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Handbook of Analytical Therapeutic Drug Monitoring and Toxicology

Edited by

Steven H.Y. Wong and Irving Sunshine

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Handbook of
ANALYTICAL THERAPEUTIC DRUG MONITORING
and
TOXICOLOGY

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I dedicate this volume to my family, Gretta, Heather, Amanda and Jonathan, whose support and understanding have sustained the lengthy process.

Steven H. Y. Wong

I wish to thank my many tutors, colleagues and friends for their continued support. Their advice and councel have sustained my efforts to promote better understanding and resolution of our common scientific problems.

Irving Sunshine

PREFACE

Adapting modern advances in analytical techniques to daily laboratory practices challenges many toxicologists, clinical laboratory and pharmaceutical scientists. Just keeping abreast of these many innovative changes is difficult for most overtaxed toxicology and clinical laboratory directors. Some of the reasons for their inability to become familiar with significant technological developments are managed care environments, which increase work-loads, decrease staff, and limit staff travel that would provide desirable scientific interchanges with professional colleagues. In an effort to help remedy these deficiencies *The Handbook of Analytical Therapeutic Drug Monitoring and Toxicology* was conceived.

In subsequent pages, competent practitioners have distilled from their special skills and experience definitive chapters on the current state of the art in their respective areas of expertise. Preview of these articles will provide readers not only with a comprehensive and well-documented survey of what other investigators have reported, but also with each author's critical evaluation of the potential and limitations of the area surveyed. Modern information retrieval procedures, if available to the reader, may capture many of the articles discussed in the reviews but time and workplace demands preclude how this material may be applied to the local scene. This volume simplifies the search process, describes many significant recent contributions, and provides evaluations and counsel that are otherwise difficult to obtain. It is each reader's prerogative to judge the value of these chapters. Careful study will facilitate a realistic judgment.

Two major topics are within this volume's domain. One deals with alternative biological specimens that heretofore have not been used extensively—hair, meconium, saliva, sweat, and vitreous humor. These specimens offer a distinct advantage in clinical practice because they (except for vitreous humor) may be obtained by noninvasive procedures. The text provides a careful assessment of the value of this attribute. Analysis of some of these specimens requires more sensitive procedures than those used for other biological specimens because the amount of sample is limited and the expected concentrations are low. How to overcome these limitations is the second major topic of this volume. Several experts describe the basic principles of these innovative techniques and detail how they can be adapted to the sensitive analysis of the alternative biological specimens. Many topics are detailed including how micellar capillary electrophoresis and hyphenated mass spectrometry satisfy the need for increased sensitivity and specificity. Supercritical fluid chromatography and extraction, and solid phase extraction improve analyte isolation techniques. Inductive coupled plasma atomic emission spectroscopy which eliminates dangerous acid digestions and simultaneously analyzes many elements minimizes personnel effort while providing reliable results. In all this development, the use of automation/robotics in the clinical laboratory enhances productivity and information transfer. Major advances in immunodiagnostics result in higher throughput, improved efficiency, and novel immunoassays which offer point of care testing capabilities.

These evaluations of the pros and cons of these many advances and how they apply to various analytes will help readers determine their suitability to a particular laboratory. Diligent study of this volume will provide readers with a cutting edge into the current mass of innovative procedures whose use may unlock present barriers to productive laboratory practice.

Finally, we would like to recognize the cooperation and understanding of all the contributors who have endured the various deadlines and corrections. In the course of completing this project, we greatly benefitted from the valuable and enthusiastic assistance of Ms. Judy Behling and Ms. Jody Bonnevier. We are most appreciative for their participation which has greatly facilitated the almost endless task of maintaining the flow of the manuscripts to be processed in a timely manner.

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ANALYTICAL THERAPEUTIC DRUG MONITORING *and* TOXICOLOGY

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DRUG MONITORING BY CAPILLARY ELECTROPHORESIS

Wolfgang Thormann

CONTENTS

I. INTRODUCTION: PRINCIPLES OF SEPARATION AND INSTRUMENTATION

Currently used methods for monitoring, screening, and confirmation of drugs in body fluids are based on the principles of spectrophotometry, immunoassays, and chromatography.[1–3] Recently, instrumentation for electrokinetic separations in fused-silica capillaries of very small ID became available and the feasibility of employing capillary electrophoresis (CE[4,5]) for drug monitoring in body fluids, including plasma, serum, saliva and urine, has been tested extensively in various laboratories.[6] The central part of a CE setup (Figure 1–1) is a fused-silica capillary of 25 to 75 μm ID and 15 to 100 cm length which is mounted between two vials which house the driving electrodes and the buffers. After filling the capillary with buffer and applying a small amount of sample at one column end, a high voltage DC electric field is applied along the column which not only induces electrophoretic transport and separations of charged compounds, but in case of a charged inner capillary wall also a movement of the entire liquid along the capillary (electroosmosis). Electroosmotic flow is characterized by a flat flow profile (a plug) and not a parabolic distribution associated with hydrodynamic flow within a capillary tube. Thus, in an electrokinetically pumped configuration hardly any solute dispersion based upon the flow profile is observed and sample zone broadening is mainly caused by longitudinal diffusion, electrophoretic dispersion due to conductivity changes, thermal effects, and solute sorption. Both electroosmosis and sample-wall interactions can be favorably influenced or minimized via permanent modification of the inner walls of capillaries or via dynamic coating of the walls with agents added to the buffer.[4,5]

