu g\ g^{-1}$. Liver, lung, blood, brain and white fat had concentrations $<0.25\ \mu g\ g^{-1}$. The reason for the variations in GHB content between the specimens analyzed is not known. A study that compared endogenous GHB concentrations in brain from human, monkey and guinea pig showed an elevated concentration compared with that seen in rat brain, 0.2–2, 0.3–1.8 and 0.1–0.6 $\mu g\ g^{-1}$, respectively (Doherty *et al.*, 1978). In this same study, endogenous GHB in CSF and blood of humans was found in only trace amounts (CSF $\sim 0.01\ \mu g\ mL^{-1}$).

The distribution of GHB into the CSF appears to lag behind that in blood or brain. After a 500 $mg\ kg^{-1}$ intravenous dose of GHB had been administered to dogs, the plasma concentration peaked within 5 min and the brain concentration peaked within 10 min, but it was 170 min before CSF concentrations reached their maximum (Shumate and Snead, 1979). This suggests a passive diffusion of GHB from serum or brain into the CSF. In alcohol-dependent patients GHB did not accumulate in the body on repeated doses nor did it exhibit any protein binding. The mean peak plasma concentrations of therapeutic oral doses of 25 and 50 $mg\ kg^{-1}$ of GHB per day given to 50 alcohol withdrawal syndrome patients were 55 $\mu g\ mL^{-1}$ (range 24–88) and 90 $\mu g\ mL^{-1}$ (range 51–158), respectively (Snead *et al.*, 1976; Ferrara *et al.*, 1993).

Absorption of GHB has been shown to be a capacity-limited process with increases in dose resulting in increases in time to peak concentration. The concentration in brain equilibrates with other tissues after approximately 30 min. GHB crosses the placental barrier at a similar rate to that in the blood–brain barrier (Van der Pol *et al.*, 1975). GHB also exhibits first-pass metabolism when given orally with about 65% bioavailability when compared with an equivalent intravenous dose.

GHB exhibits zero-order (constant rate) elimination kinetics after an intravenous dose. Since GHB exhibits zero-order kinetics, it has no true half-life. The time required to eliminate half of a given dose increases as the dose increases. A daily therapeutic dose of 25 $mg\ kg^{-1}$ has an apparent half-life of about 30 min in humans, as determined in alcohol dependent patients under GHB treatment (Ferrara *et al.*, 1992). In contrast, an apparent half-life of 1–2 h was observed in dogs when they were given high intravenous doses of GHB. In humans it has been documented that there is increased rate of absorption if GHB is administered on an empty stomach, resulting in a reduced time to reach the maximum plasma concentration of GHB (Borgen *et al.*, 2001).

As discussed previously, the absorption of GBL has been documented to occur faster than GHB. It has also been proposed that in addition to the better absorption of GBL, it may also distribute differently than GHB. An early study comparing the distribution of equimolar doses of GHB and GBL in rats found that although peak plasma concentrations were higher with GHB they remained elevated longer with GBL. In addition, concentrations of GBL in the lean muscle mass of the rat were always elevated compared with concentrations of GHB (Roth and Giarman, 1966). This suggests sequestration of GBL into lean muscle prior to its conversion to GHB. Since lean muscle does not contain the lactonase enzyme, it is conceivable that this could occur. The GBL could then redistribute into the blood to be

converted to GHB by the blood or liver lactonase enzyme. This could explain the prolonged elevated concentrations of GHB in blood that occur when GBL is given. Neither GHB nor GBL is sequestered in fat.

The absorption and distribution of 1,4BD is similar to that of GHB. It is a lipophobic or polar compound so it does not absorb faster than GHB. After its absorption, it requires a two-step enzymatic conversion to GHB that results in a slightly longer time to peak GHB concentration and also a longer time of elevated GHB concentration. The conversion process of 1,4BD to GHB can be slowed or inhibited by ethanol, pyrazole or disulfiram.

11.6 GHB INTERPRETATION ISSUES AND POST-MORTEM PRODUCTION

The most difficult aspect of GHB analysis is interpretation of the numbers that are generated. Helrich *et al.* (1964) correlated blood concentrations of GHB with state of consciousness in 16 adult human patients (Table 11.4). This study revealed that GHB blood concentrations as high as 99 $\mu g\ mL^{-1}$ could be achieved with the patient still displaying an 'awake' state, the subject spontaneously coming in and out of consciousness characterized a light sleep state. Subjects in the medium sleep state were clearly asleep but were able to be roused. At the highest concentrations studied, GHB produced a deep sleep characterized by response to stimuli with a reflex movement only. It is clear from these data that blood concentrations of GHB display a large overlap across the four states of consciousness described. For example, a subject with a blood concentration of 250 $\mu g\ mL^{-1}$ could be in a light, medium or deep sleep state. The smallest dose given, 50 $mg\ kg^{-1}$, produced peak plasma concentrations no greater than 182 $\mu g\ mL^{-1}$ and the largest dose given, 165 $mg\ kg^{-1}$, produced peak plasma concentrations $>416\ \mu g\ mL^{-1}$. Fourteen patients received doses of 100 $mg\ kg^{-1}$, resulting in peak blood concentrations ranging from 234 to 520 $\mu g\ mL^{-1}$. Twelve of the 16 patients required intubation, but the need for intubation did not necessarily correlate with those patients who received the higher doses of GHB. Metcalf *et al.* (1966) observed electroencephalographical (EEG) changes in 20 humans given oral doses of GHB in the range 35–63 $mg\ kg^{-1}$. The EEG pattern was similar to that seen in natural slow wave sleep. Profound coma was observed at approximately 30–40 min post-dose in subjects given oral GHB doses $>50\ mg\ kg^{-1}$.

Animal and human studies have demonstrated that endogenous GHB concentrations can rise post-mortem and under inappropriate specimen storage conditions. Doherty *et al.* (1978) observed an increase in the GHB concentrations in brain specimens after 6 h with a further increase if the specimens were left at room temperature. Snead *et al.* (1981) also observed an increase in GHB concentrations in CSF after 12 h of storage at room temperature. It was

Table 11.4 GHB concentrations associated with state of consciousness

State of consciousness	GHB ($\mu g\ mL^{-1}$)
Awake	0–99
Light Sleep	63–265
Medium Sleep	151–293
Deep Sleep	344–395

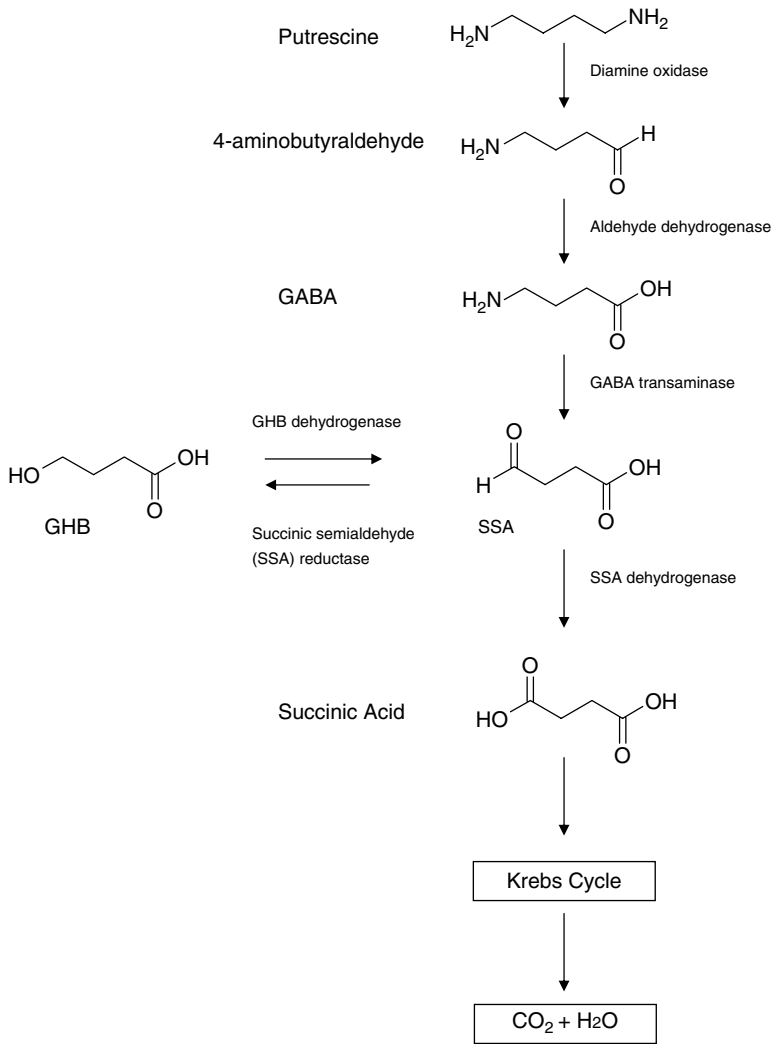

Figure 11.4 Metabolic pathway of putrescine to GHB

subsequently discovered that if animals were killed using microwave irradiation, post-mortem GHB accumulation was blocked (Eli and Cattabeni, 1983). This suggests some type of enzymatic conversion from a GHB precursor. One source of the post-mortem GHB increase is the metabolism of previously sequestered GABA that is being released from storage vesicles as the natural decomposition process occurs. Excess GABA would be exposed to the GABA transaminase enzyme, which could convert it to succinic semialdehyde, which could in turn be converted to GHB in addition to proceeding on to succinic acid (Figure 11.4).

Another source of post-mortem GHB production is putrescine (1,4-butanediamine), a biogenic polyamine initially detected in decaying animal tissues, but now known to be present in all cells, both eukaryotic and prokaryotic, where it is important for cell

proliferation and differentiation (Merck, 1996). Research on polyamine metabolism by Seiler (1980) demonstrated the formation of GABA from putrescine both in visceral organs and in the CNS of vertebrates. This is an enzymatic process in the polyamine metabolic pathway that involves diamine oxidase (DAO) and aldehyde dehydrogenase to form GABA (Figure 11.4). In organs that do not contain high activity of DAO, such as brain, an alternative pathway is available for the conversion of putrescine to GABA (Sessa and Perin, 1994). In addition, Snead *et al.* (1982) observed an 80–100% increase in GHB concentrations in rat brain after intracerebroventricular administration of putrescine. All of these theories are consistent with the observation that microwave irradiation prevents post-mortem accumulation of GHB as the exposure to the microwaves would denature the enzymes. This is also supported by the fact that excessive GHB production is not seen in blood specimens that have an enzyme inhibitor added (Stephens *et al.*, 1999).

Regardless of the source of the increased concentration of GHB post-mortem, it can be a significant problem in determination of a cause of death due to GHB toxicity. Post-mortem production of GHB can result in blood concentrations of GHB that would produce significant effects in a living person. Anderson and Kuwahara (1997) analyzed heart blood, femoral blood and urine from 96 post-mortem cases with no suspected exogenous GHB use and 50 ante-mortem blood specimens also with no evidence of GHB use. The specimens were stored at 4 °C with sodium fluoride added to the blood as a preservative. The following results were obtained for the post-mortem specimens: heart blood 1.6–36, femoral blood 1.7–48, and urine 0–14 $\mu g\ mL^{-1}$, and no detectable amount of GHB in any of the ante-mortem blood specimens with a limit of detection of 0.5 $\mu g\ mL^{-1}$. The upper end of the post-mortem blood range overlaps the range detected in blood during therapeutic application of a 25 $mg\ kg^{-1}$ per day dose of GHB, 24–88 $\mu g\ mL^{-1}$. If one compares this with the highest concentrations that were discussed earlier in ante-mortem blood and CSF, 1 $\mu g\ mL^{-1}$ in the CSF of children with myoclonic-type seizure disorder, it is obvious that these are significant concentrations. Also, the fact that there is very little difference between GHB concentrations in heart and femoral blood suggests that this is not simply a post-mortem redistribution phenomenon. In a similar study by Fieler *et al.* (1998), a range of 0–168 $\mu g\ mL^{-1}$ (average 25 $\mu g\ mL^{-1}$) in post-mortem blood was observed with no detectable GHB in post-mortem urine or ante-mortem blood or urine with a limit of detection of 1 $\mu g\ mL^{-1}$. Although these data showed a larger concentration range than the data of Anderson and Kuwahara (1997), an average of 25 $\mu g\ mL^{-1}$ for 20 blood specimens indicates that the majority of the concentrations were at the lower end of the range. Fieler *et al.* (1998) did not discuss the storage conditions of the specimens prior to analysis, which has been shown to have an effect on the amount of post-mortem GHB produced. Stephens *et al.* (1999) compared post-mortem GHB concentrations in samples under various storage conditions. They found that GHB concentrations increased by 50% if the specimen did not contain sodium fluoride even when it was stored in a refrigerator, and if the specimen was stored at room temperature without 10 $\mu g\ mL^{-1}$ sodium fluoride the concentration could double. The addition of sodium fluoride did not affect the concentration of GHB in urine specimens. Ante-mortem blood buffered with citrate (yellow top tube) has been shown to display an increase in GHB concentration over time (LeBeau *et al.*, 2000a). Ten ante-mortem citrate-buffered whole blood specimens were analyzed for GHB after various storage periods from 6 to 36 months at −20 °C. Although no exogenous GHB use was suspected, all of the specimens had concentrations of GHB ranging from 4 to 13 $\mu g\ mL^{-1}$ with a mean of 9 $\mu g\ mL^{-1}$.

The real problem this presents is in the interpretation of exogenous GHB use, GHB toxicity or GHB overdose resulting in a fatality. Since GHB is rapidly cleared from the body, even at elevated doses, if there is any survival time the blood concentrations can easily fall into the range of post-mortem production. Therefore, a urine specimen should be collected in addition to blood in suspected GHB cases. In post-mortem cases, if urine is not available, then eye fluid or CSF is indicated, especially CSF because the normal endogenous concentration of GHB in CSF is well documented. It is advised that all of the specimens collected be preserved with at least 2% sodium fluoride and stored at refrigerator temperature or frozen and that tubes containing citrate be avoided. GHB concentrations in post-mortem eye fluid and urine from decedents with no exogenous GHB exposure have been documented to be $<10\ \mu g\ mL^{-1}$ (Marinetti *et al.*, 2005). Organ specimens may also be helpful, but again there are very few published data on endogenous concentrations of GHB in various organs in humans. The cut-off concentration for reporting exogenous GHB consumption in a specimen must be set above the suspected post-mortem production or ante-mortem endogenous GHB concentration.

Data from analyses of multiple specimens and a good case history can help tremendously, especially if litigation is involved. For example, in a case that involved the death of a young girl and GHB, a post-mortem blood GHB concentration of $15\ \mu g\ mL^{-1}$ was demonstrated. This concentration is in the range of post-mortem GHB production. However, a detailed case history plus a post-mortem urine GHB concentration of $150\ \mu g\ mL^{-1}$, an ante-mortem blood concentration of $510\ \mu g\ mL^{-1}$, an ante-mortem urine concentration of $2300\ \mu g\ mL^{-1}$ and the lack of any other significant autopsy findings made the cause of death an obvious GHB fatality. The decedent had a 14-h survival time in the hospital prior to death, which explains the low post-mortem blood GHB concentration (Marinetti *et al.*, 2000). A second GHB fatality showed a heart blood concentration of 66 μg/mL, a femoral blood concentration of 77 μg/mL, an eye fluid concentration of 85 μg/mL and a urine concentration of 1260 μg/mL. This individual had an extensive history of chronic GHB use and no other cause of death (Marinetti *et al.*, 2000).

Ante-mortem blood and urine endogenous levels of GHB have been documented to be $<1\ \mu g\ mL^{-1}$ and $<10\ \mu g\ mL^{-1}$, respectively. Anderson Kuwahara (1997) analyzed 50 ante-mortem blood specimens from individuals with no evidence of GHB use. No detectable amounts of GHB were observed in any of the blood specimens, using a limit of detection of $0.5\ \mu g\ mL^{-1}$. Similarly, endogenous GHB concentrations were measured in 192 blood specimens from living subjects thought to be non-GHB users (Couper and Logan, 1999). All measurable blood GHB concentrations were below $1\ \mu g\ mL^{-1}$, using a detection limit of $0.5\ \mu g\ mL^{-1}$. LeBeau *et al.* (2000b, 2002) investigated urinary GHB levels to differentiate between endogenous and exogenous concentrations. Every urine void produced by three non-GHB using male subjects over a 1-week period was individually collected and analyzed for the presence of endogenous GHB, using a limit of detection of $0.19\ \mu g\ mL^{-1}$. Overall, 129 urine specimens were analyzed and the mean endogenous GHB concentration detected was $1.58 \pm 1.55\ \mu g\ mL^{-1}$ (range <0.19–$6.62\ \mu g\ mL^{-1}$). Individual mean concentrations in the three subjects were 0.63 ± 0.34, 3.02 ± 1.52 and $0.56 \pm 0.31\ \mu g\ mL^{-1}$, respectively. Urine specimens from females were also analyzed with an endogenous GHB range of 0.00–$1.94\ \mu g\ mL^{-1}$. Although there were significant intra- and inter-individual variations in the urinary levels of endogenous GHB, the concentrations did not fluctuate to levels that were higher than the laboratory's reporting level for urinary GHB of $10\ \mu g\ mL^{-1}$.

11.7 ANALYSIS FOR GHB, GBL AND 1,4BD

GHB does not exist in a static state, even outside the body. In solution, it exists in a dynamic state in equilibrium with its lactone, GBL (Figure 11.2). The ratio of the two forms is dependent on the pH of the matrix or the type of matrix containing the GHB. For example, in blood, the GHB acid form predominates because the lactonase enzyme converts any of the lactone to the acid. However, if the GHB is in a matrix that does not contain this enzyme, such as urine, water or juice, the two forms will reach equilibrium. The pK_as for GHB and 4-Me-GHB are 4.72 and 5.72, respectively, therefore the lactone form predominates at pH below these values and the acidic or salt form predominates above these values. Complete conversion to GBL is favored in dehydrating conditions in a concentrated acid solution (pH < 2). The rate at which the equilibrium is achieved depends on the temperature and the actual pH of the matrix.

There are two basic approaches to GHB and GBL analysis, depending on the matrix being analyzed. If a biological matrix is being analyzed, then conversion of GHB to GBL or derivatization of the GHB without conversion is acceptable, as a biological matrix should not normally contain any GBL with the exception of stomach contents. However, it is possible that a urine specimen (or any other biological specimen that does not contain the lactonase enzyme) with a low pH (5–6) might produce GBL from GHB upon long-term storage. Two studies that demonstrated this were done by Ciolino and Mesmer (2000) and Ciolino *et al.* (2001). The authors compared solutions of GHB and GBL in various matrices at various pH values and found that the relative amounts of GHB and GBL changed depending on the pH of the matrix and storage conditions, specifically time and temperature. Anderson *et al.* (2000) reported a fatality that displayed measurable concentrations of GBL in heart and femoral blood, vitreous humor, liver, bile, gastric contents and urine. Perhaps this is evidence of a massive GBL ingestion that caused saturation of the lactonase enzyme since the concentrations of GHB in this case were very high with a heart blood concentration of 1473 μg mL^{-1}. However, if 1,4BD is ingested, because it is not in equilibrium with GHB or GBL, unchanged 1,4BD may be detected in a biological specimen if enough is ingested and/or the time interval since ingestion is short (McCutcheon *et al.*, 2000). Analysis of illicit or commercial products to determine GHB or GBL require either that two aliquots of sample be analyzed if the GHB to GBL conversion is used, or that a derivatization method is used so that the percentage composition of GHB in the sample can be determined. Knowing the actual percentage of GHB and GBL in the sample may be important in the documentation of a product source in both criminal and civil litigation cases.

Biological specimen extraction can be accomplished by liquid–liquid, solid-phase or solid-phase microextraction with subsequent detection of GHB or GBL by gas chromatography–mass spectrometry (GC-MS) using electron ionization (EI), positive or negative chemical ionization (CI) or gas chromatography with flame ionization detection (GC-FID). LeBeau *et al.* (1999) describes a method that employs two aliquots of specimen. The first is converted to GBL with concentrated sulfuric acid while the second is extracted without conversion. A simple liquid–liquid methylene chloride extraction was utilized, and the aliquots were then screened by GC-FID without derivatization. Specimens that screened positive by this method were then re-aliquoted and subjected to the same extraction with the addition of the deuterated analog of GBL. The extract was then analyzed by headspace GC-MS in the full-scan mode. Quantitation was performed by comparison of the area of the

molecular ion of the parent drug (*m/z* 86) with that of the hexadeuterated analog (*m/z* 92). This method displayed linearity in both blood and urine from 5 to 1000 μg mL^{-1} with recoveries that ranged between 75 and 87%.

A liquid–liquid extraction method employed by the toxicology laboratory in the Department of the Coroner of Los Angeles County California (Anderson and Kuwahara, 1997) first converts GHB to the lactone followed by chloroform extraction. Both blood and urine were analyzed using this method with γ-valerolactone as the internal standard. Detection was by GC-MS using the selected-ion monitoring (SIM) mode. The ions monitored were GBL *m/z* 42, 56 and 86 with *m/z* 41, 56 and 85 for the internal standard. The method was linear from 5 to 300 μg mL^{-1}, with a lower limit of detection of 5 μg mL^{-1}. However, owing to the introduction of commercial products containing GVL, a different internal standard is recommended.

Couper and Logan (2000) describe a liquid–liquid extraction method that uses ethyl acetate to extract GHB, without conversion to GBL. The extract is derivatized using *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) and acetonitrile and then analyzed using GC-MS in the EI mode with SIM of *m/z* 233, 204 and 117 for GHB and *m/z* 235, 103 and 117 for the diethylene glycol internal standard. The method gave a 55% extraction recovery of GHB in blood with a limit of detection in blood and urine of 0.5 μg mL^{-1} and a linearity of 1–100 μg mL^{-1} in blood and 1–200 μg mL^{-1} in urine. Another liquid–liquid extraction that is very similar to Couper and Logan's method has been described by Elian (2000). The differences between this method and the Couper and Logan method are that it uses GHB-d_6 as the internal standard; it was evaluated only in urine specimens, and gave an 80–85% recovery with linearity from 2 to 50 μg mL^{-1}.