For electrokinetic separations, small amounts of samples (typically nL volumes or pmol to subfmol solute quantities) are introduced by electrokinetic or hydrodynamic techniques. Upon application of power (about 5 to 30 kV, 1 to 150 μA) samples are transported through the capillary by the combined action of electrophoresis and electroosmosis. Detection principles applied include, on-column direct and indirect absorbance, direct and indirect fluorescence, and occasionally, radiometry, as well as end-column or off-column monitoring employing conductivity, mass spectrometry

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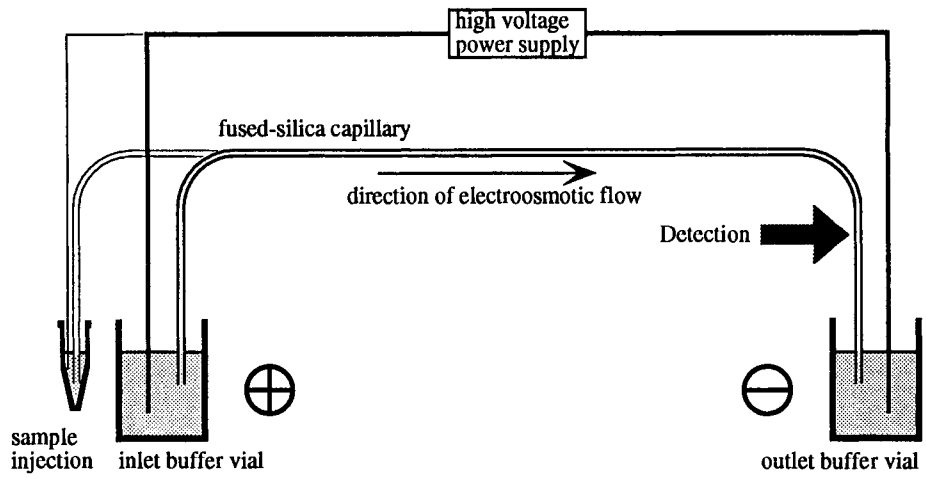


FIGURE 1–1. Overall schematic representation of a capillary electrophoresis instrument. It is assumed that the fused-silica capillary has a negative surface charge. Thus, with the provided polarity the electroosmotic flow is towards the cathode at the outlet side. All experiments whose data are shown in this paper were produced with such a configuration.

(MS), or amperometry. Until recently, most of the employed instruments have been assembled in the researchers laboratories. Whereas the first commercial instrument emerged in 1988, there are currently about a dozen companies manufacturing electrokinetic capillary instrumentation.[7] Many of these apparatuses are fully automated comprising an autosampler, as well as data gathering, and evaluation with a computerized data station.

For monitoring of drugs in body fluids, essentially two methods are employed, capillary zone electrophoresis (CZE) and an electrokinetic capillary chromatography technique called micellar electrokinetic capillary chromatography (MECC). CZE is conducted in a continuous buffer where the samples are the only discontinuities present. Under the influence of an electric field, sample zones migrate without exhibiting any steady state behavior and thus their shape and position continuously change with time.[8] In this technique, separation is based upon differences in net electrophoretic mobilities. CZE analyses can be performed in capillaries with minimized bulk flow or in the presence of electroosmotic and/or deliberately applied hydrodynamic flow, the configurations with flow permitting the simultaneous determination of cations and anions in the same run.[4] Separations primarily depend on the buffer and solvent employed, properties which determine both the charge, and the solubility of the solutes.[9] Neutral compounds cannot be separated by CZE. In MECC, a combination of electrophoresis and chromatography, two distinct phases are used, an aqueous and a pseudo-stationary phase.[10] In its most common form, the micellar phase is formed by employing buffers containing charged surfactants (e.g., sodium dodecyl sulfate (SDS)) which are added above their critical micellar concentration. Micelles are dynamic structures which are in equilibrium with the monomer. An MECC analysis is performed in a capillary which exhibits electroosmosis under current flow. Thus, both a movement of the entire liquid and migration of the charged micelles are occurring concurrently. The two phases migrate at different velocities, thus permitting chromatographic separations. Nonionic solutes partition between the two phases and elute with zone velocities between those of the two phases. For that case, separation is solely of chromatographic nature and elution order is based on the degree of partitioning. For ionic solutes separation is based on chromatography and charge effects, including electrophoresis. MECC separations are dependent on micelle formation, buffer composition and pH (typically between 7 and 10 with SDS), as well as the presence of modifiers, such as small amounts of organic solvents.[10] Instrumentation for CZE and MECC is identical.