There are three different solid-phase extraction (SPE) methods that differ with respect to the column used, pre-extraction sample treatment, post-extraction sample treatment and internal standard. All three SPE methods extract GHB from urine and blood, derivatize with BSTFA with 1% TMCS, and detect analytes by GC-MS in the EI mode with either SIM or the full-scan mode. The ions monitored for the GHB di-TMS derivative are *m/z* 233, 234 and 235 with care being taken to avoid the ions that are common between di-TMS GHB and di-TMS urea, at *m/z* 147, 148 and 149. Urea is a naturally occurring compound in urine and care must be taken that its derivative does not interfere.

The SPE method used by the Miami–Dade County Medical Examiner's Office, Toxicology Laboratory, in Miami, Florida (Andollo and Hearn, 1998) uses Chem-Elute® SPE columns, β-hydroxybutyric acid internal standard, pretreatment of urine with sulfuric acid and pretreatment of blood with sodium tungstate and sulfuric acid. The Dade County method gave an absolute recovery of 30% with a limit of detection of 2 μg mL^{-1} and a limit of quantitation of 10 μg mL^{-1}.

McCusker *et al.* (1999) described a method for urine that uses a United Chemical Technologies GHB SPE column. Urine, GHB-d_6 internal standard and phosphate buffer are combined and extracted. The final eluate is taken to dryness and then reconstituted with hexane and dimethylformamide (DMF). After a simple liquid–liquid extraction, the hexane layer is discarded, the DMF is taken to dryness and the residue is derivatized with BSTFA with 1% TMCS in ethyl acetate. The method gave a linearity range from 5 to 500 μg mL^{-1}. The limit of sensitivity and recovery of the method were not reported.

The United Chemical Technologies GHB SPE column method has been modified to include GHB and 1,4BD analysis in blood, eye fluid and tissue homogenate samples. The sample size is only 200 μL and requires a sample preparation step of extraction with

acetone prior to elution on the SPE column. The eluent from the SPE column, which is in 99:1 methanol–ammonium hydroxide, is then taken to dryness and derivatized with BSTFA with 1% TMCS. The ions monitored are at *m/z* 233, 234 and 235 for GHB di-TMS, *m/z* 239, 240 and 241 for GHB-d_6 di-TMS, *m/z* 219, 220 and 221 for 1,4BD di-TMS and *m/z* 223, 224 and 225 for 1,4BD-d_4 di-TMS (Kraner *et al.*, 2000; Crifasi and Telepchak, 2000).

The third SPE method utilizes a Multi-Prep® Anion Exchange GVSA-200 gravity flow column and is used for urine or blood analysis (Biochemical Diagnostics, 2000). This is a very simple SPE procedure that involves mixing the specimen with pH 9 Tris buffer prior to extraction and passing all solutions through the column by gravity flow. This investigational method utilized GHB-d_6 as the internal standard and displayed linearity from 1 to 500 μg mL^{-1} with a recovery >90%.

Frison *et al.* (1998) describes a method using solid-phase microextraction of GHB in plasma and urine. This is a new approach for GHB analysis that shows promise in that it is simple, sensitive and requires only 0.5 mL of specimen. The linearity range was from 1 to 100 μg mL^{-1} in plasma and from 5 to 150 μg mL^{-1} in urine with a limit of detection of 0.05 and 0.1 μg mL^{-1} for plasma and urine, respectively. The limit of detection was calculated based on aqueous solutions because the blank plasma and urine specimens had endogenous GHB concentrations of 0.1–0.2 and 0.5–1.5 μg mL^{-1}, respectively. The method required conversion of GHB to GBL with GBL-d_6 as the internal standard and detection by headspace GC-MS with spectra from both CI and EI ionization modes. Many methods are available for GHB and GBL analyses depending on the equipment and resources available to the laboratory.

For laboratories interested in measuring concentrations of GHB at or below endogenous levels, a GC-MS method with negative ionization has been described for the measurement of GHB in rat brain (Ehrhardt *et al.*, 1988). It involves a complicated liquid–liquid extraction with subsequent derivatization of GHB with MTBSTFA. This method is very sensitive, with the capability of detecting as little as 2 pg of GHB. The calibration curve showed linearity from 0 to 64 ng of GHB. Another method, described by Shima *et al.* (2005),

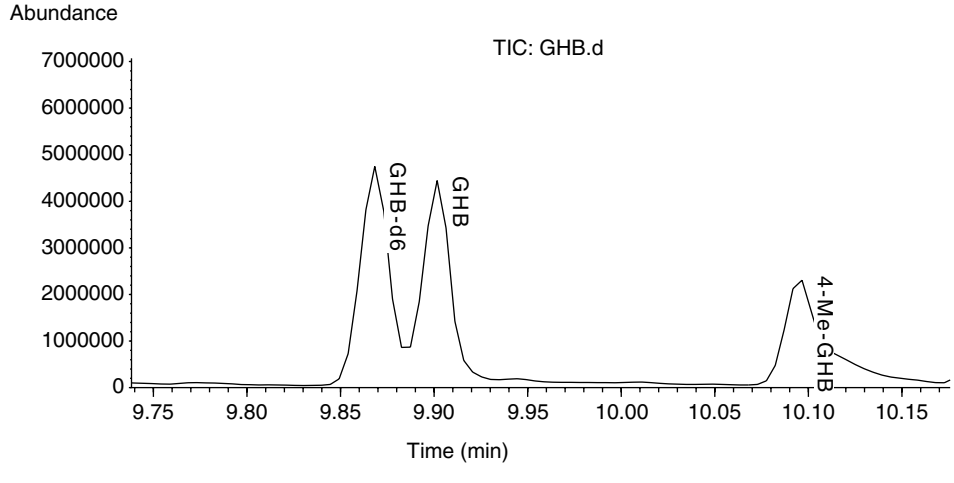

Figure 11.5 Total ion chromatogram of GHB, GHB-d_6 and 4-Me-GHB di-TMS derivatives

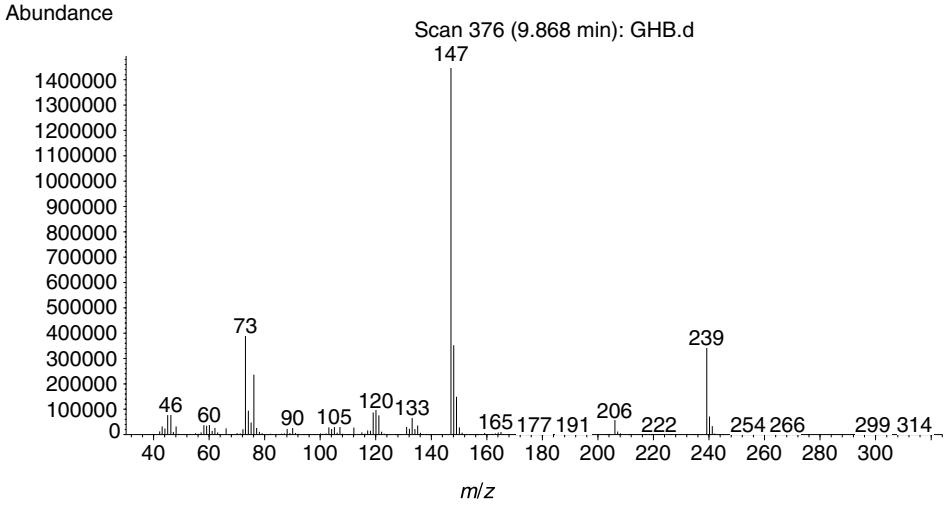

Figure 11.6 Full-scan mass spectrum of GHB-d_6 di-TMS derivative

would also be appropriate with a linear range of 0.003–3.0 μg mL^{-1}. This method involves a simple liquid extraction with subsequent BSTFA derivitization and GC-EI-MS analysis.

However, the same is not true for 1,4BD analysis. Concern over detecting and quantifying 1,4BD is just becoming an issue in the forensic community. As discussed previously, the pharmacologically active form of 1,4BD is GHB. However, determination of the concentration of 1,4BD may be useful in documenting acute intoxication. McCutcheon *et al.* (2000) utilized a simple one-step extraction at physiological pH into

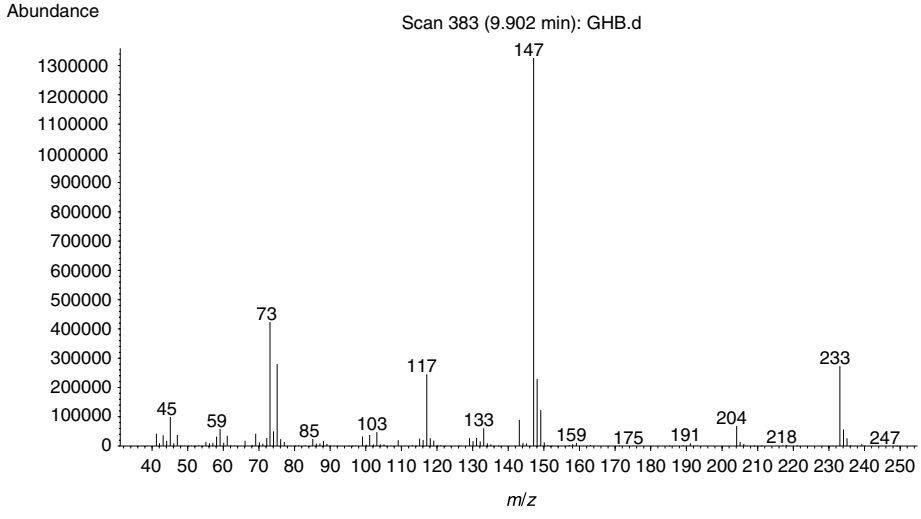

Figure 11.7 Full-scan mass spectrum of GHB di-TMS derivative

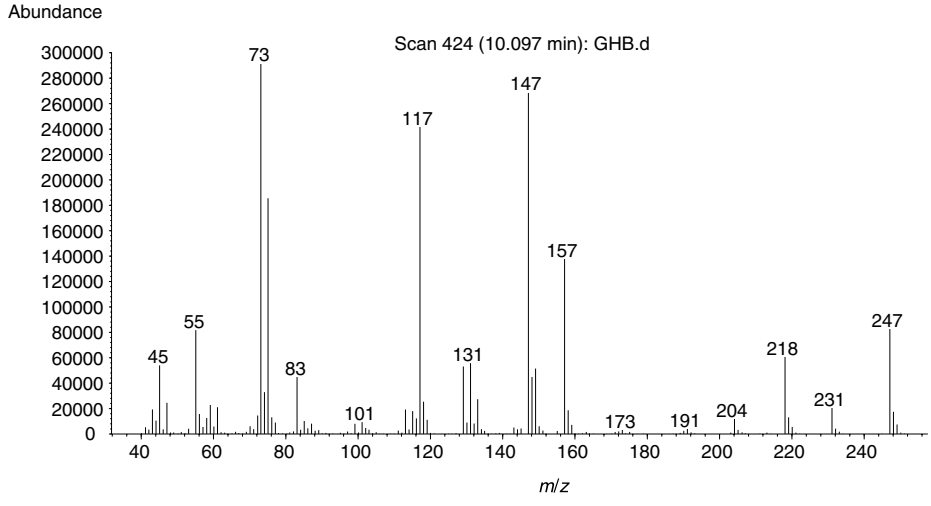

Figure 11.8 Full-scan mass spectrum of 4-Me-GHB di-TMS derivative

n-butyl chloride for the extraction of 1,4BD. The solvent was dried down to about 75 μL and subjected to GC-MS analysis. The 1,4BD eluted prior to GBL with a detection limit between 50 and 100 μg mL^{-1}. Research by the authors is under way to improve this method. A method specific for the detection of 1,4BD in liver and brain tissue was described by Barker *et al.* (1985). This method involved a complicated extraction scheme, which utilized different extraction protocols for the aqueous and lipid fractions of the tissues. Both fractions were then lyophilized, extracted again and subsequently derivatized with heptafluorobutyric anhydride (HFBA). The internal standard used was deuterated 1,4BD with identification and quantitation by GC-EI-MS in the SIM mode. Both rat and human brain tissue were analyzed in addition to rat liver. The method demonstrated a recovery of $74 \pm 10\%$ for the aqueous fraction and $88 \pm 9\%$ for the lipid fraction. The linearity range was 0.0–1.0 μg g^{-1} wet weight of tissue with a limit of detection of 0.01 μg g^{-1} wet weight of tissue.

Quantitation of 1,4BD by high-performance liquid chromatography (HPLC) with photodiode-array detection was described by Duer *et al.* (2001). The 1,4BD was extracted from the biological matrix with acetonitrile and derivatized with 3,5-dinitrobenzoyl chloride. This method has a linear range from 3 to 250 mg L^{-1} with a limit of quantitation of 10 mg L^{-1}. Also, as was mentioned previously, the United Chemical Technologies GHB SPE column method has been modified to include 1,4BD analysis (Kraner *et al.*, 2000).

Analysis for 4-Me-GHB can be accomplished along with GHB analysis. An SPE procedure for both analytes has been described by Marinetti *et al.* (2005). This procedure uses 200 μL of specimen with BSTFA derivitization and GC-EI-MS detection using GHB-d_6 as the internal standard. The ions monitored are at *m/z* 233, 234 and 235 for GHB di-TMS, *m/z* 239, 240 and 241 for GHB-d_6 di-TMS and *m/z* 231, 247 and 248 for 4-Me-GHB di-TMS (Figures 11.5–11.8). The linearity is from 5 to 150 and from 2 to 200 μg mL^{-1} for GHB and 4-Me-GHB, respectively.

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12

Liquid Chromatography with Inductively Coupled Plasma Mass Spectrometric Detection for Element Speciation: Clinical and Toxicological Applications

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12.1 INTRODUCTION

It is common knowledge that the mobility, bioavailability, retention and specific biological functions of an individual element depend on its physicochemical form. However, there are no general rules relating a chemical structure of elemental species to its behavior or biological activity in a given system. Thus, depending on their actual oxidation state, different toxicity is observed for chromium and arsenic: hexavalent chromium [Cr(VI)] presents higher toxicity than the trivalent form [Cr(III)], whereas arsenic in a lower oxidation state [As(III)] is more toxic than pentavalent arsenic [As(V)]. Furthermore, depending on the element, the organic compounds can be more or less harmful than inorganic forms (mercury and arsenic, respectively). It is clear, though, that element speciation information is of great importance in all research areas related to human health. As approved by respective IUPAC commissions, 'chemical species' is a specific and unique molecular, electronic or nuclear structure of an element. The term 'speciation analysis' refers to the measurement of the quantities of one or more individual chemical species in a sample.[1,2] In life sciences, analytical results are helpful

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in clarifying element pathways in living organisms with special emphasis on possible beneficial or toxic effects and the development of new metal-based drugs.

Element speciation in biological materials is a difficult analytical task. The challenge is to identify and/or quantify very low concentrations of few to several target species (concentration values far below the total element content) in a complex chemical matrix. Additional difficulties include similar physicochemical properties exhibited by the species of one element and their chemical lability. Very often, not all element forms in the sample are known and, finally, the list of certified reference materials (CRMs) for speciation analysis is still limited.[3,4] The two most important features of an analytical tool suitable for speciation analysis are excellent selectivity and high sensitivity. Special care should be paid to preserve the natural composition and distribution of species in the sample during the entire procedure.

A common speciation scheme after sample preparation involves a fractionation step followed by the element quantification in the fractions obtained. A clear trend exists toward using the techniques that combine separation and detection steps into one operating on-line system. In these coupled techniques, the selectivity is achieved by application of powerful separation modes (different chromatographic or electrophoretic methods), while the use of atomic spectrometric techniques assures high sensitivity of detection. It should be stressed, however, that coupled techniques with element-specific detection do not provide structural information for the species. If the appropriate standards are available, the assignment of chromatographic peaks can be accomplished by spiking experiments. On the other hand, the identification of unknown forms and/or ultimate confirmation of unexpected compounds observed in the sample require the use of complementary techniques (molecular mass spectrometry or NMR).[1,5–8]

In this chapter, the toxicologically and clinically relevant applications of liquid chromatography (LC) with inductively coupled plasma mass spectrometric (ICP-MS) detection are reviewed (a list of abbreviations used is given at the end of this chapter). After brief characterization of the analytical system, the speciation of arsenic, iodine, mercury, selenium and platinum is discussed.

12.2 LIQUID CHROMATOGRAPHY WITH INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRIC DETECTION

The choice of a separation technique is always dictated by the properties of the analyte(s) (volatility, stability, polarity, electrical charge, etc.) and also the sample composition. Since many toxicologically and/or clinically important species are not volatile, LC is often used in speciation analysis. The principal advantage of LC is the extended range of separation mechanisms, based on the convenient selection of column type and mobile phase composition.[9,10] Considering the requirement for high separation power in speciation analysis and, on the other hand, the need to preserve native element speciation, this variety of possible separation conditions is of primary importance. However, in spite of unquestionable versatility of LC, possible interactions of element species with the stationary phase and/or the components of mobile phase (buffers, organic modifiers) may cause unwanted changes in original element distribution among different species.[9,11,12] That is why, in many applications, compromised conditions have to be used, sacrificing chromatographic resolution, but assuring the integrity of species during separation. To improve the separation, a multidimensional chromatographic approach has often been explored.[13]

The chromatographic modes typically used in speciation analysis are size-exclusion, ion-exchange, ion-pair reversed-phase, reversed-phase and, to a lesser extent, micellar, vesicular, chiral and affinity LC. Detailed descriptions of their capabilities and limitations can be found in a number of comprehensive reviews.[1,2,9–11,14]

Among element-specific detectors, ICP-MS has become a primary tool in speciation analysis.[15] The unique features observed in total element analysis encompass high sensitivity (detection limits usually in the parts per billion or parts per trillion range), multielement capabilities and practically interference-free linear response over a wide concentration range (typically 4–8 orders of magnitude). Furthermore, ICP-MS detection enables: (i) monitoring effluents for their elemental composition with high sensitivity, (ii) determination of the target element with the selectivity over co-eluting elements, (iii) compensation of incomplete chromatographic resolution from complex matrices, (iv) isotope ratio and isotope dilution capabilities and (v) detection of a number of elements virtually simultaneously.[16] Recent progress in high-resolution instrumentation and collision/reaction technology has assumed high importance, since the modern instruments conveniently eliminate troublesome spectral polyatomic interferences.[7,17–19] The use of stable or radioactive artificial isotopes has expanded the potential of ICP-MS in speciation analysis, with stable isotopes being highly attractive. The major applications are isotope dilution quantification, method validation and studies on possible interconversion of species during the analytical procedure.[1] When the identity of the target species is known and the isotope-labeled species is available, the full possibilities of isotope dilution can be appreciated. In other words, possible loss and/or conversion of substance after spiking the sample with isotope labeled species have no effect on the analytical result.[20]

Finally, it should be noted that, when coupling ICP-MS with LC, factors such as the separation mechanism, composition and flow of the mobile phase and type of nebulizer and sample introduction device must be carefully considered and have consequently been topics of discussion in many papers and reviews.[9,11,16,21–23]

12.3 ANALYTICAL APPLICATIONS OF CLINICAL AND TOXICOLOGICAL RELEVANCE

Within the context of toxicological and clinical importance, speciation studies have been focused on relatively few elements, mainly aluminum, antimony, arsenic, chromium, iodine, lead, mercury, platinum, selenium and tin. However, coupled HPLC-ICP-MS has most often been used for speciation of arsenic, selenium, iodine and, to a lesser extent, mercury. The primary species of these elements include different oxidation states, alkylated metal and/or metalloid compounds, selenoamino acids and selenopeptides.[1,2] In addition, applications in studies on the pharmacokinetics of metal-based drugs (mainly platinum complexes) and metalloproteins should be included.[24,25] In the following sections, the advances in speciation studies of individual elements are reviewed.

12.3.1 Arsenic

The variety of physicochemical forms of arsenic and its well-demonstrated species-dependent toxicity have stimulated progress in speciation analysis of this element. A considerable number of analytical methods for the qualitative and quantitative analysis of

several arsenic species have been reported, most of them based on LC separation and element-specific detection.[26,27] In the context of possible health risk, such analyses have been carried out in several environmental, biological and clinical materials under different conditions of exposure. Since arsenic enters the human body in association with food products and drinking water, speciation in aquatic samples, soils, sediments and different food-related products is mandatory. Analysis of clinical samples (mainly urine, bile and liver) helps to clarify the metabolic pathways of this element.

In spite of considerable progress in analytical methodology, full information on all arsenic species contained in complex biological matrices cannot be obtained using a single analytical protocol. The main difficulty is the pretreatment step, in which a complete recovery of all species (characterized by different solubility, polarity, electrical charge, etc.) is not realistic.[27,28] However, of special interest are the species-targeted protocols that focus on (1) more efficient leaching of the species of interest, necessary for its quantification, (2) characterization of the unknown species by mass spectrometric techniques and/or (3) elucidation of the metabolic routes of the element in biological systems.[28,29]

On the other hand, real-world materials usually contain a limited number of arsenic compounds at concentration levels that allow their detection. For example, aquatic, soil and sediment samples mainly contain inorganic arsenic forms with a much lower contribution from methylated pentavalent species that are formed due to the activity of microorganisms.[30–34] Marine organisms are important in global cycling of As, since they accumulate inorganic arsenic from seawater and convert it into organic compounds. Arsenobetaine (AsB) is the main species found in fish tissues[27,35,36] and several arsenosugars have been identified in algae, marine bivalves and crustaceans.[37–42] Food-related products of terrestrial origin usually contain lower concentrations of arsenic than marine food (nanograms per gram versus micrograms per gram). However, certain plants (rice, carrots) are exceptions and speciation analysis is required.[34,43–47] Of special interest for the evaluation of health risk is the analysis of As forms in urine. Two methylated pentavalent element species (MMA and DMA) have often been reported together with smaller amounts of inorganic forms and AsB (after fish consumption). The identification of monomethylarsonous acid [MMA(III)] and dimethylarsinous acid [DMA(III)] provided a new insight into the toxicological aspects related to As biomethylation pathways. Furthermore, the conjugated forms of trivalent As species with glutathione have been identified in bile.[48–51] Worth mentioning is that the variety and quantitative distribution of As species observed in clinical samples depend on the biological species and/or subject examined and the conditions of exposure, but also depend on the analytical methodology applied.