Due to the availability of automated CE instruments, first examples of fully validated assays emerged, assays which have been applied to therapeutic and diagnostic drug monitoring (Table 1–1, Refs. 11–21) and to screening for and confirmation of drugs and metabolites in body fluids (Table

TABLE 1–1
Selected Validated CE Assays for Drugs

Drug	Body fluid	Therapeutic range (μg/mL)	Calibration range (μg/mL)	CE method	Sample prep./ detection wavelength[a]	Number of samples	Reference assay	Method correlation[b] a	b (μg/mL)	r	Ref
theophylline	serum	8–20	2.0–28.3	MECC	DSI/275	50	EMIT	0.918	–0.31	0.983	11
phenobarbital	serum	15–40	5.3–52.6	MECC	DSI/245	50	EMIT	0.962	0.44	0.981	12
phenobarbital	serum	15–40	5.3–52.6	MECC	EXI/245	50	EMIT	1.042	–0.34	0.990	13
ethosuximide	serum	40–100	19.8–99.2	MECC	DSI/220	48	FPIA	1.001	3.34	0.975	12
ethosuximide	serum	40–100	19.8–99.2	MECC	DSI/220	49	HPLC	0.995	0.92	0.972	12
ethosuximide	serum	40–100	19.8–99.2	MECC	EXI/210	43	FPIA	1.004	0.81	0.977	13
ethosuximide	serum	40–100	19.8–99.2	MECC	EXI/210	44	HPLC	1.010	–1.75	0.987	–
flucytosine	serum	20–80	20–123	MECC	DSI/210	60	Bioassay	0.963	0.63	0.957	14
antipyrine	plasma	–	1.0–40	MECC	DSI/240	72	HPLC	0.923	0.99	0.965	15
antipyrine	saliva	–	1.9–66	MECC	DSI/260	75	HPLC	1.05	0.09	0.983	16
felbamate	serum	15–135	5.0–160	MECC	DSI/214	37	HPLC	1.14	2.67	0.98	17
pentobarbital	serum	–	10–100	CZE	PP/254	9	HPLC	0.98	3.2	0.96	18
thiopental	serum	–	2.0–60	MECC	EXI/290	66	HPLC	1.07	–0.12	0.994	19
bupivacaine	drain	–	0.5–20	CZE	EXI/200	82	GC	1.00	–1.53	0.984	15
cicletanine	serum	–	0.010–1.0	MECC	EXI/214	30	HPLC	1.02	–0.005	0.998	20
retinol (vit. A)	serum	–	0.0033–0.035	CZE	UF/LIF 465	26	HPLC	0.936	0.032	0.987	21

[a] DSI: direct sample injection; EXI: extract injection after liquid-liquid extraction; PP: supernatant injection after protein precipitation; UF: ultrafiltrate injection after 30 fold dilution; LIF: laser induced fluorescence detection with excitation at 325 nm.

[b] Comparative drug levels assessed by linear regression analysis with [CE] = a × [ref. assay] + b; r: regression coefficient.

TABLE 1–2
Selected CE Screening and Confirmation Assays

Drugs, drug classes	Body fluid, tissue	CE method	Sample preparation	Reference assay(s)	Ref(s)
barbiturates	urine, serum	MECC	DSI, EXI	EMIT	22
salicylate, paracetamol, antiepileptics	urine, serum	MECC, CZE	DSI, UF	FPIA, EMIT	23
11-nor-delta^9-tetrahydrocannabinol-9-carboxylic acid	urine	MECC	H-EXI	FPIA	24
methadone and its primary metabolite	urine	CZE	DSI, EXI	EMIT, GC-MS	25
benzodiazepines	urine	MECC	H-EXI	EMIT, GC-MS	26
benzoylecgonine, opioids, methaqualone, amphetamines	urine	MECC	EXI	EMIT	27,28
cocaine and major classes of illicit and abused drugs	urine	MECC, CZE	EXI	EMIT, FPIA, GC-MS	29
cocaine, morphine	hair	MECC	EXI	HPLC	30
beta-blockers	serum	MECC	H-PP	–	31
diuretics	urine, serum	CZE	EXI	GC-MS	32
theophylline, caffeine (paraxanthine)	urine, saliva, serum	MECC	DSI, EXI	EMIT, FPIA	33
caffeine metabolites	urine	MECC	DSI, EXI	HPLC	34,35
mephenytoin, dextromethorphan, caffeine and metabolites	urine	MECC (CZE)	H	HPLC	36
dextromethorphan (dextrorphan)	urine	CZE	H	HPLC	37
mephenytoin-, phenytoin-enantiomers	urine	CD-MECC	H, H-EXI	–	38
dihydrocodeine and major metabolites	urine	MECC	DSI, EXI, H-EXI	–	39
verapamil enantiomers (norverapamil)	plasma	CD-CZE	EXI	–	40
cicletanine enantiomers	plasma, urine	CD-MECC	EXI, H-EXI	–	41
haloperidol (various metabolites)	urine	CZE-MS	EXI	–	42

Abbreviations: DSI: direct sample injection; EXI: extract injection; H-EXI: sample hydrolysis prior to extraction; H-PP: hydrolysis and protein precipitation; UF: ultrafiltration; CD: cyclodextrin; H: sample hydrolysis.

1–2, Refs. 22–42). Electrokinetic capillary technology is currently in the process of becoming applied to drug monitoring in routine laboratories.[43] This chapter provides a brief review on drug monitoring by CE. The review is restricted to CZE and MECC separations of drugs and metabolites in body fluids, particularly serum (plasma), saliva, and urine (Tables 1–1 and 1–2). It does not deal with the determination of endogenous compounds, such as creatinine and uric acid,[44] as well as the diagnosis of human diseases via monitoring of metabolic disorders,[45] nor does it review the innumerable papers reporting the use of this emerging technology for the analysis of drugs in pharmaceutical preparations.[10,46] Also, applications employing capillary isotachophoresis are not covered and interested readers are referred to the monograph of Boček et al.[47]

II. IMPORTANT ASPECTS FOR DRUG MONITORING

A. SAMPLE PREPARATION

Upon receipt of a sample, typically it has to be prepared for analysis. This stage is intended to improve specificity of the assay by removing interfering matrix compounds while concentrating the analyte, to stabilize the analyte, as well as to remove matrix particles which would block instrumental parts (e.g., capillary tubing, etc.). Sample preparation methods include simple liquid handling procedures (e.g., centrifugation, dilution, and filtering), release of the analyte from the biological matrix (e.g., hydrolysis and sonication), the removal of endogenous compounds (e.g., precipitation, ultrafiltration, and extraction), and the enhancement of selectivity and sensitivity by analyte derivatization (for a comprehensive general review refer to McDowall[48]). For CE of drugs in body fluids all of these techniques as well as direct sample injection (Figures 1–2 and 1–3) have