Representative examples of arsenic speciation performed by LC coupled with ICP-MS are presented in Table 12.1. In agreement with the abundance of As species in real-world samples and the health risk involved, most studies have been focused the separation/quantification of As(III), As(V), MMA, DMA and AsB. Depending on the pH conditions, these species can be cationic, anionic or uncharged (pK_{a_1} 9.3, 2.3, 2.6, 6.2 and 2.2, respectively[52]) allowing their separation by anion-exchange, ion-pair and, to a lesser extent, cation-exchange chromatography. Using the anion-exchange mode, good resolution was achieved using phosphate-, carbonate-, hydroxide- or phthalate-based mobile phases (pH in the range 5–11).[26,31,33,34,43,47,53–57] Ammonium salts are preferred in order to minimize the residue formed in the sampler/skimmer cones of the ICP-MS instrument. The most common elution order is AsB (minimum retention), As(III), DMA, MMA, As(V), but it is sometimes different, depending on the pH, gradient conditions, etc. The cation-exchange mode is often

Table 12.1 Applications of LC with ICP-MS detection for arsenic speciation analyses of toxicological and/or relevance

Sample	Analytical procedure	Species of interest	Toxicological and/or clinical relevance	Ref.
Drinking water	IE HPLC-ICP-MS; different mobile phases tested, of which Tris–acetate buffer enabled baseline separation	Arsenate [As(V)], arsenite [As(III)], MMA, DMA	Methodological approach: precision, accuracy, linearity and detection limits evaluated, several samples of drinking water were analyzed	30
Drinking water	Anion-exchange chromatography with a mobile phase containing 2 mmol L^{-1} NaH_2PO_4 and 0.2 mmol L^{-1} EDTA, pH 6, coupled to ICP-MS detection	Arsenate [As(V)], arsenite [As(III)], MMA, DMA	Methodological approach: baseline separation achieved within <10 min. The analytical performance characteristics studied (DL < 100 ng As L^{-1} for all the species evaluated). The feasibility for the analysis of normal As levels in drinking water was demonstrated. However, it was observed that matrix composition and the sample treatment may affect natural As(III)/As(V) distribution	31
Freshwater	Porous graphitic carbon used as the stationary phase for RP separation, elution in a gradient of HCOOH aq.; ICP-MS detection	Arsenate [As(V)], arsenite [As(III)], MMA, DMA	Methodological approach: adsorption of As(V) on the graphite surface observed, efficient separation of four species [except As(V)] within 10 min, DL ranging from 10 to 70 ng As L^{-1}; natural, arsenic-containing freshwater analyzed	32
Soil extracts	Extractions were performed with 0.3 M ammonium oxalate (pH 3), Milli-Q water, 0.3 M sodium carbonate (pH 8) and 0.3 M sodium bicarbonate (pH 11); anion-exchange separation (IonPac AS11 column) with NaOH as a mobile phase and ICP-MS detection	Arsenate [As(V)], arsenite [As(III)], MMA, DMA	Methodological approach focusing mobilization of As species from soils. The results showed that the mobilization of arsenic was pH dependent. Dramatic consequences have to be expected for pH changes in the environment especially in cases where soils contain high amounts of mobile arsenic	33

(*continued*)

Table 12.1 (*Continued*)

Sample	Analytical procedure	Species of interest	Toxicological and/or clinical relevance	Ref.
Animal feed additives, wine and kelp samples	Ion-pair HPLC on RP microbore column with mobile phase containing 5 mmol L^{-1} TBAH, pH 6.0 [separation time of As(III), As(V), MMA, DMA < 2 min], coupled on-line with ICP-MS	Phenylarsonic acids, arsenate [As(V)], arsenite [As(III)], MMA, DMA, arsenosugars	Development of analytical methodology for fast separation of As species. Application for studies on distribution and possible transformation of phenylarsonic compounds used as poultry and swine feed additives presented; different wine samples analyzed [As(III) the only species found] and arsenosugars determined in kelp samples	62,63
Poultry litter	Three ion-exchange chromatographic separations examined: (1) Dionex AS14 column using a phosphate mobile phase, (2) an AS16 column with hydroxide eluent and (3) an AS7 column with nitric acid mobile phase	ROX, *p*-ASA, arsenate [As(V)], arsenite [As(III)], MMA, DMA	Analytical methodology discriminating between organoarsenic compounds used as animal feed additives and more toxic inorganic species. The values of DL were generally <50 ng As L^{-1}. The major arsenic species in a water extract of a poultry litter sample was identified as ROX and trace concentrations of DMA and As(V) were also detected. A number of unidentified As species were observed at low concentrations, presumably metabolites of ROX	53
Chicken meat	Two anion-exchange LC columns examined, on-line ICP-MS	Arsenite [As(III)], arsenate [As(V)], MMA, DMA, AsB	Evaluation of a candidate reference material; As species found were DMA and AsB. The stability of arsenic species in a chicken meat candidate reference material for at least 12 months was demonstrated	54

Algae	Separation on anion-exchange column (Hamilton PRP-X100) with 20 mmol L^{-1} $NH_4H_2PO_4$ (pH 5.6) mobile phase and a column temperature of 40 °C [separation time of seven As species 16 min; As(V) and the ribose with the glycerol aglycone eluted in the dead volume]; on-line ICP-MS detection	Arsenosugars, arsenate [As(V)], arsenite [As(III)], MMA, DMA	Four naturally occurring arsenosugars (AsSug-OH, AsSug-PO_4, AsSug-SO_3, AsSug-SO_4), MMA, DMA and inorganic As species were separated. Extracts obtained from the brown algae *Fucus spiralis* and *Halidrys siliquosa* contained AsSug-SO_4 as the major compound (~55% of total extractable As) together with AsSug-PO_4 and AsSug-SO_3. Arsenic acid was a significant constituent of *Halidrys siliquosa* (~6.5%), but was not detected in *Fucus spiralis*	37
Algae	Multidimensional LC: SEC fractionation followed by anion-exchange LC and further purification by RP-HPLC. The ICP-MS detector was coupled on-line and ESI-MS and ESI-MS-MS analyses were additionally carried out for identification of the eluted compounds	Up to 14 standards of arsenic species (including arsenosugars) plus identification of new species	Identification and quantification of As species in algae extracts were undertaken. Several arsenosugars were found, accounting for >99% of the arsenic present in the extract. The identities of all the species, were confirmed and/or assigned by matching the retention times of chromatographically pure (after the 3rd LC dimension) species with standards and by ESI-MS-MS	40,41
Algae and shrimp	Weak anion-exchange column (Dionex AS4A) with elution in a gradient of HNO_3 allowed the separation of 8 species within 12 min; on-line ICP-MS.	arsenate [As(V)], arsenite, [As(III)], MMA, DMA, AsB, TMAO, AsC, TETRA	Methodological approach: very low background in the ICP-MS detection was obtained using nitric acid mobile phase, which enabled for very low DL (30–1600 ng As L^{-1}). Application to real sample analysis shown	38
Lobster tissue	Different extraction procedures tested (Soxhlet, MW-assisted, SFE); anion-exchange column (Hamilton PRP-X100), gradient elution with the two mobile phases containing $(NH_4)_2CO_3$ at different concentrations (separation time 27 min); on-line ICP-MS detection	AsC, AsB, DMA, MMA, arsenite [As(III)], arsenate [As(V)]	Methodological approach, focusing the efficiency of extraction and baseline separation of 6 species. The values of DL were in the range 17–29 ng As $kg,^{-1}$ and AsB was a primary species found in the lobster tissue	39

(*continued*)

Table 12.1 (*Continued*)

Sample	Analytical procedure	Species of interest	Toxicological and/or clinical relevance	Ref.
Fish, crustacean and sediment samples from estuary in the tin mining area	Enzymatic hydrolysis (trypsine) carried out for fauna samples; sediments were treated with 1 mol L^{-1} H_3PO_4 in an open focused MW system; separation on anion-exchange column (Hamilton PRP-X100); step gradient elution with a phosphate-based mobile phase, pH 6–7.5; on-line ICP-MS	AB, DMA, MMA, arsenite [As(III)], arsenate [As(V)]	In order to assess possible health risk for population living in contaminated area, the important As species were determined in fish and crustaceans which contribute to the local diet. The presence of more toxic inorganic forms of arsenic in both sediments and biota samples has implications for human health, particularly as they are readily 'available'	186
Fish and rice	Water–methanol extraction (for rice α-amylase treatment) followed by IP on an anion-exchange column (IonPac Ag7 + AS7); gradient elution with two mobile phases containing HNO_3, benzene-1,2-disulfonic acid dipotassium salt and 0.5% methanol; on-line ICP-MS	Arsenite (As(III)), arsenate (As(V)), DMA, MMA, AsC, TMAO, AsB, AsSug-PO_4, AsSug-SO_3, AsSug-SO_4, AsSug-OH	Health risk associated with dietary arsenic intake: the elevated levels of toxic inorganic As species (up to 90% in rice) found in ~250 food samples. The value of DL in rice was 2 ng As g^{-1}. In marine organisms, inorganic As was mainly present as As (V) whereas in rice As(III) predominated	44
Rice	Water–methanol (1:1) extraction; As(III) and AB separated on cation-exchange column (Hamilton PRP-X200) in pyridine formate 4 mmol L^{-1}, pH 2.8; As(V), MMA and DMA separated on anion-exchange column (Hamilton PRPX-100) with phosphate mobile phase 10 mmol L^{-1}, pH 6; on-line ICP-MS	Arsenite (As(III)), arsenate (As(V)), MMA, DMA, AsB	Study on arsenic species in rice, a candidate reference material representative for terrestrial biological samples. The values of DL expressed as As in dried rice wheat were 2, 3, 3 and 5 ng As g^{-1} for As(III), AsB, As(V), MMA and DMA, respectively. Effect of different processing steps, storage time and temperature conditions on species stability was studied. MMA and As(V) species were not stable under any storage conditions, probably due to microbiological activity	45

Rice	Sample hydrolyzed with 2 mol L^{-1} TFA, 6 h, 100 °C (extraction efficiency 92%); three AE separation conditions applied: (1) Waters IC-Pak Anion HR with carbonate mobile phase at pH 10; (2) Dionex AS7 and AG7 with HNO_3 (pH 1.8); and (3) Hamilton PRP-X100 with phosphate-based mobile phase at pH 6.3	Arsenite (As(III)), arsenate (As(V)), MMA, DMA, AsB, AsC	Different procedures examined for the extraction of arsenic species, their separation and quantification. The DL ranged from 6 to 17 ng As g^{-1}. Owing to the reduction of As(V) to As(III) during TFA treatment, the proposed procedure allowed for measuring total inorganic As. Inorganic As accounted for 11–91% of total As in rice. Lower concentrations of DMA were also detected	43
Carrots grown on arsenic-contaminated and non-contaminated soil	Extraction carried out with 1 mmol L^{-1} $Ca(NO_3)_2$ aq, As species separated and detected using anion-exchange HPLC coupled with ICP-MS.	Arsenite (As(III)), arsenate (As(V)), MMA, DMA	Study on the soil-to-carrot uptake rate of As (bioavailability). Inorganic arsenic species were prevalent in soil. The ingestion of the potentially toxic inorganic arsenic via consumption of carrots grown in soil contaminated at 30 µg As g^{-1} was conservatively estimated at 37 µg per week	34
Carrots	ASE (extraction yield 80–102%), weak anion-exchange column, isocratic elution with aqueous NH_4NO_3, on-line ICP-MS	Arsenite [As(III)], arsenate [As(V)], MMA, DMA, AsB	Toxicological consideration: inorganic As(III) and As(V) were the only species found in samples that contained less than 400 ng g^{-1} of total arsenic. MMA and an unidentified arsenic compound were present in some of the samples with higher total arsenic content	46
Apples	Overnight treatment with α-amylase and sonication with acetonitrile–water (40:60), followed by AE separation with phosphate-based mobile phase; on-line ICP-MS	Arsenite [As(III)], arsenate [As(V)], MMA, DMA	Health risk associated with dietary arsenic intake: total arsenic concentrations in the freeze-dried apple samples ranged from 8.2 to 80.9 µg As g^{-1}. The three most abundant species found were As(III), As(V) and DMA	55

(*continued*)

Table 12.1 (*Continued*)

Sample	Analytical procedure	Species of interest	Toxicological and/or clinical relevance	Ref.
Mushrooms	MW-assisted extraction, ion-exchange HPLC, on-line ICP-MS	Arsenite [(As(III)], arsenite [As(VI)], DMA, AsB, TMAO	DMA was the primary species found in mushrooms from contaminated and non-contaminated soil (68–74% of total extracted As). The results showed that, in contaminated soils, mushrooms or their associated bacteria were able to biosynthesize DMA from inorganic As	187
Nuts	As species were extracted with chloroform–methanol (2:1). After addition of DI, polar species concentrated in aqueous layer and analyzed by AE chromatography (8 min) with ICP-MS detection	Arsenite [As(III)], arsenate [As(V)], MMA, DMA	Health risk associated with dietary arsenic intake: significantly higher contribution of total As (2.6–16.9 ng As g^{-1}) was found in nut oil as compared to defatted material. The primary species found in the oil extracts were As(III) and As(V). Lower concentrations of two methylated species were observed in several nut types	56
Peanut butter	Sonication extraction with 2-butoxyethanol (recovery for spiking experiments 40–122%) followed by ion-exchange HPLC-ICP-MS or HG-ICP-MS	Arsenite [As(III)], arsenate [As(V)], MMA, DMA	Health risk associated with dietary arsenic intake: total As content ranged from 6.5 to 21.4 ng As g^{-1}. Inorganic As was primary element form in the samples analyzed with lower contribution of methylated species	188
Processed infant food products	TFA treatment, 6 h, 100 °C (extraction yield 94–128%), anion-exchange HPLC and ICP-MS detection	Arsenite [As(III)], arsenate [As(V)], MMA, DMA	Health risk associated with dietary arsenic intake: total As levels in the rice-based cereals were in the range 63–320 ng As g^{-1} and in the other food products below 24 ng As g^{-1}. Inorganic As and DMA were the main species found in rice-based and mixed rice/formula cereals. These species were also present in freeze-dried sweet potatoes, carrots, green beans, and peaches. MMA and DMA were detected in minute amounts only in few samples	47

Fish tissues and urine	Sample acidification with H_3PO_4, IP chromatographic separation (Altima C_{18}) with a mobile phase containing citric acid and hexanesulfonic acid at pH 4.5 (separation of 5 species in <4 min); on-line ICP-MS	Arsenite [As(III)], arsenate [As(V)], MMA, DMA, AsB	Methodological approach focusing fast and reliable speciation analysis in environmental and biological samples. The QL for As(V), MMA, As(III), DMA and AsB were 44, 56, 94, 64, 66 ng As L^{-1}, respectively. The procedure was tested using two reference materials (DORM-2 dogfish muscle tissue, NIST SRM 2670 freeze-dried urine, normal level, and then applied to realworld samples	36
Marine organisms, urine	Anion-exchange column with bicarbonate (pH 10.3 with aqueous ammonia) mobile phase and on-line ESI-MS	Arsenosugars, AsB, DMA, MMA	The ability of LC-ESI-MS for both quantitative analysis of As species and structural elucidation of new As compounds in biological samples was demonstrated	42
Urine, hair, fingernail, blood from people exposed to As-contaminated drinking water	Hot water extraction (TCA treatment for blood plasma), AE separation (polymer-based Shodex Asahipak ES-502N 7C column) with non-buffering mobile phase (15 mmol L^{-1} citric acid adjusted to pH 2 with HNO_3); ICP-MS detection	AsC, AsB, DMA(III), DMA, MMA(III), MMA, arsenite [As(III)], arsenate [As(V)]	Monitoring environmental exposure to As: seven As species, including MMA(III) (6.6%) and DMA(III) (13%) were found in urine samples. Primary species in fingernail was As(III) (62.4%) and lower concentrations of As(V), MMA, DMA(III), DMA were also observed. No organic trivalent As species were found in hair and blood. Inorganic arsenic was predominant in hair and DMA in blood plasma. Arsenic in urine, fingernails, and hair correlated positively with water As, suggesting that any of these measurements could be considered as a biomarker to As exposure. Status of urine and exogenous contamination of hair urgently need speciation of As in these samples, but speciation of As in nail is related to its total As content. Therefore, total As concentrations of nails could be considered as biomarker to As exposure in the endemic areas	189–191

(*continued*)

Table 12.1 (*Continued*)

Sample	Analytical procedure	Species of interest	Toxicological and/or clinical relevance	Ref.
Rat bile	Anion-exchange column (Shodex RSpak JJ50-4D) with 20 mmol L^{-1} oxalic acid (pH 2.3 with aqueous ammonia) and on-line ICP-MS	Arsenite [As(III)], arsenate [As(V)], MMA, MMA(III), DMA, $As(GS)_3$, $CH_3As(GS)_3$	Study on the stability of As–glutathione complexes in rat bile and the role of GSH in stabilizing these complexes. The results obtained suggest that GSH plays an important role in preventing hydrolysis of As–GSH complexes and generation of well-known toxic trivalent arsenicals	49
Rat bile and urine after oral or intravenous exposure to As compounds	RP separation (Intersil ODS-3 column, 5 mmol L^{-1} TBAH + 3 mmol L^{-1} malonic acid, 5% methanol mobile phase); ICP-MS detection	Aresenite [As(III)], arsenate [As(V)] DMA, MMA, $As(GS)_3$, $CH_3As(GS)_2$	Metabolic speciation studies revealed that, after exposure to inorganic As, the element was excreted into bile as $CH_3As(GS)_2$ and DMA. Arsenic in rats exposed to methylated species was mostly excreted into urine in the unchanged chemical forms. It was demonstrated that biliary and urinary arsenic excretion and speciation are affected by the route, dose and chemical forms of arsenical administration, and GSH plays a key role in arsenic metabolism	50
Organs and body fluids of rats after intravenous injection of As(III)	Anion- and cation-exchange separation; on-line ICP-MS	Arsenite [As(III)], MMA, DMA, MMA(III), As conjugates with GSH	The metabolic balance and speciation studies suggested that As(III) is methylated in the liver during its hepato-enteric circulation through the formation of the GSH-conjugated form $As(GS)_3$, and MMA(III) and MMA are partly excreted into the bile, the former being in the conjugated form $CH_{(3)}As(GS)_2$. DMA is not excreted into the bile but into the bloodstream, accumulating in RBCs, and then excreted into the urine mostly in the form of DMA in rats	58

Organs and body fluids of rats after intravenous injection of MMA and DMA	Urine and bile analyzed on AE column (ES-502N 7C) with citric acid (15 mmol L^{-1}, pH 2) as a mobile phase; liver supernatants, plasma and red blood cells lysates on gel filtration column (GS 220 HQ) with ammonium acetate buffer (50 mmol L^{-1}, pH 6.5); on-line ICP-MS	Arsenite [As(III)], DMA, MMA, AsB, possible As conjugates with GSH	Study on species-dependent metabolic pathway of As in rats: DMA and MMA were mostly excreted into urine in the intact forms. Their uptake to organs/tissues was lower with respect to As(III) exposure. The unidentified metabolites were excreted not into the bile but into the bloodstream	51
Urine from four APL (acute promyelocytic leukemia) patients treated with arsenic trioxide	HPLC-ICP-MS, no details given	Arsenite [As(III)], arsenate [As(V)], MMA, DMA, MMA(III), DMA(III)	Pharmacokinetics and metabolism of As(III) in APL patients studied. The intermediate MMA(III) and DMA(III), were detected in most urine samples when diethyldithiocar-bomate, was added to these samples for stabilization of As species. The major urine species As(III), MMA and DMA, accounting for >95% of the total arsenic excreted in urine. On the other hand, these species accounted for 32–65% of the total arsenic, suggesting other pathways of As excretion, such as through the bile, may play an important role in removal of arsenic from the human body when challenged by high levels of As(III)	192
Human urine and blood after ingestion of Chinese seaweed Laminaria	AE column (Hamilton, PRP-X100) with different phosphate-, oxalate- and acetate-based mobile phases containing 3–20% of methanol; ICP-MS detection and ESI-MS-MS for species identification and/or confirmation	Arsenosugars, arsenite [As(III)], DMA, MMA, DMAE	Study on metabolic pathway of As species (mainly arsenosugars) ingested with algae. Total arsenic and speciation analysis revealed no marked increase in arsenic blood, serum and packed cells levels up to 7 h after ingestion. As species identified in urine were DMA, MMA and DMAE. Another 5 species remained unknown. In simulated gastric fluid incubated with algae, a degradation of arsenosugars into a compound with a mass of 254 Da was observed	193

(*continued*)

Table 12.1 (*Continued*)