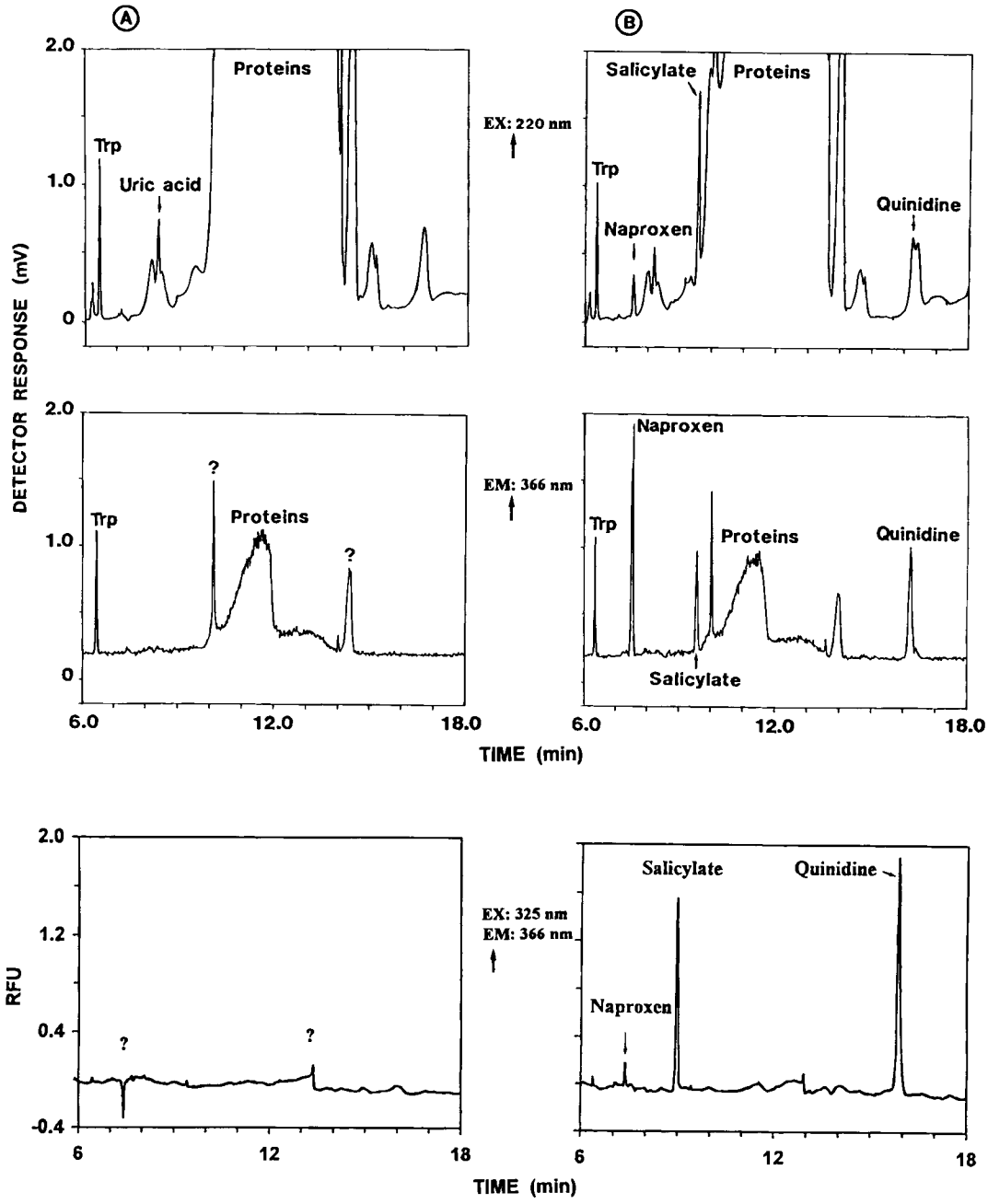


FIGURE 1–2. MECC data obtained with different detection modes for direct injection of A) serum blank and B) serum blank spiked with naproxen, salicylate and quinidine and using a buffer composed of 75 mM SDS, 6 mM $Na_2B_4O_7$ and 10 mM Na_2PO_4 (pH ~ 9.2) in untreated fused-silica capillaries of 75 μm ID and 50 cm effective (distance between inlet and detector) length. Top and center panels represent simultaneously collected UV absorbance data measured at the excitation wavelength of 220 nm and fluorescence data obtained with a 366 nm bandpass filter, respectively, using a homemade instrumental setup with 70 cm total capillary length (described in detail elsewhere[58]) and drug spiking levels of 5, 75 and 13 μg/mL, respectively. Sample application occurred manually via gravity through lifting the anodic capillary end, dipped into the sample vial, some 34 cm for 2 s. The applied constant voltage was 20 kV. The data presented in the bottom panels were obtained with drug concentrations of 0.5, 7.5 and 1.5 μg/mL, respectively, and having LIF detection on the Beckman P/ACE System 5510 instrument which was interfaced with a 325 nm (10 mW) Liconix HeCd laser and a 366 nm bandpass filter. The total capillary length was 57 cm, injection was effected by application of pressure (0.5 psi) for 1 s and the applied voltage was 20 kV.

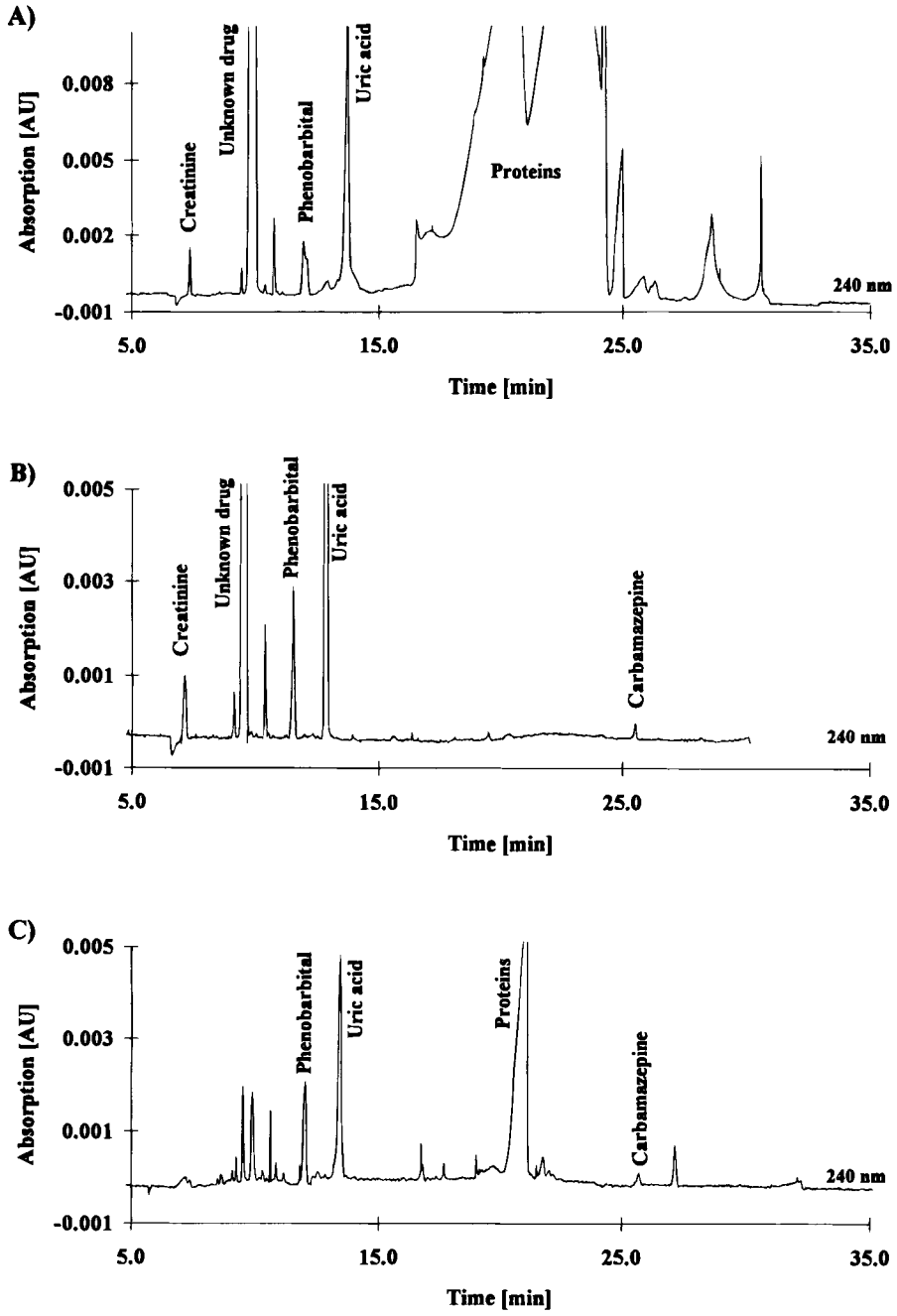


FIGURE 1–3. Single-wavelength (240 nm) MECC data of A) directly injected patient serum containing 23.9 μg/mL and 3.78 μg/mL of phenobarbital and carbamazepine, respectively, B) the ultrafiltered serum, and C) saliva of the same patient under multiple anticonvulsant drug therapy obtained in a homemade instrument featuring a 75 μm ID capillary of 70 cm effective (90 cm total) length.[51] Sample application was effected by gravity (see Figure 1–2) during 5 s. Buffer as for Figure 1–2. The applied voltage was a constant 20 kV (current about 80 μA).

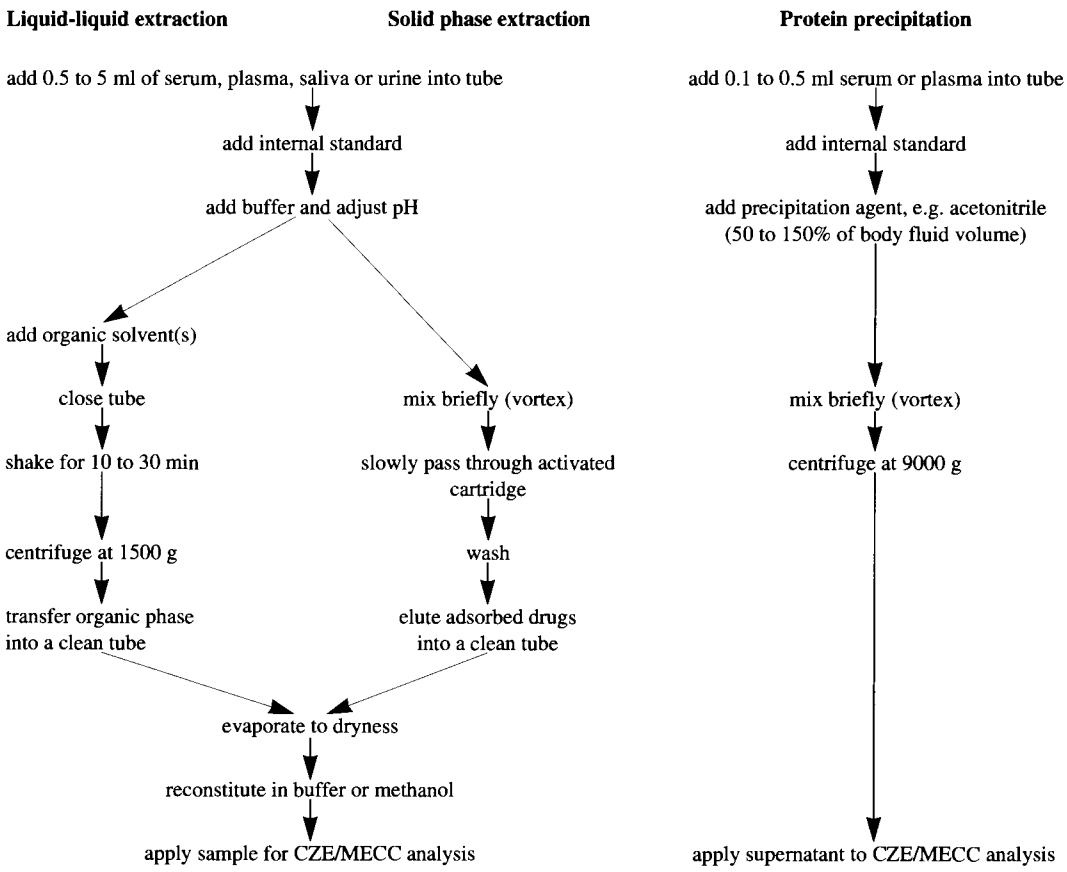
been applied. Selected examples are listed in Tables 1–1 and 1–2.

Both liquid-liquid and solid phase (solid-liquid) extraction schemes are attractive approaches for two reasons. Firstly, they are typically quite selective thereby greatly simplifying the sample matrix and secondly the analyte can simultaneously be concentrated by 1–2 orders of magnitude. Due to the

relatively high sample concentrations required for electrokinetic capillary analysis, the latter effect is very important. On the other hand, liquid-liquid and somewhat also solid-phase extractions are quite time consuming. Typical protocols are listed in Table 1–3. In most of the applications reported thus far, standard approaches were employed. Liquid-liquid extractions were executed in glass tubes and solid phase extraction was performed with disposable cartridges, processes which are standard practice in chromatography.[48] The in-column use of solid phase material for on-line preconcentration of the analyte[49] and the application of solid phase cartridges for electrochromatographic extraction[50] have also been described. Alternatively, the sample matrix can be simplified by protein precipitation (e.g., with acetonitrile, methanol or trichloroacetic acid) and injection of the supernatent after centrifugation. This approach is quick, simple and inexpensive (Table 1–3). It provides, however, diluted samples and is thus only applicable to high concentrations and/or with highly sensitive detection.[18] Furthermore, ultrafiltration can be employed to remove proteins (Figure 1–3B), a method which leads to the determination of the free (unbound) fraction of a drug. The successful use of disposable cartridges which can be put into a conventional laboratory centrifuge for the determination of drug intoxications has been reported.[23,51]

One interesting and appealing feature of electrokinetic capillary techniques is the possibility of directly injecting a tiny amount of a body fluid onto the capillary at one end and detecting drugs and metabolites as they pass an on-column absorbance or fluorescence detector placed towards the other capillary end (Figure 1–2). Depending on the capillary used, their entire volumes are between 0.1 and 5 µL, and the sample plugs applied are in the 1–10 nL range. Thus, this technology truely allows