Sample	Analytical procedure	Species of interest	Toxicological and/or clinical relevance	Ref.
Urine from children and adults exposed to As in drinking water	HPLC-ICP-MS, no details given	Arsenite [As(III)], arsente [As(V)], DMA, MMA	Health risk assessment: average total urinary arsenic was higher in children than in adults and total arsenic excretion per kg body weight was also higher for children than adults. The values of ratio MMA/(inorganic As) for adults and children were 0.93 and 0.74, respectively, suggesting that the first methylation step could be more active in adults. On the other hand, the values of ratio DMA/MMA in children were higher than in adults (8.15 and 4.11, respectively) indicating that the 2nd methylation step is more active in children than adults. It was concluded that children retain less arsenic in their body than adults, which could explain why children did not show skin lesions compared with adults that had been exposed to this same contaminated water	194
Urine reference material	Samples diluted 1 + 3 and analyzed by gradient elution AE (ICSep ION120 column, carbonate-based mobile phase at pH 10.3) or cation exchange (ChromPack Ionospher 5C with pyridinium ion-based mobile phase) LC with ICP-MS detection	Arsenite [As(III)], arsenate (As(V)), MMA, DMA, AsB, TMAO, TMAs, TMAP, DMAE	Methodological development focusing the separation/quantification of nine As species in urine matrix (reference urine NIES No. 18 and NIST SRM2670a). AE chromatographic mode gave baseline separation of AsB, DMA, As(III), MMA, As(V) in 15 min. Additionally, using a cation-exchange column AsC, TMAs, TMAO and TMAP were separated	59

Human urine	Separation on RP column (GL Sciences Inertsil ODS) with TEAH, 4.5 mmol L^{-1} malonic acid, 0.1% methanol mobile phase at pH 6.8 (with HNO_3), on-line ICP-MS detection	Arsenite [As(III)], arsenate [As(V)], MMA, DMA, AsB, arsenosugars	Urinary excretion of As species was evaluated during a 3-day period after a meal of blue mussels, *Mytilus edulis*. The effect of cooking on the arsenic speciation was also studied. AsB and DMA were the major arsenic metabolites found in the urine samples. Significant amounts of unknown metabolites were also detected	195
Human urine reference materials (NIST SRM 2670E and 2670N)	AE microbore column (150 × 1 mm, Nucleosil SB 100-5; 5 μm) with phosphate-based mobile phase, pH 5.25 at a flow rate 100 μL min^{-1}. Three nebulizers (DIN, MCN and CFN) were compared for hyphenation of HPLC with ICP-MS detection	Arsenite [As(III)], arsenate [As(V)], MMA, DMA	A miniaturized speciation method was developed, which is suitable for analysis of the main As metabolites in urine. Enhanced analytical performance was obtained with the low-flow nebulizers (DIN and MCN). The values of DL were 0.2–0.6 ng As mL^{-1}	65
Urine from from the population exposed to As	Cation- and anion-exchange columns in series, gradient elution with $(NH_4)_2CO_3$ (between 10 and 50 mmol L^{-1}) and ICP-MS detection (separation time <30 min)	Arsenate [As(V)], arsenite [As(III)], AsC, AsB, MMA, DMA	As species were determined in human urine ($n = 256$) in a population-based exposure assessment survey	60

(*continued*)

Table 12.1 (*Continued*)

Sample	Analytical procedure	Species of interest	Toxicological and/or clinical relevance	Ref.
Urine NIST SRM 2670	AE separation (ION-120 InterAction chromatography column) coupled with ICP-MS through DN or HG. For DN: gradient elution using 40 and 70 mmol L^{-1} $(NH_4)_2CO_3$ (pH 10.5, at 60 °C). For HG: isocratic elution with 40 mmol L^{-1} $(NH_4)_2CO_3$. Photo-reactor interface between the column and the HG facilitated the detection of non-hydride active As species	AsB, arsenite [As(III)], DMA, MMA, arsenate [As(V)]	Two procedures for As speciation were proposed that differ in the dilution factor, sample introduction and separation conditions. For DN, resolution among the early eluting species [AsB, DMA, As(III)] was improved at higher column temperatures and lower eluent molarity, but the separation of Cl^- has to be assured. Using HG mode, chloride is eliminated in gas–liquid separator. The high sensitivity of this mode enables 50-fold dilution of urine matrix prior to analysis. The results obtained in the analysis NIST SRM 2670 revealed statistically significant differences, mainly in DMA concentrations	57
Urine in occupational exposure to As_2O_3	As speciation: a weak AE column with the mobile phase containing $NH_4H_2PO_4$, CH_3COONH_4, CH_3COOH and methanol; on-line ICP-MS	Arsenite [As(III)], arsenate [As(V)], DMA, MMA, AsB	The increased urinary excretion of some porphyrin homologues, correlated with As(III) levels, was observed in exposed workers, which confirms the role of inorganic As in the inhibition of URO-decarboxylase in the heme biosynthesis pathway	196

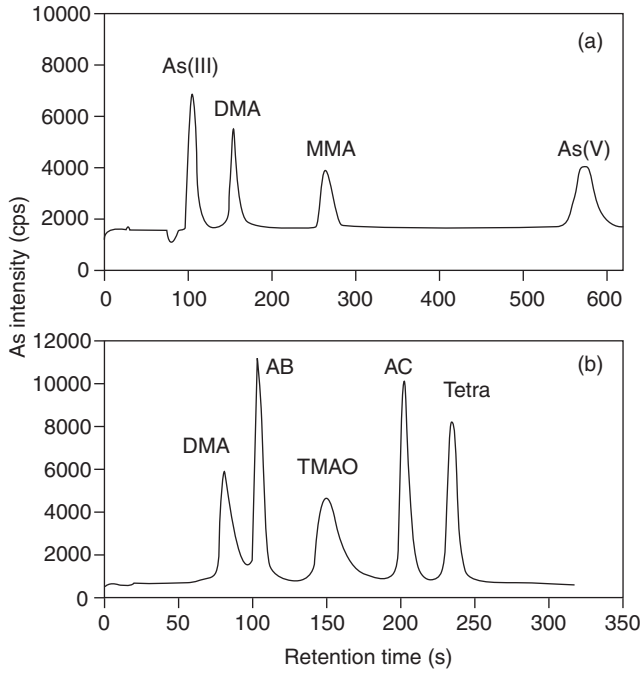

Figure 12.1 HPLC-ICP-SFMS chromatograms of arsenic species (0.5 ng mL^{-1}), obtained using a conventional concentric nebulizer and low-resolution sector field ($m/\Delta m \approx 300$): (a) anion-exchange chromatography (Hamilton PRP-X100, 20 mmol L^{-1} $NH_4H_2PO_4$, pH 5.6); (b) cation-exchange chromatography (Zorbax 300-SCX, 20 mmol L^{-1} pyridine, pH 2.31). From Zheng, B., Hintelmann, H. Hyphenation of high performance liquid chromatography with sector field inductively coupled plasma mass spectrometry for the determination of ultra-trace level anionic and cationic arsenic compounds in freshwater fish. *J. Anal. At. Spectrom.* 2004, **19**, 191–195. Reproduced by permission of The Royal Society of Chemistry

used to separate AsB, AsC, TMAO and TMAs. These separations are achieved at generally lower pH values (<4) with the mobile phases containing formate or pyridine/formate.[45,48,58–60] In Figure 12.1, the anion- and cation-exchange chromatograms of arsenic species are shown.[61] On the other hand, different ion-pairing agents (mainly tetrabutylammonium ion and various alkylsulfonates) have been used for separation of both charged and neutral species in one chromatographic run within a relatively short time (4–20 min).[26,36,62,63] Typically, separations were achieved on reversed phase columns, but ion-exchange columns were also used.[44,64] Both, isocratic[30,36,43,45,46,53–56,62,63,65] and gradient elution[38,39,44,57,59,60,66] modes were reported. Logically, gradient elution is often preferred, since it offers efficient separation in a shorter time. However, the ICP tolerance to high salt content is limited and the sensitivity of ICP-MS detection can be affected by changes in the composition of the solution entering the plasma (mobile phase). Finally, for the characterization of new As species, a multidimensional chromatographic approach was used prior to electrospray ionization mass spectrometry analysis (ESI-MS).[40,41,67]

The most common interference in As determination by ICP-MS is caused by chloride ($^{40}Ar^{35}Cl^{+}$). Since arsenic is monoisotopic, chromatographic separation of chloride ions or

their elimination prior to ICP-MS detection by means of hydride generation is necessary.[36,57,68] On the other hand, high-resolution mass spectrometry and collision cell technology have been shown to be effective in suppressing chloride-based polyatomic interferences.[68–72]

12.3.2 Iodine

Unlike arsenic, iodine is an important human nutrient. It is utilized by the thyroid gland for the biosynthesis of the thyroid hormones thyroxine (T4) and triiodothyronine (T3), necessary for human growth and development.[2] Deficiency of this element leads to iodine deficiency disorders (IDD). Today, about 30% of the world's population is at risk of IDD, 750 million people suffer from goiter, 43 million have IDD-related brain damage and mental retardation and 5.7 million are afflicted by cretinism, the most severe form of IDD.[73] The knowledge about the variety and distribution of different iodine species [mainly monoiodothyronine (T1), diiodothyronine (T2), T3 and T4] in serum or urine can give information about a malfunction of the thyroid gland and may explain other T4/T3-influenced metabolic abnormalities.[74] Addition of iodine to table salt has become a common practice in order to prevent element deficiency. Several investigations have been performed on iodine speciation in human body fluids and in food-related products, predominantly using chromatographic separations.[10,74–78] Such analyses were also performed in pharmaceutical formulations using ICP-MS as the primary detection tool.[79,80] Even though the determination of halogens by ICP-MS has been somewhat difficult, the relatively low first ionization potential of iodine (10.46 eV) makes it suitable for ICP-MS analysis.[19] As demonstrated by Kannamkumarath *et al*., the key advantage of this detection system over spectrophotometric detection (225 nm) is its high sensitivity (detection limits for seven iodine species are about 200 times lower with ICP-MS), which permitted the quantification of the degradation products of T4 at trace levels.[79] The chromatograms of seven iodine compounds obtained in the cited work with the two detection systems are shown in Figure 12.2.[79]

The applications of LC coupled to ICP-MS in iodine speciation have been reviewed elsewhere[2,10,19] and some representative examples are presented in Table 12.2.[74–79,81]

12.3.3 Mercury

Where health hazard is concerned, the main requirement for speciation analysis is to distinguish the inorganic forms of mercury from its short-chain alkyl species (MMM, MEM) in environmental, food and clinical matrices. A critical step in any analytical procedure is the extraction of Hg species from the original sample, aiming for a complete recovery of the element without species interconversion. Relatively aggressive treatments have been used, such as acid extraction (mostly combined with solvent extraction), distillation or alkaline extraction.[82] Even though water vapor distillation seems to be a method of choice for complete recovery, the formation of methylated mercury artifacts has been discussed.[83–85] Separation of the Hg species is usually carried out by gas chromatography (GC), after convenient derivatization (hydride generation or alkylation).[86,87] However, the applications of LC have been increasing, since it simplifies sample preparation (no precolumn derivatization needed).[88] As already mentioned, there is a significant risk of mercury

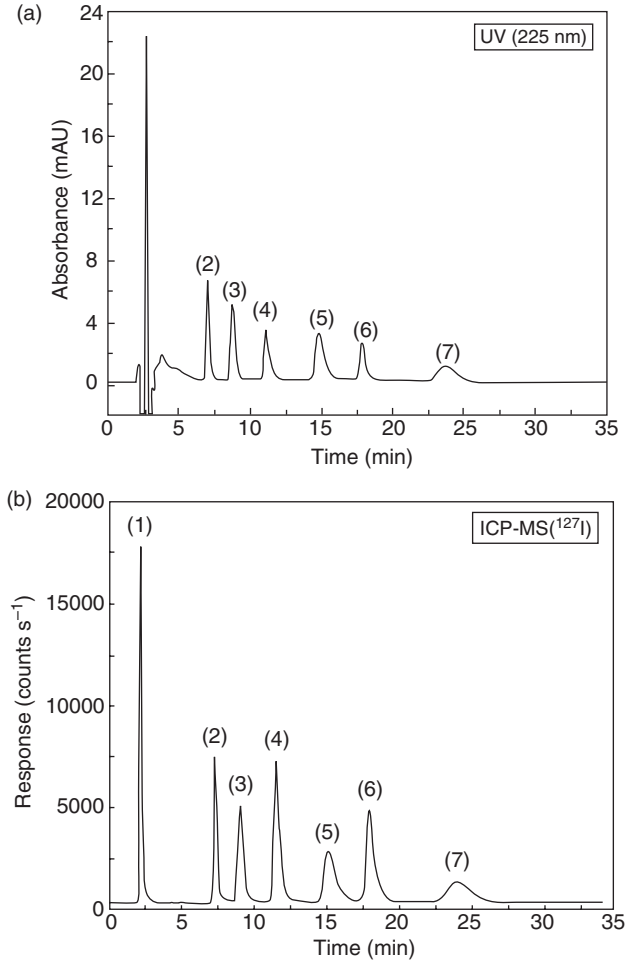

Figure 12.2 Comparison of (a) UV absorbance detection at 225 nm and (b) ICP-MS detection (^{127}I) for the analysis of iodine species in levothyroxine pharmaceutical tablets. Iodine species: (1) inorganic iodine; (2) T2; (3) TTAA2; (4) T3; (5) TTAA3; (6) T4; (7) TTAA4. The concentration of each iodine species was 200 mg L^{-1}. From Kannamkumarath, S. S., Wuilloud, R. G., Stalcup, A., Caruso, J. A., Patel, H., *et al*. Determination of levothyroxine and its degradation products in pharmaceutical tablets by HPLC-UV-ICP-MS. *J. Anal. At. Spectrom.* 2004, **19**, 107–113. Reproduced by permission of The Royal Society of Chemistry

methylation/demethylation during different steps of analytical procedure. Thus, in the particular case of mercury speciation, ICP-MS detection not only provides high selectivity and sensitivity but also offers the opportunity to perform isotope dilution analysis in order to compensate for changes of the original species distribution in the sample. Several representative applications of LC coupled to ICP-MS for mercury speciation are given in Table 12.3.[83–85,89-94] The methodological development has been focused on efficient separation and interference-free, sensitive detection. Typically, reversed-phase columns

Table 12.2 Analytical applications of HPLC-ICP-MS for iodine speciation

Sample	Analytical procedure	Species of interest	Toxicological and/or clinical relevance	Ref
Milk and infant formulas	Milk whey obtained after centrifugation of fresh milk (with SDS) was analyzed by SEC with 30 mmol L^{-1} Tris–HCl buffer as a mobile phase and ICP-MS detection (40 min)	HMW versus LMW iodine species	Iodide was found as the primary iodine species in fresh milk, whereas in the infant formulas more than 50% of element was bound to compounds presenting MW >1000 kDa	77
Milk	Different pretreatment procedures tested, centrifugation finally adopted, separation carried out ion-exchange column (Dionex AS14) with carbonate buffer as the mobile phase	Iodine, iodate	The values of DL were <2 μg I L^{-1}. Iodide was identified as the main iodine species in milk, but in a few samples also traces of iodate and several unidentified, presumably organoiodine, compounds were observed.	81
Commercial seaweeds	Multidimensional chromatographic approach: alkaline extracts analyzed by SEC (Superdex 75HR, 0.03 mol L^{-1} Tris–HCl, pH 8.0) and by AE chromatography (Ion Pac AS-11, Dionex, 5 mmol L^{-1} NaOH) coupled to ICP-MS; Enzymatic digests (Proteinase K) were analyzed by RP-HPLC (Altima C_{18}, elution with 0.01 mol L^{-1} Tris–HCl, pH 7.3 in a gradient of methanol)	Association of iodine with HMW compounds, iodide, T1, T2,	The association of iodine with both LMW and HMW fractions was observed, which was dependent on the biological species analyzed. Iodine was a primary species found in Kombu, whereas T1 and T2 (bound to proteins) were detected in Wakame. The results obtained suggest that, in different seaweeds, iodine follows different metabolic pathways. Since the bioavailability of iodide is better than any other form of iodine, Kombu seaweed would be preferred as a natural dietary supplement	76

Levothyroxine tablets	RP-HPLC [Cyano-Spherisorb narrow bore column, 22% (v/v) acetonitrile; 0.08% (v/v)trifluoroacetic acid, pH 2.3] with UV or ICP-MS detection. For ICP-MS, post-column, on-line dilution (1:3.3) of the chromatographic eluent with a 2% (v/v) nitric acid (separation time 26 min).	T4, T3, T2, T1, TTAA4, TTAA3, TTAA2	The detection limits obtained with UV detection ranged from 28.9 to 34.5 $\mu g\,I\,L^{-1}$, whereas those obtained with ICP-MS were about 175–375 times lower. Effect of different common diluents used in the formulation of levothyroxine tablets was studied and iodine species were determined in commercial levothyroxine sodium tablets	79
Human serum and urine	Enzymatic digests (protease) analyzed by RP-HPLC (Phenomenex CAPCELL-C_{18} column, elution with 10 $mmol\,L^{-1}$ Tris–HCl, pH 7.3, in methanol gradient 1–50% (v/v), at 15 °C)], post-column dilution with dilute HNO_3 and ICP-MS detection (separation time 26 min)	T4-TBG, T1, T2, r-T3, T3, T4. iodide	The values of DL were in the range 0.08–1.5 $\mu g\,I\,L^{-1}$. The concentrations of iodide, T1, T2, r-T3, T3 and T4 found in 'normal' sera were 11, 1.6, 2.1, 3.9, 5.9 and 60 $\mu g\,L^{-1}$, respectively. The method proved to recognize abnormalities in a pathological serum, having reversed -T3 (r-T3) as the predominant species. Iodine was a primary species found in urine	74,75
Homogenates of adult male and female zebrafish	Enzymatic digests (pronase E) were analyzed by RP-HPLC-ICP-MS using the method developed by Michalke *et al.*[74,75]	T1, T2, r-T3, T3, T4	Male and female zebrafish (*D. rerio*) and tadpoles of the African clawed frog (*X. laevis*), at two different development stages, were taken for analysis. The speciation results were different between two biological species and also for different stages of development. Five unknown iodine species were detected	78

Table 12.3 Applications of LC with ICP-MS detection for speciation analysis of mercury

Sample	Analytical procedure	Species of interest	Toxicological and/or clinical relevance	Ref
Seawater, tap water	RP separation (Spherisorb ODS column) with 0.5% cysteine (pH 5) as mobile phase (separation time <6 min), post-column CV ($NaBH_4$) and ICP-MS	Hg(II), MMM, MEM	Methodological approach: the feasibility of post-column CV generation prior to ICP-MS detection was demonstrated. The values of DL were in the range 0.03–0.11 ng Hg mL^{-1}	89
Spiked urine	Separation on C_{18} column with mobile phase acetonitrile–water (20:80) with addition of 5 mmol L^{-1} ammonium pentasulfonate, pH 3.4, flow rate 0.1 mL min^{-1}. Column effluent introduced to ICP-MS through DIN	Hg(II), MMM, MEM, MPhM	Methodological approach: the DLs in urine were 0.018 and 0.016 ng Hg mL^{-1} of MEM and MPhM, respectively	90
Biological tissues	Separation on C_{18} column with an aqueous mobile phase containing 0.08% ammonium acetate and 0.02% L-cysteine, coupled on-line to ICP-MS detection. SSID calibration using enriched isotope standards: ^{198}Hg in MMM and ^{201}Hg in inorganic Hg(II)	MMM, Hg(II)	Methodological approach: abiotic methylation/demethylation process during treatment with TMAH at controlled pH (citric acid) was studied. Depending on the type of sample matrix, up to 11.5% of added Hg(II) was methylated and up to 6.26% MMM was demethylated to Hg(II). The adjustment of pH after samples treatment with TMAH was recommended in order to minimize changes in mercury speciation during analytical procedure	83
Rat tissues after oral intake of $HgCl_2$	A home-made preparative SEC Sephadex G-75 column (30 × 1.9 cm) used for the purification of MT fractions and, after desalting, further separation accomplished by RP-HPLC-UV, ICP-MS and ES-MS detection	Hg binding to MTs	The role of MTs in the detoxification of mercury after its oral intake in mammals was studied. One major and several minor peaks were observed in the HPLC traces of the MT fraction for the kidney sample. Hg binding to MTs observed in kidney but not in liver extracts. Further characterization with ESI-MS allowed the identification of several complexes, containing only Hg and/or Hg and Cu	91

Fish and sediment CRM	RP separation on Hypersil ODS column with acetonitrile–water (65:35) mobile phase adjusted to pH 5.5 with CH_3COONH_4. Column effluent introduced to ICP-MS through HHPN	MA, MMM, MEM, MPhM, Hg(II)	Methodological approach: the DLs were 0.02, 0.015, 0.02, 0.02 and 0.01 ng of Hg as MA, MMM. MEM, MPhM and Hg(II), respectively	92
Fish tissues, DORM-2	An open-vessel MW extraction, RP separation with a mobile phase containing L-cysteine and 2-mercaptoethanol, CV generation and ICP-MS detection	Hg(II), MMM, MEM	Methodological approach: post-column CV generation was used to enhance the detection power (DL in the range 0.05–0.09 ng Hg mL^{-1}).	93
Operationally defined soil extracts	RP-HPLC on Supelcosil LC-18 column with water–methanol (70:30, v/v) mobile phase containing 0.001% (v/v) 2-mercaptoethanol and 0.2 mol L^{-1} CH_3COONH_4; on-line ICP-MS (separation time 11 min)	Hg(II), MMM, MEM	Methodological approach focusing selective determination of toxic mercury species: 'mobile and toxic' fraction was extracted by with acidic ethanol solution (2% HCl + 10% ethanol solution); further SPE fractionation yielded 'soluble inorganic Hg' and 'alkyl-Hg species'	94
Different food products, plants and sediments	SSID (^{200}Hg(II)), steam distillation ($NaCl–H_2SO_4$), preconcentration on Hypersil in form of SPDC complexes, RP separation (Hypersil ODS) with acetonitrile–water (65:35, v/v), UV irradiation and on-line introduction to ICP-MS through ultrasonic nebulizer	Hg(II), MMM	Methodological approach: application of SSID for the elimination of analytical errors caused by possible interconversion of species. Absolute DL 12 pg of Hg (*m/z* 202). Methylation of inorganic mercury contained in the sample caused positive errors in the determination of MMM in sediment (54%) and plant materials (0.3–18.7%). No error was detected in the analyses of fish tissues	84,85

have been used with water–acetonitrile or methanol, L-cysteine or 2-mercaptoethanol mobile phases with separation times below 15 min. Size-exclusion chromatography (SEC) was applied for studying mercury–metallothionein complexes.[91] Since the quantification of methylmercury (MMM) in biological samples has become necessary, quality assurance strategies are emerging. There are few CRM materials available for MMM in marine matrices and sediments (fish tissue, CRMs 473 and 464; sediment, CRM 580).[3,4,95] It is worth mentioning that, among other methods, HPLC-ICP-MS was used in interlaboratory studies and certifications.[4]

12.3.4 Platinum

The capability of ICP-MS for the sensitive and specific detection of elements makes this technique suitable for studies on the uptake and metabolism of metal-based drugs.[19] In particular, cisplatin has become one of the most widely used chemotherapy drugs. Its mechanism of action relies on the ability of drug to modify the DNA structure in cancer cells, hence causing their apoptosis. However, it presents severe toxicity and its anticancer activity is limited to a small group of tumor cells. In further development, several other platinum complexes (mainly oxaliplatin, carboplatin, satraplatin, JM-216 and ZD0473) have been synthesized and tested for their possible anticancer activity.[96–100] The challenge is to obtain drugs active against a broad spectrum of tumor cells, to minimize their toxicity and to avoid the development of resistance mechanisms in target cells.[101]

After intravenous or oral administration of drug, it undergoes several biotransformations that activate its toxicity (tumor cells in addition to nephro- and neurotoxicity). A number of speciation studies have been reported that were focused on the characterization and quantification of the parent drug and its possible metabolites *in vitro* and *in vivo*. Typically, LC separation was carried out and platinum was determined in column effluents by atomic absorption sperctrometry[102,103] or by ICP-MS.[96,98,99,104–110] Several applications of mass spectrometry for the structural characterization of platinum compounds have also been reported.[96,101,103,111] The analytical figures of merit for platinum aqua species in plasma were compared for HPLC-ICP-MS system and HPLC with triple-quadrupole MS.[111] Obviously, MS-MS and MS-MS-MS measurements permitted species characterization, but the advantage of HPLC-ICP-MS was its better sensitivity (detection limit lower by an order of magnitude with respect to that achieved by triple-quadrupole instrumentation). The details of analytical procedures, together with toxicological and/or clinical importance of the results obtained, are summarized in Table 12.4.