TABLE 1–3
Typical Flow Diagrams for Sample Preparation



drug determinations to be performed in nanoliter samples of body fluids. However, although sampling from 0.1 μL[52] and single cells[53] has been described, current commercial instrumentation employs vials which typically require between 20–200 μL of sample. MECC with dodecyl sulfate was shown to allow the direct injection of proteinaceous fluids, such as serum, plasma or saliva, an approach which bears great similarity to high-performance liquid chromatography (HPLC) with micellar mobile phases.[54] The proteins are solubilized by dodecyl sulfate and elute (as a very broad zone) after uric acid. Drugs which elute outside the solubilized proteins can be recognized by UV absorbance detection.[55,56] This is illustrated with the data presented in the top graphs of Figures 1–2B and 1–3. Fundamental work on MECC with direct injection of proteinaceous material has been predominantly done with serum and plasma samples.[13,57] Strategies for proper buffer selection have been established,[51,57] the impact of physico-chemical drug properties on peak shape have been elucidated,[13] the fact that total rather than partial drug concentrations are determined has been experimentally proven,[13] and the advantages of using simultaneous absorption and fluorescence detection have been discussed.[58] Furthermore, fully validated serum assays for phenobarbital, ethosuximide, flucytosine, antipyrine, felbamate, and theophilline have been published (Table 1–1). Similarly, drugs can be analyzed in ultrafiltered serum (Figure 1–3B), saliva[16,33] (Figure 1–3C) and also in urine (Figure 1–4).[25,35,36,59] Comparison of the pherograms shown in Figure 3 reveals a number of interesting points. First, removal of the serum proteins allows the determination of free carbamazepine (panel B), a compound which could not be detected after direct serum injection (panel A). The same applies for the experiment with direct injection of saliva (panel C). Second, peak shapes of certain compounds, such as phenobarbital and uric acid,[13] and detection times[51] are shown to be sample matrix dependent, the latter aspect calling for solute identification by multiwavelength detection.[13] For detection by on-column absorbance, direct injection of a body fluid requires that

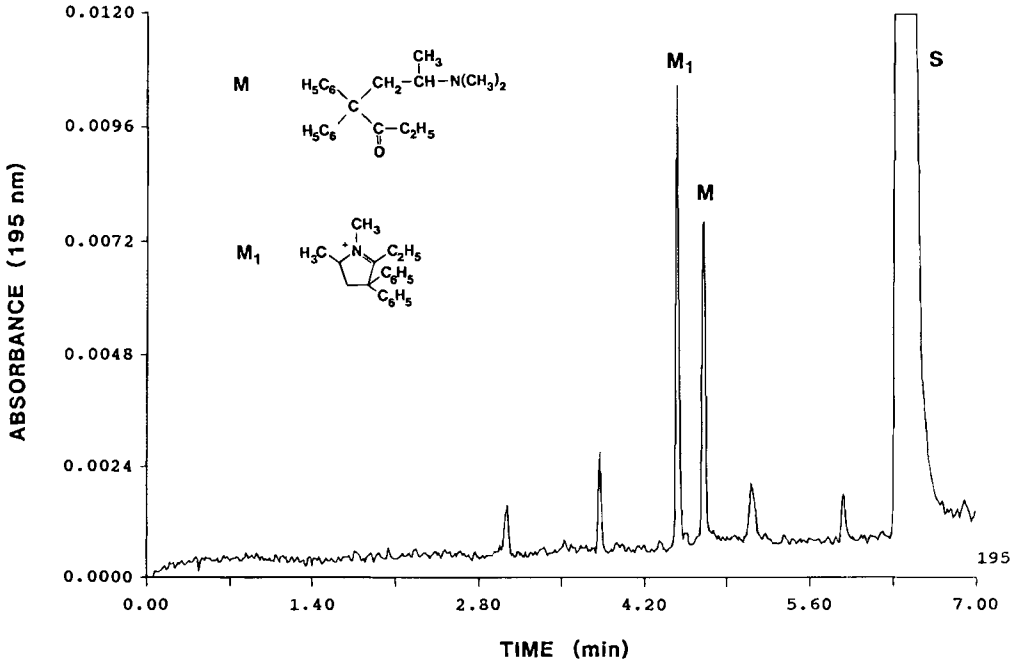


FIGURE 1–4. CZE data at 195 nm for methadone (M, ~16.5 μg/mL) and its primary metabolite (M_1, ~19.9 μg/mL) obtained after direct injection of a patient urine which tested positively for methadone using EMIT. The data were generated on the Bio-Rad's BioFocus 3000 apparatus which featured an untreated 50 μm ID capillary of 50 cm total length (45 cm to the detector) and having a 50 mM tetraborate buffer (pH 8.9). Pressure sample injection was effected by application of 5 psi/s, the temperature of the cartridge was maintained at 20°C and the applied voltage was 15 kV (current about 39 μA). For more details refer to Ref. (25).

drug concentrations be at or higher than the μg/mL (μM) level.