Since clinical treatment is always based on the administration of a single platinum compound, simultaneous analysis of different drugs has rarely been undertaken. On the other hand, the metal drugs and their metabolites are released into communal waste water. The environmental impact of this discharge becomes an issue and also a challenge for speciation analysis. Recently, the separation of cisplatin, monoaquacisplatin, diaquacisplatin, carboplatin, and oxaliplatin in environmental and biological samples was studied by Hann *et al*.[98] Owing to different properties of target compounds (neutral and positively charged species), pentafluorophenylpropyl-bonded silica stationary phase was used, providing both reversed-phase and ion-exchange-based retention. The elution was carried out with a mobile phase containing 10 mmol L^{-1} ammonium formate (pH 3.75) and methanol. With increasing concentration of organic modifier, the retention of cisplatin, carboplatin and oxaliplatin

Table 12.4 Summary of HPLC-ICP-MS procedures for platinum speciation in analyses of clinical and/or toxicological relevance

Sample	Analytical procedure	Species of interest	Toxicological and/or clinical relevance	Ref.
Model solutions	IP-HPLC separation on C_{18} column using SDS or sodium heptanesulfonate as ion-pairing agents, on-line ICP-MS detection	Cisplatin, the products of its hydrolysis and reactions with SMet, SCys and GSH	Methodological development: the value of DL evaluated for cisplatin was 1 ng Pt mL^{-1}	104
Dog plasma after ZD0473 administration	HPLC-ICP-MS, HPLC–MS-MS and HPLC–MS-MS-MS	ZD0473 and its 'aqua' compounds	Methodological development: HPLC-ICP-MS procedure was compared with ICP-MS-MS showing extended linear range and superior sensitivity (limit of quantification 0.1 ng Pt mL^{-1} by ICP-MS versus 5 ng Pt mL^{-1} using MS-MS)	111
Platinum drug (JM-216) and blood plasma samples from clinical trial on a new drug	Plasma ultrafiltrates were diltued 1:50 and analyzed by RP-HPLC (column packed with PLRP-S, Polymer Laboratories) with solvent gradient elution (acetonitrile–water from 15:85 to 90:10, 30 min), coupled to ICP-MS through a novel interface, allowing efficient elimination of acetonitrile	JM-216 and its possible metabolites	Methodological development: thanks to the application of the new LC–ICP-MS interface, a gradient of organic solvent could be used and the separation of five metabolites was achieved. The DL was 0.60 ng Pt mL^{-1}. The results obtained in the analysis of clinical samples indicated the complete decomposition of JM-216 in the human body with formation of at least five metabolites	106
Blood plasma after treatment with JM-216	Methanol extracts of clinical samples were analyzed by RP-HPLC (Phenomenex Prodigy C_8 column); elution gradient of methanol (mobile phase A, 25% methanol–0.01% H_3PO_4 at pH 2.5; mobile phase B, 100% methanol, 20 min); ICP-MS detection	JM-216 and its–biotransformation products: JM-118 (reduction product), JM-518 (hydrolysis product), JM-383	Methodological development: an increasing concentration of methanol in the mobile phase caused the suppression of platinum counts for later eluted species by ~70%. However, the quantification of platinum compounds was achieved with good intra- and inter-assay precision (range 1–13% RSD) and the DLs evaluated were in the range 1–2 ng Pt mL^{-1}. The analysis of clinical samples revealed relatively fast degradation of JM-216 with formation of JM-118 and an unidentified Pt species	107

(*continued*)

Table 12.4 (*Continued*)

Sample	Analytical procedure	Species of interest	Toxicological and/or clinical relevance	Ref.
Red blood cells incubated *in vitro* with satraplatin	Plasma ultrafiltrates and methanol extracts analyzed by RP-HPLC (Phenomenex Prodigy C_8 column) with 0.85% H_3PO_4 mobile phase (pH 2.5), elution in methanol gradient from 20 to 40% (v/v); off-line ICP-MS detection. The fractions were also analyzed by SDS-PAGE ICP-MS	Satraplatin and its possible metabolites	Studies on the biotransformation of satraplatin: the rapid disappearance of drug from human blood *in vitro* was observed (half-life 6.3 min), which was dependent on the presence of RBCs. Two new platinum-containing species observed on HPLC-ICP-MS traces confirmed that satraplatin undergoes rapid biotransformation in whole blood. The unknown metabolite was analyzed by SDS-PAGE and ICPMS and identified as a platinated protein with a similar electrophoretic mobility as serum albumin	99
Pooled serum incubated with Pt or Ru drug	SEC separation (Pharmacia Superdex 75 HR 10/30 or Toso Haas Progel TSK-GEL PWXL column) with 30 m mol L^{-1} Tris–HCl buffer mobile phase (pH 7.0); ICP-MS detection (^{194}Pt,^{195}Pt,^{196}Pt,^{100}Ru, ^{101}Ru,^{102}Ru,^{63}Cu,^{114}Cd and ^{31}P)	2 platinum (incuding cisplatin) and 3 ruthenium drugs	A rapid and simple method was developed, suitable for estimation of (1) the speed of the drug binding to proteins, (2) the efficiency of its transport in the blood stream and (3) possible efficiency of 'releasing agents' (e.g. citrate, EDTA, tartrate)	108
Incubates of cisplatin with guanosine 5-monophosphate	Ion-exchange HPLC (Dionex AS14 column) with mobile phase containing 17.5 mmol L^{-1} Na_2CO_3, 5 mmol L^{-1} $NaHCO_3$ and 5% acetonitrile at pH 10.7 (45 °C), on-line ICP-sector field MS (^{31}P,^{115}In,^{195}Pt)	Cisplatin and its GMP adducts	The time-dependent reaction course of the cisplatin–GMP system was followed by monitoring the decrease in the concentration of GMP and the increase in the concentration of adducts formed. The use of high-resolution MS for simultaneous quantification of P and Pt in chromatographic effluent provided unambiguous stoichiometric information about the major GMP adduct.	109

Incubates of platinum complexes with guanosine 5-monophosphate (GMP)	Anion-exchange HPLC (Nucleosil 5SB column) with 50 mmol L^{-1} formic acid–triethylamine in methanol–water (5:95) mobile phase at pH 2.6 and 30 °C; cation-exchange separation (Nucleosil 5SA column) with triethylamine in acetonitrile–water–acetic acid (12.5:82.5:5) mobile phase at pH 3.9 and 6 °C; on-line ESI-MS analysis of the column effluents	Cisplatin and four structurally related platinum(II) complexes	The formation of conjugates between Pt complexes and GMP was studied in order to gain a better insight into possible binding of platinum chemotherapy agents with DNA. Three different types of platinum(II)–mono-GMP adducts could be detected via cation-exchange chromatography. Using anion-exchange chromatography, isomers of bis-GMP adducts could be identified	101
Cisplatin incubated with GSH, cysteinylglycine, or *N*-acetylcysteine	HPLC with off-line atomic absorption spectrometric detection of Pt or MS analysis	Cisplatin and its conjugates	Studies on the nephrotoxicity of cisplatin: the diplatinum and monoplatinum conjugates of the drug were structurally characterized. The toxicity of the preincubated solutions was tested with time and the results obtained showed an increase in toxicity, which correlated with the formation of the monoplatinum conjugate, whereas prolonged preincubation decreased toxicity and correlated with the formation of the diplatinum conjugate	103
Urine from cancer patients	Diluted urine (1:20) was analyzed by adsorption chromatography on a Thermo Hypersil–Keystone Hypercarb column (2.1 mm i.d.) with elution in gradient of NaOH; ICP-sector field MS; IDA analysis (^{196}Pt)	Cisplatin, monoaquacisplatin, diaquacisplatin	The DLs for cisplatin and its metabolites were in the range 0.65–0.74 μg Pt L^{-1}. Diluted urine from a cancer patient contained the parent drug cisplatin and a considerable fraction of highly active monoaquacisplatin, and also several unknown platinum species	110

(*continued*)

Table 12.4 (*Continued*)

Sample	Analytical procedure	Species of interest	Toxicological and/or clinical relevance	Ref.
Human urine	RP-HPLC separation (Supelco Discovery HS F5 microbore column), step-gradient elution with three mobile phases: (A) 20 mmol L^{-1} ammonium formate–methanol (4%, v/v), (B) water and (C) methanol at 45 °C (separation time 23 min); on-line ICP-MS detection	Cisplatin, mono-aquacisplatin, diaqua-cisplatin, carboplatin, oxaliplatin	For cisplatin, carboplatin and oxaliplatin the DLs were 90.0, 100 and 150 ng Pt L^{-1}, respectively. The urine sample contained more than 17 different reaction products, which demonstrates the extensive biotransformation of the compound	98
Human urine	RP-HPLC separation (YMC ODS-AQ microbore column); gradient elution with two mobile phases: (A) 5 mmol L^{-1} aqueous ammonium acetate with 0.01% (v/v) acetic acid and (B) methanolic 5 mmol L^{-1} ammonium acetate with 0.01% (v/v) acetic acid (separation time 16 min); on-line ESI-MS-MS	ZD047	The assay was developed for IDA–validated RP HPLC-MS quantification of platinum drug in urine. The limit of quantification in urine (100 μL) was 200 ng Pt mL^{-1}. A novel platinum adduct was formed during the storage of ZD0473 in human urine. The addition of 50% (w/v) sodium chloride to the urine prevented the formation of this adduct during storage.	96

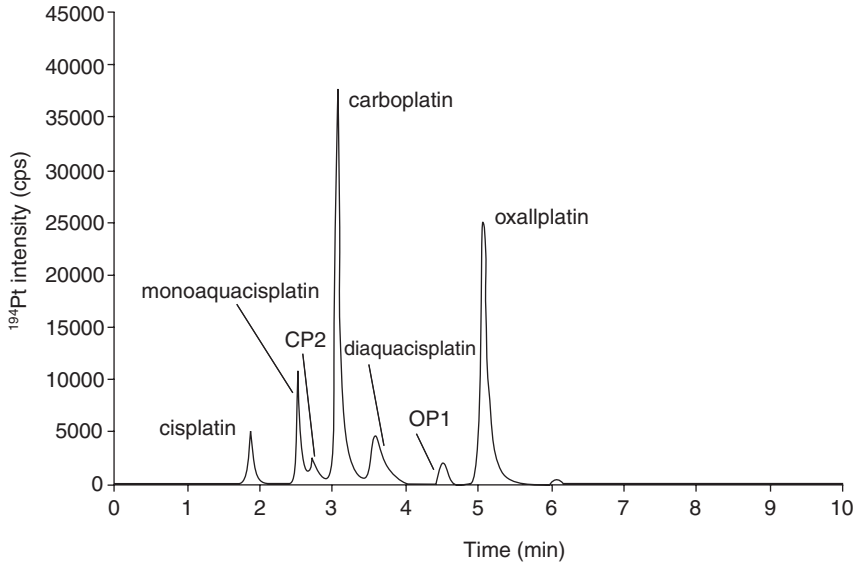

Figure 12.3 Chromatogram obtained from a 24-h mixture of cisplatin ($t_R = 1.9$ min), carboplatin ($t_R = 3.1$ min) and oxaliplatin ($t_R = 5.1$ min) in sub-boiled water. In addition to the parent drugs, the chromatogram shows the major degradation products of cisplatin (monoaquacisplatin, $t_R = 2.5$ min; diaquacisplatin ($t_R = 3.6$ min)), the product of carboplatin (CP2) ($t_R = 2.7$ min) and the product of oxaliplatin (OP1) ($t_R = 4.5$ min). The void volume of the separation system was equivalent to a retention time of 1.5 min. From Hann, S., Stefanka, Z., Lenz, K., Stingeder, G. Novel separation method for highly sensitive speciation of cancerostatic platinum compounds by HPLC-ICP-MS. *Anal. Bioanal. Chem.* 2005, **381**, 405–412, with kind permission of Springer Science and Business Media

decreased (non-polar compounds), whereas the retention of ionic species (monoaquacisplatin, diaquacisplatin) was controlled by the ionic strength. In Figure 12.3, the chromatogram of standards, obtained under optimized conditions (Table 12.4), is presented.[98]

12.3.5 Selenium

Selenium is an element of fundamental importance to human health. Depending on its chemical form and total concentration, it may exhibit essential or toxic effects with a very narrow tolerance band.[112] Thus, the recommended selenium reference nutrient intake (RNI) is 75 and 60 μg day^{-1} for adult males and females, respectively.[113] According to the UK Department of Health, an intake of 750–900 μg is toxic and the maximum safe intake is 450 μg day^{-1} for adult males.[114] The specific biological role of selenium is not fully understood. However, selenocysteine has been characterized as the 21st proteinogenic amino acid, which forms an active center of a number of proteins (selenoenzymes include glutathione peroxidase, iodothyronine deiodinase and thioredoxine reductase and selenoprotein P, among others).[112,115,116] Experimental evidence exists on selenium bioactivity in cancer prevention.[117,118] The ability of the element to reduce toxic effects of heavy metals

was also observed.[119] On the other hand, the toxicity of selenium originates in its high affinity to sulfur, which results in non-specific competition of the two elements for sulfur-binding sites in different biomolecules.[120] Consequently, the characterization and selective determination of the particular selenium species in biological and/or clinical materials are necessary in order to understand its metabolism and biological significance in clinical chemistry, biology, toxicology and nutrition.

Several health conditions require supplementation with selenium (element deficiency in many geographical regions, cancer chemoprevention, geriatric treatment). Full characterization of selenium forms in the supplement to be used, their stability and species-dependent bioavailability and pharmacokinetics in the human body are of primary importance. Food products naturally rich in selenium should also be considered. On the other hand, the risk of intoxication due to excessive selenium intake needs to be minimized.[121] Selenium-rich dietary products containing relatively non-reactive, organic element forms are generally preferred, because of their better bioavailability and favorable pharmacokinetics with respect to the inorganic species.[122–124] The biological activity of different selenium species for tumor prevention has been studied in several experimental models.[125–127] The results obtained showed the highest activity for methylselenocysteine followed by selenite, selenocystine and dimethyl selenoxide.[125,128] In addition to inorganic forms and possible selenium metabolites, the activity of synthetic compound [1,4-phenylenebis(methylene)selenocyanate] has been tested.[129,130] A survey of analytical methodology based on HPLC coupled to ICP-MS for selenium speciation in food-related products and in different supplements is presented in Table 12.5. The full list of selenium species is composed of over 30 compounds that comprise inorganic forms, low molecular weight methylated species, Se-amino acids, Se-sugars and Se-containing proteins. Since pure standards of the compounds are not always available, mass spectrometry with soft ionization sources has been used to obtain structural information.[131–134] The feasibility of ion-pairing HPLC coupled to ICP-MS detection for the separation of up to 30 Se compounds was demonstrated.[135,136] However, in applications to real-world samples, a smaller number of species has been found, especially when using one pretreatment and a single chromatographic condition. Logically, the analytical results are determined by the variety of naturally occurring selenium compounds. On the other hand, the design of the pretreatment procedure may enhance the biologically relevant information obtained in speciation analysis.[28] Furthermore, better characterization of species can be achieved by combining different chromatographic approaches.[134,137–142] SEC has typically been used for discrimination between LMW and HMW selenium compounds.[134,137–139,142–145] For LMW inorganic and organic species, ion-exchange[134,139,140,146–149] and ion-pair chromatographic[127,134–136,141–144,150–152] modes have been reported. Anion-exchange columns have been applied for the separation of two inorganic forms [Se(IV) and Se(VI)] and also SeMet, SeCys, SeMC and TMSe in plant and yeast extracts.[2,147,153] A number of organic selenium compounds (selenoamino acids, peptides, TMSe, etc.) are cations, or can be easily converted to cationic species by lowering pH of the solution, thus facilitating separation by a cation-exchange mechanism. Up to 10 selenium compounds (SeMC, allylselenocysteine, propylselenocysteine, SeMet, SeEt, SeCys, dimethylseloniumpropionic acid, SeHcy, TMSe, SeMM) were resolved with different mobile phases at pH 2–5.7 (pyridine, ammonium formate, etc.).[11,35,154] The conjunctive use of anion- and cation-exchange HPLC was shown to provide information on the ionic nature of the retained and separated selenium species with a high degree of selectivity.[140] The majority of selenium speciation analyses in food-related products have been done with ion-pair

Table 12.5 Analytical applications of HPLC-ICP-MS for selenium speciation

Sample	Analytical procedure	Species of interest	Toxicological and/or clinical relevance	Ref
Se-enriched plants and yeast	Hot water and enzymatic extracts were analyzed by IP chromatography on a Waters Symmetry Shield RP8 column using water–methanol (99:1) mobile phases that contained different perfluorinated carboxylic acids as ion-pairing agents. 0.1% HFBA in the mobile phase allowed the separation and ICP-MS detection of >20 Se compounds in 70 min. For ESI-MS analyses, 0.1% TFA was used	23 Se species, among them selenate [Se(VI)], selenite(IV), TMSe, SeMet, SeCys, SeEt, SeHcy, AdoSeHcy, SeMC, γ-glutamyl-SeMC	Methodological development focusing Se speciation in biological materials. Limits of quantification for HPLC-ICP-MS were in the range 2–50 ng Se mL^{-1} and for HPLC–ESI-MS these values were about 100 times higher. Between 60 and 85% of compounds observed in real-world samples were identified by ESI-MS. Primary species found were SeMet, SeMC, γ-glutamyl-SeMC and selenocystathionine, depending on the biological sample and extraction procedure applied	135,150
Se-enriched ramps (leeks)	Hot water and enzymatic (protease XIV) extractions, followed by ion-pairing IP-HPLC-ICP-MS (^{82}Se)	Inorganic Se, organic species with anti-cancer activity	Primary species found in extracts: SeMC (35–50%) and Se(VI) (15–42%), lower levels of Se-cystathionine, γ-glutamyl-SeMC. In rats fed with the selenized ramps, the reduction in chemically induced tumors was observed	127
Se-enriched onion leaves	Aqueous extracts (0.1 mol L^{-1} NaOH) were analyzed by SEC-UV–ICP-MS (Superdex peptide HR 10/30 column, CAPS 10 mmol L^{-1}, pH 10.0); methanol–chloroform–water (12:5:3) extracts or enzymatic digests (proteinase K, protease XIV) analyzed by IP-HPLC-ICP-MS [Altima C_8, 5 mmol L^{-1} citric acid, 5 mmol L^{-1} hexanesulfonic acid, pH 4.5)–methanol (95:5)]; ESI-MS and ESI-MS-MS used for species identification and/or confirmation	Selenite [Se(IV)], SeMet, SeCys, SeMC	Uptake, distribution and speciation of selenium in green parts of onion were evaluated. The results obtained indicated that onion leaves could be an alternative dietary source of SeMC	143,144

(*continued*)

Table 12.5 (*Continued*)

Sample	Analytical procedure	Species of interest	Toxicological and/or clinical relevance	Ref.
Se-yeast and SeMC-based supplements	ASE (water) and enzymatic digestion; SEC performed on a Supelco TSK gel G 3000 PWxL column with 10 mmol L^{-1} CH_3COONH_4 (pH 8.5) mobile phase; AE separation on a Hamilton PRP-X 100 column with 5 mmol L^{-1} ammonium citrate (pH 5.9) mobile phase containing 2% (v/v) methanol; IP-HPLC on an Agilent Zorbax Rx-C8 column with 0.1% TFA, 2% (v/v) methanol. On-line ICP-MS detection, sample introduction via USN; IP-HPLC coupled on-line to ESI-MS-MS	SeMet, SeMC	The proposed procedure permitted enhanced separation selectivity and very low DL (ng Se L^{-1} levels). The identification and quantification of SeMC (minor yeast species) were reported	134
Se-yeast	Homogenization of fresh cells and precipitation of proteins (0. 4 mol L^{-1}, $HClO_4$, 0 °C); IP-HPLC (hexanesulfonic acid) with UV (254 nm) and ICP-MS detection; ESI-MS and MS-MS used for identification/ confirmation of Se species	AdoSeMet, AdoSeHcy, SeCis	AdoSeMet and AdoSeHcy were identified in yeasts extract and the presence of SeCis was also observed. A significantly lower ratio between AdoSeMet and AdoSeHcy was observed in yeast extracts compared with the ratio between sulfur analogs. This observation could contribute to further understanding of the selenium role in biomethylation processes	151
Se-yeast	Water extract analyzed by two-dimensional LC (SEC and RP on porous graphitic carbon stationary phase) with ICP-MS detection. Two Se-containing fractions were isolated and analyzed by nanoESI-MS and MS-MS	Se-containing glutathione *S*-conjugates	Two selenium species identified: selenodiglutathione (GS-Se-SG) and the mixed selenotrisulfide of glutathione and cysteinylglycine (GS-Se-SCG)	137,138

Se-yeast	Different chiral separation techniques investigated: HPLC on a β-cyclodextrin (β-CD) column, CD modified micellar electrokinetic chromatography (MEKC), GC on a Chirasil-L-Val column and HPLC on a Chirobiotic T column	D- and L-enantiomers of SeMet and SeEt	The analytical results for D- and L-SeMet obtained in selenized yeast were presented [HPLC on a Chirobiotic T column, water–methanol (98:2, v/v), separation time 8 min]	156
Se-enriched onion, garlic and yeast	Water and a pepsin enzymatic extract analyzed on chiral crown ether column [Daicel Crownpak CR(+)] with 0.1 mol L^{-1} $HClO_4$ as mobile phase; on-line ICP-MS detection	Enantiomers of SeMet, SeMC, SeCys, γ-glutamyl-SeMC, SeEt, SeHcy; Se-lanthionine, Se-cystathionine	Separation/determination of optical isomers of SeMet and SeCys was studied. Baseline separation of all enantiomers in one chromatographic run was not achieved; however, enhanced resolution was observed at higher temperatures	158
Se-nutritional supplements	HPLC separation on a chiral crown ether stationary phase with 0.10 mol L^{-1} $HClO_4$ as mobile phase and UV or ICP-MS detection (separation time 60 min, 22 °C)	D,L- SeCys, SeMet, SeEt	Absolute DL obtained with UV detection ranged from 34.5 to 47.1 ng of Se, whereas those obtained with the plasma detector were ca 40–400 times better	159
Se-yeast candidate reference material	Sequential extraction yielding watersoluble and non-soluble fractions; 2-dimensional chromatography (SEC on Superdex 75 HiLoad 16/60 column and AE on Dionex AS10 column with gradient elution, pH 8) and on-line UV-ICP-MS (280 nm, ^{78}Se, ^{82}Se); SDS-PAGE-ETV-ICP-MS	Inorganic Se, SeMet, SeCys	The analytical approach focusing characterization of all Se forms/species in Se-yeast	139
^{77}Se-labeled and enriched yeast	Two enzymatic digestions examined, separation by cation-exchange HPLC (Ionosphere-5C, Chrompack International) with gradient elution using pyridinium formate mobile phase (separation of 30 Se compounds within 60 min); ICP-DRC-MS detection (^{77}Se, ^{80}Se).	LMW Se species biosynthesized by the yeast during fermentation	A batch of ^{77}Se-labeled and enriched yeast was characterized with regard to isotopic composition and content of selenium species for later use in a human absorption study based on the method of enriched stable isotopes. SeMet constituted 53% of the total Se content in the yeast. Oxidation of SeMet to selenomethionine-Se-oxide (SeOMet) occurred during sample preparation	146

(*continued*)

Table 12.5 (*Continued*)

Sample	Analytical procedure	Species of interest	Toxicological and/or clinical relevance	Ref.
Yeast and wheat flour	Enzymatic hydrolysis (protease, lipase) followed by AE separation (Hamilton PRP-X100 column) with 5 mol L^{-1} ammonium citrate in 2% (v/v) methanol and post-column IDA-ICP-MS with octapole reaction system	Selenite [Se(IV)], selenate [Se(VI)], SeCys, SeMet, SeCM	Analytical approach to more reliable quantification of Se in biological materials. Results showed that SeMet was the main Se species in both matrices (59% of total Se for wheat flour and of 68% for yeast). Inorganic Se was detected in yeast samples, but not in wheat flour	147
Se-yeast	Enzymatic hydrolysis (protease XIV) followed by anion-exchange separation (Hamilton PRP-X100 column) in phosphate gradient (from 10 to 100 mmol L^{-1}, pH 7) in the presence of 2% (v/v) of methanol and SSID-ICP-MS with octapole reaction system (separation time 15 min)	SeMet	Biosynthesis of ^{77}Se-enriched SeMet by *Sacchaoromyces cerevisiae* investigated, SSID method applied for the determination of SeMet in a candidate reference yeast material	148
Selenite-, SeMet- and Se-yeast nutritional supplements	Hot water extracts analyzed by RP-HPLC [Nucleosil 120 A C_{18} column with 30 mmol L^{-1} ammonium formate, pH 3.0, 5% (v/v) methanol] and IP HPLC (10 mM TBAA in the RP mobile phase) with on-line ICP-MS detection. Three nebulizers were tested (Meinhard, HHPN, MCN) and a hexapole collision reaction cell was used	Selenate [Se(VI)], selenite [Se(IV)], SeMet, SeCys, SeEt, TMSe	Performance of different nebulization and chromatographic systems for the speciation of Se investigated. For selenite- and SeMet-based supplements, the analytical results confirmed the information given by the manufacturer (extraction efficiency 80–95%). For the Se-yeast supplements (extraction efficiency 10–25%), more than 20 selenium compounds were detected, among them SeMet, TMSe and SeEt	152

Se-nutritional supplement	IP-HPLC or LiChrosorb RP18 column using a mixed ion-pair reagent (2.5 mmol L^{-1} sodium 1-butanesulfonate and 8 mmol L^{-1} TMAH); on-line introduction of effluent to ICP-MS through USN and pneumatic nebulizer (separation time 18 min)	Selenite [Se(IV)], selenate [Se(VI)], SeCys, selenourea, SeMet, SeEt, SeCM, TMSe	Methodological development focusing fast separation of 30 Se-species in biological samples. SeMet confirmed as the primary Se compound in supplement analyzed	136
Se-yeast, Se-garlic	HPLC-ICP-MS and ESI-MS for the identification/confirmation of species	SeMet, γ-glutamyl-SeMC	The primary Se species in enriched yeast and garlic were characterized (SeMet and γ-glutamyl-SeMC, respectively) and quantified (73 and 85% of total Se) and certain biological activities of these two materials compared. In the rat feeding studies, supplementation with Se-garlic caused a lower total tissue selenium accumulation compared with Se-yeast. On the other hand, Se-garlic was significantly more effective in suppressing the development of premalignant lesions and the formation of adenocarcinomas in the mammary gland of carcinogen-treated rats. The metabolism of SeMet and γ-glutamyl-SeMC was discussed in an attempt to elucidate how their disposition in tissues might account for the differences in cancer chemopreventive activity	126

(*continued*)

Table 12.5 (*Continued*)

Sample	Analytical procedure	Species of interest	Toxicological and/or clinical relevance	Ref.
Se-yeast and algae	Enzymatic digestion of yeast (commercial β-glucosidase and peptidases); freeze-drying and TCA extraction (0 °C) for algae samples. Cation-exchange separation (Ionosphere-C, Chrompack column and gradient elution with piridinium formate pH 3.0–3.2) and anion-exchange HPLC (ION-120 interaction chromatography column and isocratic elution with sodium salicylate at pH 8.5); ICP-MS detection with DRC (^{80}Se)	12 Se species comprising Se-amino acids and inorganic Se	Methodological development focusing the optimization of IC-HPLC. SeMet, SeOMet and SeMC were detected in yeast digests (complementary ESI-MS analyses). Algae extracts contained dimethylselon-iumpropionate, allylSeCys and SeEt	140
Se-yeast intervention agents in cancer prevention	Proteolytic hydrolysis, ion-exchange HPLC coupled to ICP-MS detection	SeMet and unidentified species	Speciation and bioavailability of selenium in yeast-based intervention agents: the results revealed different SeMet contents, depending on the product source, batch and age. Differences in intake, speciation or bioavailability of selenium from the yeast-based supplements could be responsible for different increases in Se in blood plasma observed in the clinical trials	149
In vitro gastric and intestinal digests of Se-yeast supplements	HPLC-ICP-MS and HPLC–ESI-MS-MS	Se-amino acids	The main compound extracted by both gastric and intestinal fluid was SeMet, which is also the primary Se species in yeast proteolytic digest. Two other minor compounds could be identified as SeCis and SeOMet	197

Nuts	LMW fraction extracted with 0.4 mol L^{-1} $HClO_4$; proteins solubilized with 0.1 mol L^{-1} NaOH; enzymatic digestion (proteinase K). HMW compounds separated by SEC (Superdex Peptide HR 10/30 acetate buffer containing 0.2% SDS at pH 4.5); LMW compounds separated by IP-HPLC [Altima C_8, 5 mmol L^{-1} citrate buffer, 5 mmol L^{-1} hexanesulfonic acid, pH 4.5, with 5% (v/v)] methanol. For HMW compounds, on-line UV and ICP-MS, for LMW ICP-Ms and additional ESI-MS analyses	Inorganic Se, SeMet, SeCys, SeEt	Distribution and speciation of Se in different types of nut studied: ~12% of Se in Brazil nuts weakly bound to proteins, ~3–5% found in cytosol, the primary species found in enzymatic digests was SeMet (19–25% of total Se for different types of nuts). Peptide structure studied by ESI-MS and MS-MS. Brazil nuts proposed as naturally rich dietary source of SeMet	141,142
Tuna and mussel tissues	Two-step enzymatic hydrolysis with a non-specific protease (subtilisin); separation on a Spherisorb 5 ODS/AMINO column using two different chromatographic conditions, namely phosphate buffers at pH 2.8 and 6.0 as mobile phases; on-line ICP-MS	Selenate [Se(VI)], selenite [Se(IV)], TMSe, SeCis, SeMet, SeEt	Characterization of Se species in marine food: only organic selenium species were found in the samples (TMSe, SeMet and several unknown species). The sum of identified selenium species in the sample was about 30% of the total selenium present in the enzymatic extract	198
Cod muscle	Four different extraction procedures tested; speciation analysis by SEC and RP-HPLC followed by post-column ID-ICP-MS with octapole reaction system	SeMet	The highest Se recoveries were obtained when using enzymatic digestions whereas only 5% of total Se in cod was extracted when a 'soft' extraction procedure (MeOH-HCl) was used. SeMet was the main seleno compound found in enzymatic hydrolyzates. In MeOH-HCl extracts, SeMet was absent, indicating that Se is mainly incorporated into proteins. A number of unidentified Se species were also detected in cod muscle tissue	145

(*continued*)

Table 12.5 (*Continued*)

Sample	Analytical procedure	Species of interest	Toxicological and/or clinical relevance	Ref.
Rat organs and body fluids after ^{82}Se-enriched selenate, selenite or SeMet treatment	Se distribution in organs determined by ICP-MS, speciation studied by SEC HPLC (Asahipak GS 520 or GS 320 column, elution with 50 mmol L^{-1} Tris–HCl buffer at pH 7.4) and ICP-MS detection	Inorganic and organic species relevant to Se metabolism in rats	Metabolic, nutritional and toxicological aspects of Se were studied. The transport of Se within the organism and the urine metabolites observed were different, depending on the form of Se administred. Rapid and efficient incorporation of Se into SeP in the liver and excretion into the plasma followed by the slow and steady incorporation of Se into GPx in the kidneys and excretion into the plasma described. The formation of Se-methyl-*N*-acetylselenohexosamine in liver and its excretion via urine were reported. The mechanisms underlying the interaction between Se and mercuric ions in the bloodstream were explained by the formation of a ternary complex, $[(HgSe)_n]_m$–selenoprotein P	160–163
Rat urine after administration of Se(IV) or Se-garlic	Sample homogenization followed by separation by multi-mode capillary gel filtration (CShodex GS320AM5D and GS320A-M8E, 50 mmol L^{-1} CH_3COONH_4, pH 6.5) and ICP-MS detection (microflow total consumptionnebulizer)	Selenite [Se(IV)], SeMet, SeMC, γ-glutamy l-SeMC, TMSe, SeGal	Methodological development: the advantages of the capillary HPLC over conventional HPLC was demonstrated as follows: (1) lower sample requirement (as low as 100 nL); (2) improved signal-to-noise ratio; and (3) shorter time of analysis. Separation of SeMC and γ-glutamyl-SeMC directly in homogenates of Se-garlic achieved in 15 min. Urine SeGal and TMSe resolved in 16 min	176
Human volunteer urine	Filtered samples analyzed by IP-HPLC on LiChrosorb RP18 column with a mobile phase containing 2.5 mmol L^{-1} sodium 1-butanesulfonate and 8 mmol L^{-1} TMAH (separation time 15 min); on-line ICP-MS	Selenate [Se(VI)], selenourea, SeMet, SeEt, TMSe	The method developed (DL 0.6–1.5 μg Se L^{-1}) was used for the analysis of urine samples: TMSe and two unknown metabolites were detected. On the other hand, SeMet, selenourea and SeEt were not observed even at a total Se concentration higher than 100 μg Se L^{-1}.	169

Human volunteer urine	Diluted (1 + 1) or purified (by benzo-15-crown-5-ether extraction) samples were analyzed by cation-exchange LC (Ionpac CS5 column with a mobile phase containing 10 mmol L^{-1} oxalic acid and 20 mmol L^{-1} K_2SO_4 at pH 3); on-line ICP-MS detection (^{78}Se and ^{82}Se)	Selenite [Se(IV)], selenate [Se(VI)], SeMet, TMSe	Methodological development: for diluted urine, different retention behavior of TMSe in urine versus standard solution and co-elution of SeMet and Se(VI) were observed. Analyzing the purified urine, six chromatographic peaks were observed, two of them assigned as SeMet and TMSe. Crown ether extraction did not improve the separation. Hence, apart from SeMet and TMSe, at least three more unknown selenium-containing species were present in urine	167,168
Rat urine	Methanol extraction; separation by SEC HPLC (Shodex Asahipak GS-320 7G column with 50 mmol L^{-1} Tris-HCl at pH 7.4 or GS-220 HQ with 50 mmol L^{-1} CH_3COONH_4 at pH 6.5) and on-line detection by ICP-MS detection (^{77}Se and ^{82}Se); ESI-MS and MS-MS analyses for species identification	TMSe, Se-sugars and their possible oxidation products	Identification of major Se metabolite in urine as methylselenium-*N*-acetylselenohexosamine. Methodological development focusing possible effect of storage and pretreatment conditions on speciation results. It was proposed that monome-thylseleninic acid observed in purified urine could be the oxidation product of Se-sugar	175,199
Human urine before and during supplementation with SeMet	Urine purified by SPE (C_{18} cartridge conditioned with HFBA) and analyzed by IP-HPLC [microbore Luna C_8 column with different mobile phases containing perfluorinated carboxylic acids and 20% (v/v) methanol]; ICP-MS detection with DIN	SeMet, SeMM, SeMC, SeGaba, TMSe	Methodological development aiming characterization of Se metabolites in urine. The DLs were in the range 0.8–1.7 μg Se L^{-1}. Several chromatographic peaks were observed and, in some samples, co-elution of species with TMSe, SeMM or SeMet occurred. The major Se compound remained unidentified	171
Human urine	RP-HPLC separation with ICP-MS detection; column fractions were analyzed by ESI-MS and MS-MS for structural information	SeMet, SeCystamine	Six Se-containing fractions were collected from the RP column and the first two fractions were identified specifically as SeMet and selenocystamine, estimated to be present at ~11 and 40 μg L^{-1}, respectively	178

(*continued*)

Table 12.5 (*Continued*)

Sample	Analytical procedure	Species of interest	Toxicological and/or clinical relevance	Ref.
Human urine	Urine purified by SPE (C_{18} cartridges modified with hexanesulfonic acid) and acidified ($HClO_4$, pH 2.0) was analyzed by IP-HPLC [Altima C_8 with a mobile phase containing 2 mmol L^{-1} hexanesulfonic acid, 0.4% acetic acid, 0.2% triethanolamine (pH 2.5) and 5% methanol]; ICP-MS detection (^{77}Se and ^{78}Se), ESI-MS and MS-MS analyses for structural information	SeMet, TMSe, AdoSeMet, SeMM, SeEt	Se species identified in urine were: SeMet, TMSe and AdoSeMet. The identification of AdoSeMet seems to confirm that SeMet enters the metabolic pathway of its sulfur analog in the activated methylation cycle	172
Human urine (volunteers supplemented with Se-yeast)	SPE and multidimensional chromatography (IP, RP and SEC) with ICP-MS detection. Purified fractions analyzed by APCI-MS and MS/MS	Main urinary metabolite of Se	The major selenium metabolite from human urine identified as Se-sugar (methylseleno-*N*-acetylselenohexosamine)	174
Rat urine after feeding with Se(IV)	SEC HPLC (GS 320 column with 50 mmol L^{-1} Tris–HNO_3 buffer at pH 7.4) coupled to ICP-MS detection (^{77}Se,^{82}Se)	SeGal, TMSe	The changes in the urinary SeGal and TMSe were investigated to clarify the relationship between the dose and two main urinary metabolites. It was also examined whether the metabolites are related to age. In young rats, SeGal was always the major urinary metabolite and TMSe increased with a dose higher than 2.0 μg Se mL^{-1} drinking water. In adult rats, TMSe increased only marginally despite the fact that the rats suffered much more greatly from the Se toxicity, suggesting that TMSe cannot be a biomarker of Se toxicity	179

Human urine (after a single dose of Se-yeast)	Three pretreatment procedures tested: (1) preconcentration through evaporation in N_2 stream; (2) evaporation and methanol extraction followed by the analysis of two fractions; (3) as (2), but using 100 mL of urine. Two chromatographic systems compared: RP-HPLC [microbore Luna C_{18} (2), 2 mmol L^{-1} CH_3COONH_4 with 5% methanol) and IP-HPLC [microbore Luna C_8 (2), 0.2% HFBA with 20–30% methanol]; on-line ICP-MS with MCN and modified DIN	TMSe, SeMC, SeMM, SeGal, SeGlu, SeEt, SeMet Se-cystamine	Pretreatment procedures did not affect native species distribution in urine. For large urine volumes (preconcentration factor 100), at least 10 selenium compounds were separated. The elution behavior of some species was slightly changed in the presence of a urine matrix, as observed in spiking experiments. Only two species were identified in the analyzed samples: SeGal – primary species and SeGlu – about 2% of SeGal; TMSe, SeMet, SeMM, SeCM and Se cystamine were not detected	177
Serum and prostate tissue from prostate cancer patients	HPLC-ICP-MS	SeMet	The cancer patients had total Se levels in serum and in prostate tissue in a range expected for normal Se intake. There was a significantly higher total Se level found in peripheral versus transitional zone tissues. This is the first time that Se-Met has been detected in prostate tissue	164
Human serum (healthy volunteers and hemodialyzed patients)	Anion-exchange HPLC (Mono Q HR 5/5 column) and affinity chromatography (Hi-Trap Heparin and Hi-Trap Blue-Sepharose columns) and IDA-ICP-MS with an octapole reaction system ($^{78}Se/^{77}Se$ and $^{80}Se/^{77}Se$)	Se-containing proteins	Fractionation of serum proteins was achieved by affinity chromatography: three main selenium fractions corresponded to SeP, albumin and GPx. Mass balance performed under different experimental conditions showed quantitative selenium recovery. The distribution of selenium between plasma GPx (~20%), SeP (~55%) and albumin (~20%) was similar in both populations	14
Human serum	Derivatization of the SeCys residues with iodoacetamide, enzymatic digestion (lipase, protease), purification of Seamino acid fraction by SEC-HPLC and separation by capillary HPLC; SSID–ICP-MS with DRC ($^{77}Se/^{78}Se$)	Se-amino acids	A ^{77}Se-labeled SeMet was added to serum for species quantification. The accurately determined SeMet was used as an internal standard for the SeCys determination from the same chromatogram	165

HPLC.[141,152] Different perfluorinated carboxylic acids have been examined as the ion-pairing agents (pH 2.5–4.5) in the analyses of enriched yeast and vegetable extracts.[135,153] Alkylsulfonic acids have been successfully used in speciation analysis carried out on nuts, onion leaves and yeast.[142,143,151,155] Owing to their different biological activities, optical enantiomers of Se-amino acids were analyzed by several chiral separation techniques.[156–159]

The distribution and speciation of selenium in different organs, tissues and body fluids have been investigated by HPLC-ICP-MS for a better understanding of selenium metabolism[14,160–165] (Table 12.5). In particular, the characterization of selenium metabolites in urine has often been undertaken in the context of element metabolism and evaluation of possible health risk. Depending on the element intake, its chemical form, total urine selenium and the pretreatment procedure applied, different compounds were observed. Inorganic selenium and TMSe have often been detected, confirming the biomethylation route of element elimination.[166–170] In recent studies, significant progress in species characterization has been achieved by application of different clean-up and extraction procedures and the use of collision and reaction cells in ICP-MS.[170–174] Thus, selenosugars were identified as primary selenium species both in a rat model[175] and in human urine.[174,176,177] Less abundant metabolites (SeMet, SeMC, Se-cystamine, AdoSeMet) were also identified and confirmed by ESI-MS.[168,169,171,172,178] Based on the results obtained, a putative pathway and dose-dependent excretion of selenium in human organism have been proposed, which is presented in Figure 12.4.[177] Dose- and age-related changes in the excretion of SeGal and TMSe in rats were studied by Suzuki *et al.*[179] As can be observed in Figure 12.5, in young rats, selenosugar was always the major urinary metabolite and TMSe increased with a dose higher than 2.0 μg Se ml^{-1} drinking water.[179] On the other hand, in adult rats, TMSe increased only marginally despite the fact that the rats suffered much more greatly from the Se toxicity, suggesting that TMSe cannot be a biomarker of Se toxicity. The results suggested that sources of the sugar moiety of selenosugar are more abundant in adult

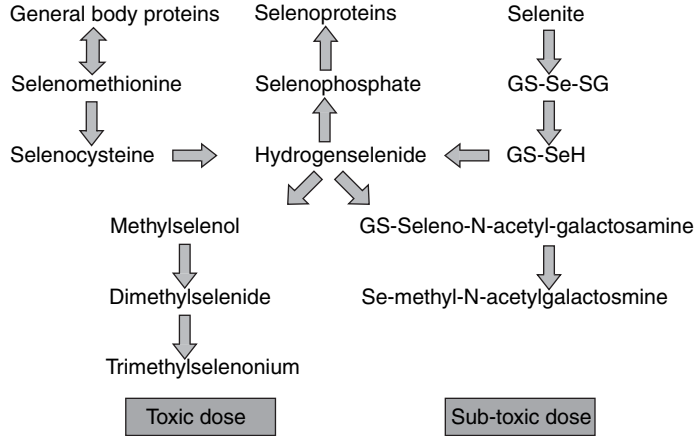

Figure 12.4 The proposed selenium metabolism (GS-: glutathione conjugate). From Gammelgaard, B., Bendahl, L. Selenium speciation in human urine samples by LC- and CE-ICP-MS separation and identification of selenosugars. *J. Anal. At. Spectrom.* 2004, **19**, 135–142. Reproduced by permission of The Royal Society of Chemistry

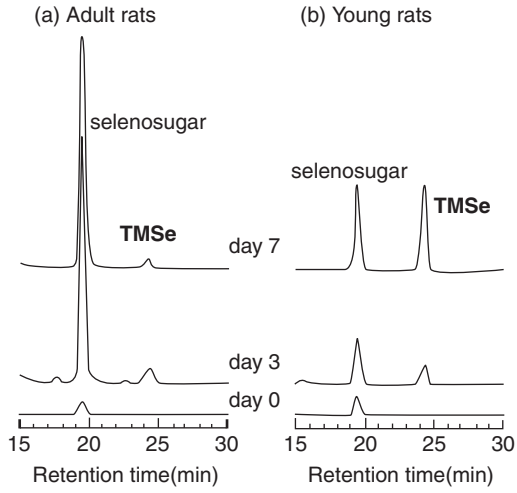

Figure 12.5 Changes in the distribution of Se in the urine of adult (a) and young (b) rats fed selenite at the concentration of 5.0 μg Se mL^{-1} drinking water for 7 days. Male Wistar rats of 36 (a) and 5 (b) weeks of age (three rats/group) were fed selenite at the concentration of 5.0 μg Se mL^{-1} drinking water *ad libitum* for 7 days, and 24-h urine was collected daily for each rat maintained in a plastic cage for metabolism. Urine samples from three rats in each group were combined and applied to a GS 320HQ column, and the column was eluted with 50 mM Tris–HNO_3 (pH 7.4, 25.8 °C). Reprinted from *Toxicology and Applied Pharmacology*, **206**, Suzuki, K.T., Kurasaki, K., Okazaki, N., Ogra, Y. Selenosugar and trimethylselenonium among urinary Se metabolites: Dose- and age-related changes, 1–8. Copyright 2005, with permission from Elsevier

than in young rats. Chondroitin 4-sulfate did not affect the ratio of the two urinary metabolites, suggesting that the sugar source is of endogenous origin and that it increases with age.[179]

Regarding element-specific detection in selenium speciation analyses, the conventional ICP-MS instruments pose two important difficulties: (1) low ionization efficiency (about 33% of atoms entering plasma) caused by relatively high ionization potential of selenium [9.8 eV, close to the first ionization potential of argon (15.8 eV)][180] and (2) spectral interference on the most abundant selenium isotope (^{80}Se, 49.61%) caused by argon dimer ion. Hence the sensitivity of selenium speciation is often compromised by incomplete ionization and the necessity of monitoring less abundant isotopes (^{77}Se or ^{78}Se). Furthermore, the effect of the chromatographic mobile phase composition on selenium response in ICP-MS should be mentioned.[151] Consequently, the reported detection limits for selenium species by ICP-MS coupled to chromatographic separation range from 0.5 to 50 μg L^{-1} (as selenium concentration in the solution introduced on to the column).[140,150,181,182] The progress in ICP-MS instrumentation and, in particular, the commercial availability of high-resolution instruments (HR-ICP-MS) and collision/reaction cell technology, have offered improved conditions of selenium determination.[19,140,145,147,148,165,183,184] Isotope dilution analysis has been explored for accurate speciation results.[14,147] In particular, the species-specific ID mode (SSID) permits correction for analyte losses and/or conversion during the entire analytical procedure and reliable quantification.[148,165,185]

12.4 CONCLUSIONS AND FUTURE TRENDS

Element speciation studies are emerging in the life sciences. Such analyses are necessary for assessing pharmacokinetic aspects of elements, for elucidation of their biological pathways and for better understanding of possible cellular mechanisms responsible for the observed health effects. The list of toxicologically and clinically relevant elements/species has gradually grown larger as a result of methodological developments and the progress in our knowledge of element-specific biological functions. Coupling of LC and ICP-MS is well established and considered as a primary speciation tool for non-volatile compounds. The high separation power and versatility of HPLC make this technique particularly suited for the separation of element species. Owing to different molecular size, polarity and/or electrical charge of target compounds, size-exclusion, ion-exchange, reversed-phase and ion-pair reversed-phase separation mechanisms are commonly used. On the other hand, ICP-MS offers outstanding detection conditions in terms of sensitivity and multielement and isotopic capabilities. The use of high-resolution instruments and collision/reaction cell technology eliminates troublesome polyatomic interferences and offers enhanced performance for non-metal determinations. Several elements can be monitored in one chromatographic run, providing information on their possible binding in the sample. However, for the identification and/or confirmation of species, structural analysis is needed. On-line coupling of HPLC with ICP-MS helps to minimize possible analyte loss or sample contamination, to avoid problems related with the storage of fractions, provides higher reproducibility and permits automation. The importance of compatibility between column effluent and plasma and the importance of state of the art interface designs should not be under-emphasized. Within this context, optimization of mobile phase composition, multidimensional separations, the development and applications of both low-flow separation systems and high-consumption nebulizers have been addressed. Finally, the toxicological and clinical importance of speciation results call for more reliable analytical results. Thus, an increase in applications of isotope dilution and, in particular, species-specific isotope dilution can be expected.

12.5 ABBREVIATIONS

AdoSeHcy	selenoadenosylhomocysteine
AdoSeMet	selenoadenosylmethionine
APCI	atmospheric pressure chemical ionization
AsB	arsenobetaine
AsC	arsenocholine
ASE	accelerated solvent extraction
AsSug-OH	arsenosugar: glycerol–ribose
AsSug-PO_4	arsenosugar: phosphate–ribose
AsSug-SO_3	arsenosugar: sulfonate–ribose
AsSug-SO_4	arsenosugar: sulfate–ribose
$As(GS)_3$	arsenic triglutathione
CFN	cross-flow nebulizer
$CH_3As(GS)_2$	methylarsenic diglutathione

Cisplatin	*cis*-diamminedichloroplatinum(II)
CRM	certified reference material
CV	cold vapor
DI	deionized water
DIN	direct injection nebulizer
DL	detection limit
DMA	dimethylarsinic acid
DMA(III)	dimethylarsinous acid
DMAE	dimethylarsinoylethanol
DN	direct nebulization
DRC	dynamic reaction cell
ESI-MS	electrospray ionization mass spectrometry
ETV	electrothermal vaporization
GMP	guanosine 5-monophosphate
GPx	glutathione peroxidase
GSH	glutathione
HFBA	heptafluorobutanoic acid
HG	hydride generation
HHPN	hydraulic high-pressure nebulizer
HMW	high molecular weight
HPLC	high-performance liquid chromatography
ICP-MS	inductively coupled plasma mass spectrometry
IDA	isotope dilution analysis
IEC	ion-exchange chromatography
IP	ion-pair
JM-216	bisacetatoamminedichlorocyclohexylamineplatinum(IV)
LC	liquid chromatography
LMW	low molecular weight
MA	mersalylic acid
MCN	micro flow nebulizer
MEM	monoethylmercury
MMA	monomethylarsonic
MMA(III)	monomethylarsonous acid
MMM	monomethylmercury
MPhM	monophenylmercury
MT	metallothionein
Oxaliplatin	(1*R*, 2*R*)-1,2-cyclohexanediamine-*N*,*N*′-oxalato(2−)-O,O′-platinum
p-ASA	*p*-arsanilic acid (4-aminobenzenearsenic acid)
ROX	roxarsone (4-hydroxy-3-nitrobenzenearsenic acid)
RP	reversed-phase
Satraplatin (JM216)	bisacetatoamminedichlorocyclohexylamineplatinum(IV)
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	size-exclusion chromatography
SeCis	selenocystine
SeCys	selenocysteine
SeEt	selenoethionine

SeGaba	seleno-γ-aminobutyric acid
SeGal	Se-sugar: methylseleno-*N*-acetyl-D-galactosamine
SeGlu	Se-sugar: methylseleno-*N*-acetyl-D-glucosamine
SeHcy	selenohomocysteine
SeMC	methylselenocysteine
SeMet	selenomethionine
SeMM	methylselenomethionine
SeOMet	selenomethionine oxide
SeP	selenoprotein P
SPDC	sodium pyrrolidinedithiocarbamate
SPE	solid-phase extraction
SSID	species-specific isotope dilution
T1	monoiodothyronine
T2	3,5-diiodothyronine
T3	3,3′,5-triiodothyronine
T4	3,3′,5,5′-tetra-iodothyronine
T4–TBG	thyroxin–thyroxin-binding globulin
TBAA	tetrabutylammonium acetate
TBAH	tetrabutylammonium hydroxide
TCA	trichloroacetic acid
TEAH	tetraethylammonium hydroxide
TETRA	tetramethylarsonium ion
TFA	trifluoroacetic acid
TMAH	tetramethylammonium hydroxide
TMAO	trimethylarsine oxide
TMAP	trimethylarsoniopropionate
TMAs	tretramethylarsonium ion
TMSe	trimethylselonium cation
TTAA2	3,5-diiodothyroacetic acid
TTAA3	3,3′,5-triiodothyroacetic acid
TTAA4	3,3′,5,5′-tetraiodothyroacetic acid
USN	ultrasonic nebulizer
ZD0473	*cis*-amminedichloro(2-methylpyridine)platinum(II)

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13

Application of Gas Chromatography-Mass Spectrometry to the Determination of Toxic Metals

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13.1 INTRODUCTION

Determination of toxic metals such as lead (Pb), cadmium (Cd), mercury (Hg) and arsenic (As) in human samples is necessary to determine the extent of occupational or non-occupational exposure to these metals. Historically, analytical methods were designed to measure the total amount of toxic metal in the biological specimen, but greater understanding of the mechanisms of toxicity of specific chemical forms of these metals has made it important to know the nature/form of the toxic metal as it exists in biological tissue. As an example, methylmercury is much more toxic to humans than inorganic Hg. Similarly, As(III), As(V) and other organic forms of As such as arsenocholine and arsenobetaine have different toxicities. Therefore, it is essential to obtain information on the type of species present in the sample and their individual concentrations, in addition to determining the total concentration of the toxic metal.

A number of methods have been described for determining the concentrations of toxic metals in biological samples.[1–4] These methods include graphite furnace atomic absorption spectrometry (GF-AAS), electroanalytical techniques such as anodic stripping voltammetry (ASV), neutron activation analysis (NAA), and mass spectrometry (MS). Amongst these techniques, mass spectrometry occupies a unique role due to its potential to measure the

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isotopic composition of the metal, which is useful for discriminating among various sources of contamination. Moreover, the use of isotope dilution for quantitation in mass spectrometry makes this technique less susceptible to matrix effects that often limit the accuracy of other analytical techniques. Another advantage of the isotope dilution technique is that the results are not affected by incomplete recovery of the analyte from the matrix. However, isotope dilution depends on the availability of at least two stable isotopes of an element and therefore cannot be used for the determination of monoisotopic elements such as As. In cases where no stable isotope is available, another element may be used as an internal standard. This approach requires the development of a suitable calibration curve to account for the effect of different response factors of the two elements in the mass spectrometer. The use of hyphenated techniques such as gas chromatography–mass spectrometry (GC-MS) and liquid chromatography–mass spectrometry (LC-MS) offers the potential to perform the speciation analysis for toxic elements.[5] High-performance liquid chromatography (HPLC) coupled to inductively coupled plasma mass spectrometry (ICP-MS) has been widely used for determining the concentrations of different species of various toxic metals in biological samples. Recently, capillary electrophoresis (CE) has also been employed in conjunction with ICP-MS. The advent of electrospray ionization mass spectrometry (ESI-MS) has also provided an opportunity to distinguish among the different species of toxic metals. HPLC-ICP-MS requires a dedicated mass spectrometer, which may not be available in many toxicological and biomedical laboratories. The use of a general-purpose instrument, such as GC-MS, may be more practical in many laboratories.

This chapter presents the results of studies carried out for the determination of Pb,[6,7] Cd,[8] Hg[9] and As[10] using GC-MS. Since GC-MS requires a volatile form of the metal, suitable chelating agents were identified, prepared, and examined for the cross-over (memory) effect. Also the validation of isotope dilution GC-MS for the determination of these toxic metals in biological samples was carried out using suitable NIST certified reference materials.

13.2 INSTRUMENTATION

In principle, any mass spectrometer coupled with a gas chromatograph can be used to measure toxic metals by preparing suitable metal chelates. During the early stages of this work at the University of Virginia, we used a Finnigan MAT 8230 reverse-geometry, double-focusing mass spectrometer coupled to a Varian 3700 gas chromatograph. The instrument was calibrated using methyl stearate and measuring the isotope ratios for m/z values 299:298, 300:298, 301:298 and 302:298. These ratios vary by four orders of magnitude, providing a good measure of the overall linearity of the mass spectrometer. A resolution of about 1000 with a sampling frequency of 2 Hz was found to be optimum (resolution is defined as the separation of two masses, M and $M + \Delta M$. The calculated resolution is then $M/\Delta M$.) The instrument was operated in the electron ionization (EI) mode with 70-eV electrons, a source temperature of 200 °C, the conversion dynode at –5000 V and the secondary electron multiplier at 2400 V. The source and the collector slit widths were adjusted to obtain trapezoidal peaks with flat tops. The GC-MS interface was at 280 °C and high-purity He was used as a carrier gas. Data were acquired in the selected-ion monitoring (SIM) mode using voltage peak switching and the quantitation was based on peak areas.

During later development of these methods at the VA Medical Center in San Diego, CA, a Finnigan MAT 4500 mass spectrometer equipped with a quadrupole mass analyzer

interfaced to a Hewlett-Packard 5890 gas chromatograph and a Finnigan A200S autosampler was used. In this instrument, electron ionization (EI), positive chemical ionization (PCI) and electron-capture negative chemical ionization (ECNCI) were used as the ionization modes and the electron energy was set at 70 eV. Methane was used as the reagent gas for chemical ionization, at an ionizer pressure of 0.4 ± 0.05 Torr (1 Torr = 133.3 Pa) and at an analyzer pressure of 1.4×10^{-5} Torr. A conversion dynode and a secondary electron multiplier were operated at ± 3000 and 1250 V, respectively. The ionizer temperature was 100 °C and the manifold was kept at 106 °C. Perfluorobutylamine (PFTBA) was used for tuning mass and peak shape for EI, PCI and ECNCI. The GC injector was operated in the splitless mode and the data were acquired using SIM.

13.3 EXPERIMENTAL PROCEDURE

One of the main requirements of using GC-MS for the determination of metals is the preparation of a suitable metal chelate, which should have adequate thermal stability and should also be volatile. Further, the metal chelate should not introduce any memory effect or carry-over effect when injecting sequential samples of different isotopic compositions. We have been able to identify suitable metal chelates for the toxic metals Pb, Hg, Cd and As. Another requirement for the determination of toxic metals in biological samples is the development of a suitable digestion procedure, without introducing any contaminants from the reagents employed.

13.3.1 Preparation of internal standard solutions

The concentration of the toxic metal can be determined by isotope dilution mass spectrometry (IDMS), provided that another stable isotope of the element is available to be used as an internal standard. Since IDMS compensates for most matrix effects, it has the potential of being incorporated into a definitive analytical technique. In addition, the IDMS results are mostly unaffected by incomplete recovery of the analyte during specimen digestion and extraction, which are ordinarily required owing to the complex nature of the biological matrix. The internal standard solution is added to the biological sample prior to carrying out any chemical digestion of the sample. An optimal amount of internal standard is required to obtain the most accurate concentration determination for minimizing random errors in the isotope ratio measurements in spiked samples. A 1:1 ratio of internal standard to analyte provides optimal accuracy in the quantitative measurements.

13.3.2 Digestion of biological sample

Urine samples for Pb and Cd analysis were digested with H_2O_2 (50%, stabilized, from Fisher) and HNO_3 (67–70%, double sub-boiling quartz-distilled in Teflon bottles, NIST) to destroy most of the organic material present. Initially, the urine sample (1 mL) was treated with concentrated HNO_3 and was allowed to stand at room temperature for about 1 h. The specimen was subsequently heated at 50 °C on a hot-plate to reduce the volume

to ~50 μL. Then 100 μL of 50% H_2O_2 were added and the solution was again heated gently on the hot-plate. The digestion with H_2O_2 was performed 4–5 times until a white residue remained on evaporating the solution. The dry residue was dissolved in 2 mL of deionized water and the solution was extracted with 2 mL of CH_2Cl_2, discarding the organic phase to remove undigested lipids. This solution was then ready for metal chelate formation.

For determination of Pb in whole-blood samples, 1 mL of blood sample was mixed with 2 mL of 4 M HNO_3, which was added to each of the samples dropwise, with constant vortex mixing. The digest was centrifuged for 30 min and the supernatant was further digested with $HNO_3 + H_2O_2$ and prepared for chelate formation as described for urine samples.[6] A simpler extraction procedure that eliminated the time-consuming hot digestion procedure is described below for blood Pb[7] and Hg.[9]

The simpler method for blood Pb required 200 μL from a whole blood sample drawn into a metal-free tube containing EDTA as an anticoagulant. This sample is mixed with internal standard and vortex mixed briefly. Then, with gentle vortex mixing, 400 μL of 4 M HNO_3 are added dropwise. The sample is centrifuged at 1760 *g* for 10 min and the supernate transferred to a clean tube. The pH is adjusted to 7–9 with concentrated NH_4OH. This solution is then ready for the chelate preparation step.

Blood Hg required 200 μL from a whole blood sample drawn into a metal-free tube containing EDTA as an anticoagulant. The sample was mixed with 200 μL of 0.6 M HCl and vortex mixed to free any Hg originally bound to protein thiol groups. Then 1 mL of 0.2 M borate buffer was added. This sample could then be chelated.[9]

13.3.3 Preparation of metal chelate

For Pb, lithium bis(trifluoroethyl)dithiocarbamate [Li(FDEDTC)], synthesized in our laboratory, was tested as a derivatizing agent for GC-MS analysis of $Pb(FDEDTC)_2$. However, this approach proved unsuccessful owing to strong memory effect (carryover). The procedure was modified by reacting this derivative with with the Grignard reagent 4-fluorophenylmagnesium bromide (4-FPMgBr), 2.0 mol L^{-1} in diethyl ether, to produce $Pb(FC_6H_4)_4$.[6] $Pb(FC_6H_4)_4$ can also be prepared by using inexpensive commercially available sodium diethyldithiocarbamate or ammonium pyrrolidine dithocarbamate.[7]

The $Pb(FDEDTC)_2$ chelate was prepared at pH 3, using the digested urine or blood samples mixed with 1 mL of acetic acid–sodium acetate buffer and 100 μL of a 20 mmol L^{-1} solution of Li(FDEDTC) in deionized water. The sample was vortex mixed for 2 min before extracting the $Pb(FDEDTC)_2$ chelate into 1 mL of toluene. The organic extract containing the Pb chelate was evaporated to dryness at 60 °C under a stream of argon gas in a laminar-flow hood. The dried residue was dissolved in 200 μL of diethyl ether and approximately 200 μL of 4-FPMgBr were added. Care was taken to add the Grignard reagent quickly, because it is very reactive. The solution in the tube was shaken to allow complete mixing. Excess Grignard reagent was neutralized by adding 100 μL of 100 mL L^{-1} isopropyl alcohol in toluene (i.e. 10%, v/v) and then adding 1 mL of 1 M HNO_3. The $Pb(FC_6H_4)_4$ chelate was extracted with 1 mL of toluene, evaporated to dryness and reconstituted in 10–50 μL of CH_2Cl_2 for GC-MS analysis.

For Cd and Hg, $Cd(FDEDTC)_2$ and $Hg(FDEDTC)_2$ chelates were prepared with Li(FDEDTC) as the chelating reagent, using the procedure described above.

For As, two different derivatizing agents, *N-tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) with 1% *tert*-butyldimethylchlorosilane (*t*-BDMS) and 4-fluorophenylmagnesium bromide (4-FPMgBr), were used. The chelates of As(III), As(V) and organic arsenic species such as dimethylarsenic acid (DMAA, also known as cacodylic acid) were prepared. For this purpose, an aqueous solution of As(III) as As_2O_3 in 10% HNO_3, a methanolic solution of As(V) as As_2O_5 and a methanolic solution of DMAA were used. First, *t*-BDMS derivatives were prepared by treating the digest directly with MTBSTFA. Second, the *t*-BDMS derivatives were prepared by first synthesizing the pyrrolidinedithiocarbamates of As(III), As(V) and DMAA. The toluene solutions containing these derivatives were evaporated to complete dryness at 40 °C under a steady stream of N_2 gas. The dried residues were dissolved in 200 μL of ethyl acetate and 50 μL of MTBSTFA were added to generate the As derivatives.

13.4 GC-MS STUDIES

13.4.1 Memory effect evaluation

Memory effect refers to the carryover of analyte from a previously injected sample, which compromises the reliability of quantitative results by variably contributing to the measured ion intensities. This memory effect can result from poor volatility of a metal derivative in the GC injection port, high reactivity of the metal derivative with active sites on the GC column, or both. One approach that can be used to evaluate the memory effect is sequential analyses of an unaltered specimen, a specimen enriched with isotope (internal standard), followed by the unaltered sample again. Figures 13.1–13.3 show the results of such studies carried out for Pb, Cd and Hg. As Figure 13.1a demonstrates, there is a strong memory effect when sequentially injecting two $Pb(FDEDTC)_2$ chelate preparations with isotope ratios differing by a factor of 10. In contrast, Figure 13.1b shows that when using the $Pb(FC_6H_4)_4$ derivative in the two samples (one unaltered and the other enriched with ^{204}Pb) with isotope ratios differing by a factor of 300, there was no measurable memory effect. Figure 13.2 shows the results obtained on the evaluation of memory effect for Cd using $Cd(FDEDTC)_2$ chelate. It is clear that a strong memory effect exists across sequential analyses of specimens containing endogenous amounts of Cd and specimens enriched with ^{106}Cd internal standard. The concentration of Cd in the enriched specimen, however, is much greater than typical Cd concentrations in urine and whole blood specimens. A good design would maintain the isotope ratios in different samples within reasonable limits, making it possible to determine these isotope ratios experimentally without significant contributions from the memory effect. A small memory effect was observed when analyzing two synthetic mixtures with Cd isotope ratios differing by a factor of about 10, demonstrating that the chelating agent Li(FDEDTC) could be used for Cd determination by GC-MS provided that an optimum sample to internal standard ratio is maintained in the spiked samples. Alternatively, the analysis may be carried out over a limited range of isotope ratios, or the analyses may be run in replicate to detect whether significant carryover has occurred. Figure 13.3 shows the results of Hg analyses using $Hg(FDEDTC)_2$ when two mixtures with isotope ratios differing by a factor of about 40 were injected sequentially into the GC-MS system. No measurable memory effect was observed for the $Hg(FDEDTC)_2$ chelate. The memory effect associated with As cannot be assessed using this method, since it is a monoisotopic element.

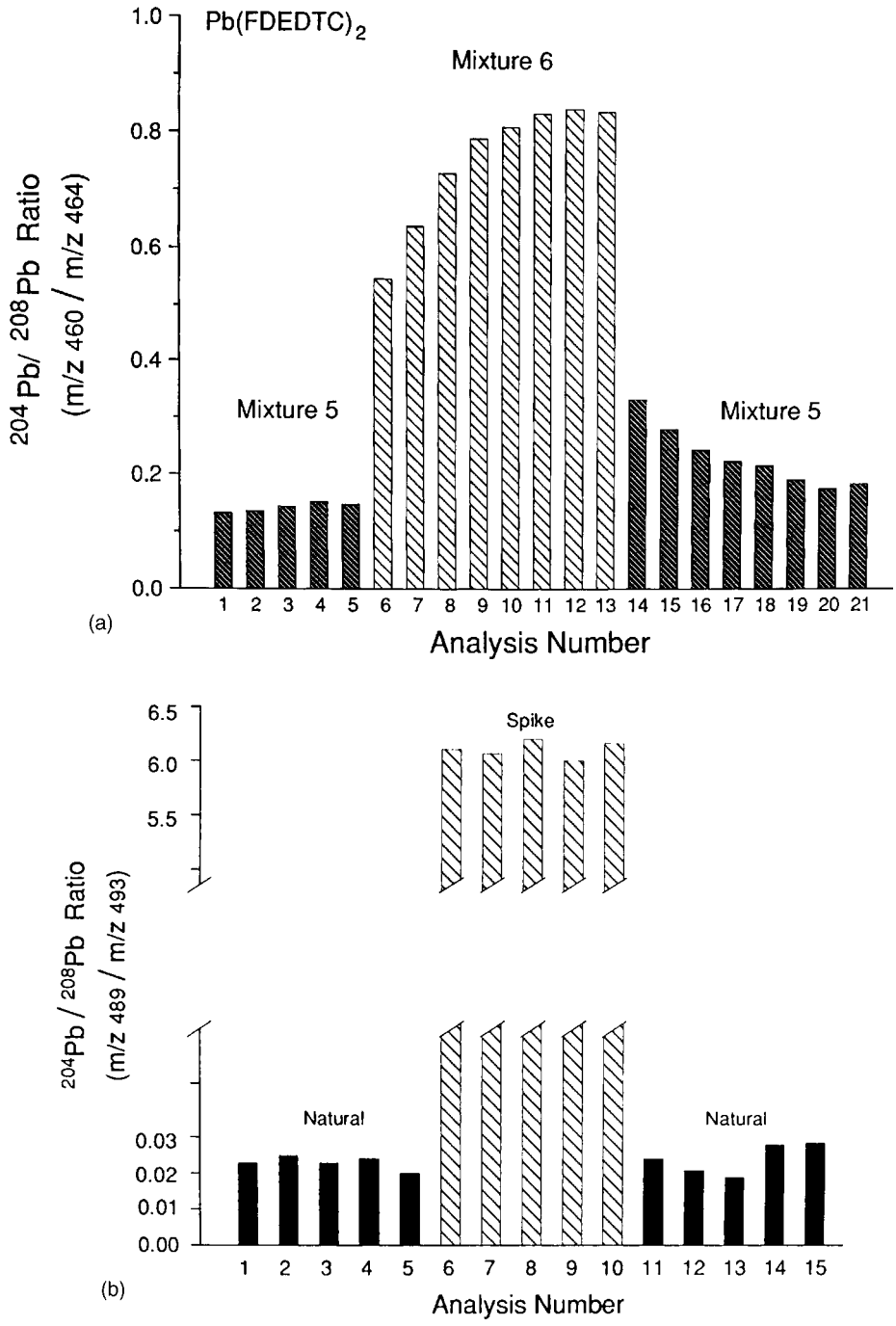

Figure 13.1 (a) Evaluation of cross-contamination in consecutive analyses of two $Pb(FDEDTC)_2$ samples with isotope ratio differing by a factor of ~8 (Ref. 6). (b) Evaluation of cross-contamination in consecutive analyses of natural Pb and enriched ^{204}Pb internal standard by using $Pb(FC_6H_4)_4$ with *m/z* 489:493 ratio differing by a factor of ~300 (Ref. 6)

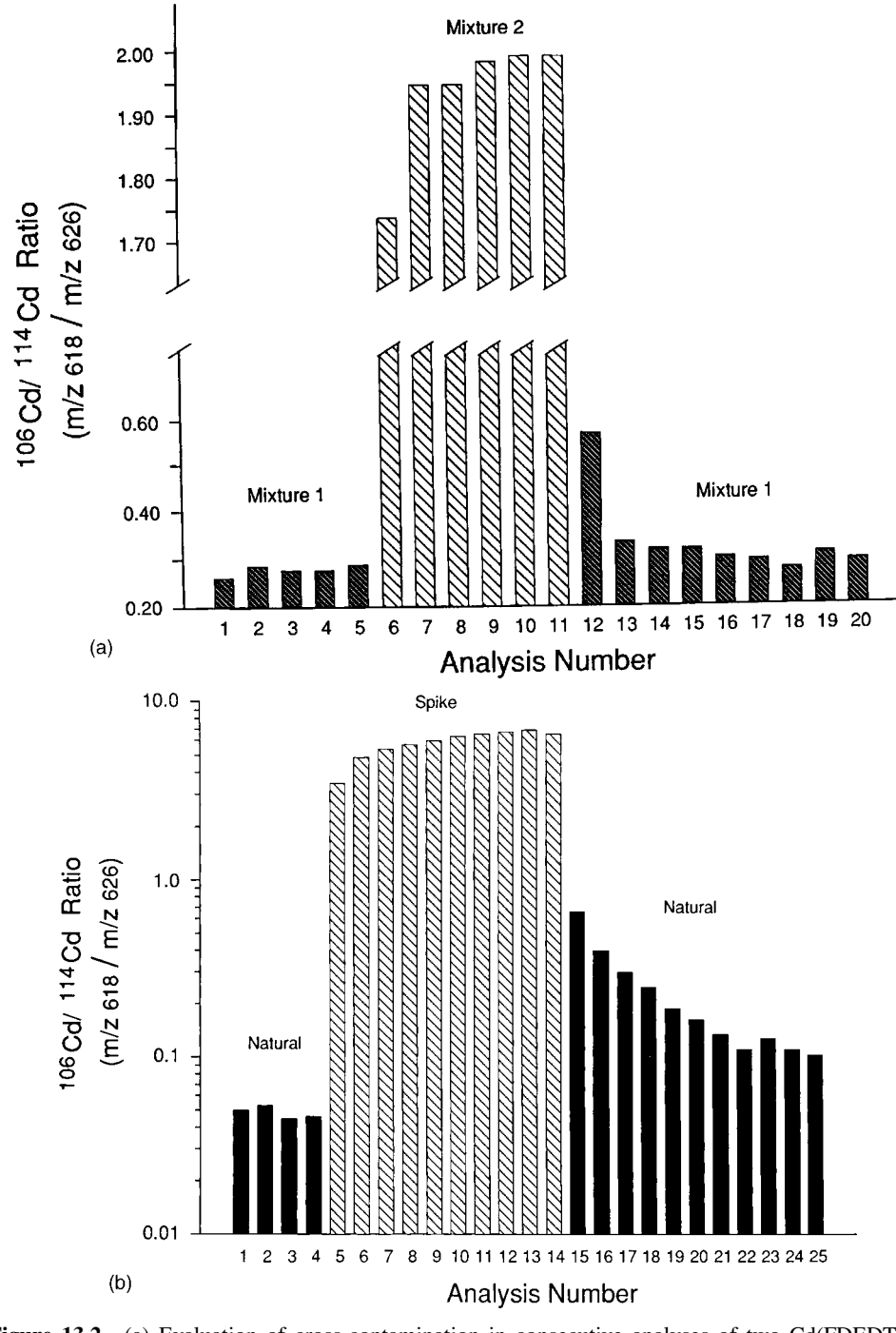

Figure 13.2 (a) Evaluation of cross-contamination in consecutive analyses of two $Cd(FDEDTC)_2$ samples with isotope ratio differing by a factor of ~10 (Ref. 8). (b) Evaluation of cross-contamination of consecutive analyses of two $Cd(FDEDTC)_2$ samples with isotope ratio differing by a factor of ~200 (Ref. 8)

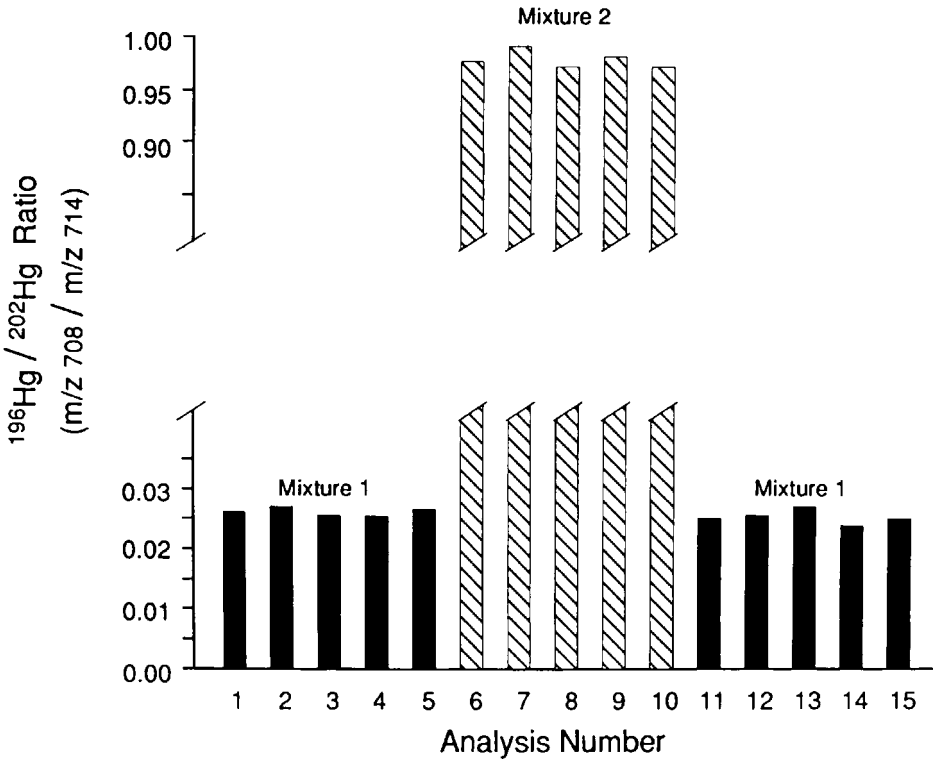

Figure 13.3 Evaluation of cross-contamination of consecutive analyses of two $Hg(FDEDTC)_2$ samples with isotope ratio differing by a factor of 40 (Ref. 4)

13.4.2 Precision and accuracy in measuring isotope ratios

Table 13.1 lists the different metal chelates used for various toxic metals analysis by GC-MS. Table 13.2 summarizes the data on precision and accuracy for isotope dilution measurement of different metals using these metal chelates. These data were generated by injecting 1 μL of the chelate solution containing a few nanograms of the toxic metal. The atom% abundances of different isotopes in an unaltered sample were compared with the calculated abundances of the corresponding ions in the metal chelate, considering the contributions of isotopes of carbon, nitrogen, sulfur, etc., in the molecule. In all cases, there was good agreement between the calculated and experimentally measured abundances of the different isotopes. The data in Table 13.2 are observed values that have not been corrected for any differences in mass discrimination among the isotopes, since any mass discrimination factor would be canceled in isotope dilution. The precision of various isotope ratio measurements was evaluated with chelated endogenous metal over several days. Overall precision was calculated by combining the within-run and between-run precisions. Representative precision data are also included in Table 13.2. It can be seen that typical overall relative precision values of 1–2% are possible using GC-MS of volatile metal chelates when isotope ratios are 0.1 or higher.

Table 13.1 Metal chelates used for different toxic metals in GC-MS[a]

No.	Element	Chelating agent	Metal chelate	Ions containing information on metal in EIMS	Ref.
1	Pb	4-FPMgBr	$Pb(FC_6H_4)_4$	$\mathbf{(M-L)^+}$ **(493)**; $(M-3L)^+$ (308); M^+ (208)	6,7
2	Cd	Li(FDEDTC)	$Cd(FDEDTC)_2$	$\mathbf{M^{+\cdot}}$ **(626)**; $(M-L)^+$ (370)	8
3	Hg	Li(FDEDTC)	$Hg(FDEDTC)_2$	$\mathbf{M^{+\cdot}}$ **(714)**; $(M-L)^+$ (321)	9
4	As	4-FPMgBr	$As(FC_6H_4)_3$	$M^{+\cdot}$ (360); $\mathbf{(M-2L)^+}$ (170)	10

[a] $M^{+\cdot}$ and $(M-zL)^+$ denote the molecular ion and ion obtained after the loss of z ligands, respectively. Ion of the highest intensity is given in bold with m/q value in parentheses, referring to the isotope with the highest percentage abundance in natural element. Li(FDEDTC) and 4-FPMgBr are lithium bis(trifluoroethyl)dithiocarbamate and 4-(fluorophenyl)magnesium bromide, respectively.

The accuracy of this method for determining isotope ratios by GC-MS was evaluated by comparing the results obtained on different synthetic mixtures of Pb with an alternative technique, quadrupole-based inductively coupled plasma mass spectrometry (Q-ICP-MS). Table 13.3 lists the results of comparison studies for Pb. The calculated ratios for m/q

Table 13.2 Precision and accuracy in isotope ratio measurement of toxic metals by GC-MS[a]

No.	Isotope	Atom %	Ion (m/z)	Calculated abundance (%)	Measured abundance (%)	Isotope ratio	Mean of mean values	Within-run precision [CV (%)]	Between-run precision [CV (%)]	Overall precision [CV (%)]
1	Pb-204	1.40	489	1.27	1.31	489/493	0.0256	3.2	8.1	8.7
	Pb-206	24.10	491	21.96	23.26	491/493	0.4528	1.6	0.4	1.7
	Pb-207	22.10	492	24.57	24.04	492/493	0.4679	1.3	0.7	1.5
	Pb-208	52.40	493	52.20	51.38	*	*	*	*	*
2	Cd-106	1.25	618	1.00	1.05	618/626	0.0388	1.8	6.6	6.8
	Cd-108	0.89	620	0.90	0.96	620/626	0.0354	1.3	6.0	6.1
	Cd-110	12.49	622	10.17	10.46	622/626	0.3851	1.2	2.7	3.0
	Cd-111	12.80	623	11.79	12.02	623/626	0.4427	1.2	2.0	2.3
	Cd-112	24.13	624	22.77	22.05	624/626	0.8122	0.6	0.8	1.0
	Cd-113	12.22	625	14.62	15.00	625/626	0.5526	0.9	1.7	2.5
	Cd-114	28.73	626	28.24	27.15	*	*	*	*	*
	Cd-116	7.49	628	10.51	11.30	628/626	0.4161	1.1	2.5	2.7
3	Hg-196	0.14	708	0.11	0.12	708/714	0.0041	10.1	–	10.1
	Hg-198	10.02	710	7.96	8.00	710/714	0.2808	1.6	0.8	1.8
	Hg-199	16.84	711	14.55	14.86	711/714	0.5217	2.1	0.7	2.2
	Hg-200	23.13	712	21.86	21.77	712/714	0.7644	1.5	0.4	1.6
	Hg-201	13.22	713	15.99	16.21	713/714	0.5692	1.5	0.6	1.6
	Hg-202	29.80	714	29.11	28.48	*	*	*	*	*
	Hg-204	6.85	716	10.42	10.56	716/714	0.3709	1.8	1.5	2.3

[a] Asterisks denote the highest abundant isotope in the natural element, used as a reference to represent isotope ratios.

Table 13.3 Comparison of Pb isotope ratios in synthetic mixtures by GC-MS and Q-ICP-MS

Synthetic mixture	$^{204}Pb/^{208}Pb$ atom ratio by Q-ICP-MS	*m/z* 489:493		
		Calculated from ICP-MS data	Measured by GC-MS	Measured (GC-MS)/ calculated (ICP-MS)
1	0.3177	0.290	0.302	1.042
2	0.4884	0.445	0.452	1.015
3	0.8269	0.752	0.751	0.999
4	1.1511	1.045	1.032	0.988
Mean				1.011

489:493 in $Pb(FC_6H_4)_4$ were obtained by using the experimentally determined Pb isotope ratios by Q-ICP-MS and including the contributions of carbon isotopes. It is apparent from data in Table 13.3 that results obtained using the GC-MS method agree closely with the same measurements by ICP-MS.

13.4.3 Results of concentration determination of toxic metals in biological samples

The IDMS method, using GC-MS, was validated by determining the toxic metals in NIST certified reference materials (SRM 2670 Urine). Prior to formation of the chelate, a suitable wet digestion procedure was used, taking care to eliminate any adventitious contamination from reagents, laboratory ware, environment and personnel. A procedure based on $HNO_3 + H_2O_2$ was used for urine samples. For serum, the reference samples were deproteinated using concentrated nitric acid added dropwise with continuous vortex mixing of the mixture. For Pb and Hg determination, the whole blood specimens were not deproteinated. Table 13.4 summarizes the results obtained for some of the toxic metals by isotope dilution GC-MS; the results obtained were very close to the certified values.

Table 13.5 presents the results of Pb measurements in whole blood specimens obtained from College of American Pathologists (CAP) proficiency testing program and from NIST SRM 955a Lead in Blood. The results demonstrate excellent agreement between GC-MS

Table 13.4 Toxic metals determined by isotope dilution GC-MS in NIST Standard Reference Materials

Element	Matrix	Concentration ($\mu g\,L^{-1}$)	
		Determined	Expected
Pb	Urine	105 ± 4 ($n = 4$)	109 ± 4
	Blood	160 ± 5 ($n = 3$)	150 ± 40
		431 ± 9 ($n = 3$)	430 ± 40
Cd	Urine	94 ± 10 ($n = 6$)	88 ± 3
		9.3 ± 1 ($n = 2$)	8.4 ± 0.3

Table 13.5 Determination of Pb in proficiency and reference standards ($\mu g\,L^{-1}$)

Sample source	Expected value	ID-GC-MS ($n = 3$)	CV (%)	Difference (%)
CAP	105	102	4.9	−2.9
	209	210	0.1	0.5
	316	313	5.4	−0.9
	435	455	1.8	4.6
	483	514	5.1	6.4
NIST SRM 955a	50.1	50.1	11.0	0
	135.3	140.5	4.1	3.8
	306.3	307.3	1.1	0.3
	544.3	547.2	0.6	0.5

and expected values over the range of clinically relevant Pb concentrations. These data were collected using electron-capture negative chemical ionization (ECNCI) and quadrupole mass spectrometry, and involved ambient temperature extraction of Pb from whole blood without hot digestion.

13.5 CONCLUSIONS

GC-MS is a useful analytical technique for the determination of toxic metals in biological specimens. It has the potential to provide relatively high throughput and can be adapted to quadrupole mass spectrometers commonly available in clinical and environmental toxicology laboratories. The isotope dilution GC-MS methodology has been validated for determination of Pb and Cd using NIST certified biological reference materials, e.g. urine and blood. Hg was validated using a standard additions method and ID-MS.

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