B. SAMPLE INTRODUCTION, STACKING, AND DETECTION

A small aliquot of sample (typically a few nL) is introduced by hydrodynamic techniques via short time application (typically 1–10 s) of: 1) gravity provided by a height differential between the fluid levels in the sample vial and the buffer vial at the column end, 2) pressure at the inlet side of the capillary, or 3) vacuum at the outlet side and having the capillary inlet immerced into the sample vial. For electrokinetic sample injection, high voltage is applied between the electrodes in the sample vial and the outlet vial (Figure 1–1). With short time application of power and without electroosmosis, either positively or negatively charged solutes are electrophoretically introduced by a bias based upon differences in electrophoretic mobilities. Furthermore, in presence of electroosmotic pumping towards the capillary outlet, a small amount of sample solvent (nL quantity) with uncharged and oppositely charged solutes is inserted as well. Aspects of the two sampling approaches are illustrated with the data presented in Figure 1–5 which originate from cationic CZE experiments performed in a fused-silica capillary filled with a citrate buffer at pH 2.5. Almost no differences in responses were observed with hydrodynamic sampling (application of vacuum for 2 s) and dissolution of the three dipeptides in the citrate buffer or water (right electropherograms). It should be noted, however, that for the sample in water and longer injection times, appreciable sample stacking and somewhat higher peaks would be expected (see below). Having electrokinetic injection at 5 kV for 4 s, a strong difference in injection between the two samples was noted (left electropherograms). When the solutes are dissolved in water, the compound with highest mobility (1) and an impurity (I) are much more strongly injected compared to dipeptides 2 and 3.

Electrophoretic mass transport is highly regulated, thus allowing charged solutes to be concen-

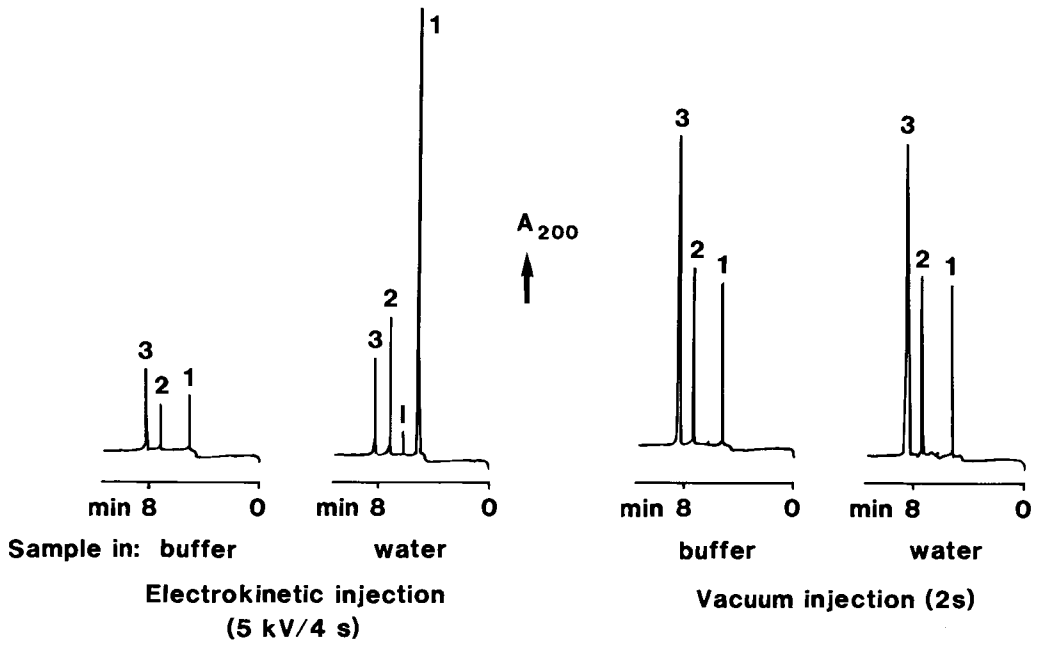


FIGURE 1–5. CZE data of three cationic dipeptides obtained with electrokinetic (left electropherograms) and vacuum injection at 5 inchHg (right electropherograms) and dissolving the analytes in running buffer (left graphs in each group) and water (right graphs). A 20 mM citrate buffer (pH 2.5) was employed. The data were generated using the ABI 270A capillary electrophoresis instrument equipped with an uncoated fused-silica capillary of 50 μm ID and 50 cm effective (70 cm total) length. The applied voltage was a constant 30 kV (current about 36 μA), the temperature was set at 35°C and solute detection was effected at 200 nm. Key: (1) L-his-L-phe (~0.3 mM)), (2) D-ala-D-phe (0.4 mM) and (3) L-phe-L-asp (1.0 mM), (I) impurity.

trated (stacked) across an electrolyte discontinuity,[8] including that produced initially between sample and running buffer. In CE, this inherent and exclusive feature of electrophoresis may take place when the sample compounds encounter isotachophoretic concentration (isotachophoretic sample stacking is based upon differences in electrophoretic mobilities)[8,60] or when the conductivity of the sample is smaller than that of the buffer (field amplified sample stacking).[8,61] After hydrodynamic sample introduction, stacking techniques are not only dependent upon sample composition, but also on the sample volume injected and thus limited by the capillary volume. Experimentally determined enhancement factors associated with these in-column stacking techniques typically do not exceed 100. Head-column field amplified sample stacking (also referred to as field amplified sample injection)[62] associated with electrokinetic sample introduction takes place at the tip of the column and has no limited injection volume. It is best performed with a sample of low conductivity (Figure 1–5, left electropherograms) and a short plug of water at the capillary inlet. During electroinjection, analytes are stacked at the interface between the low-conductivity zone and the running buffer. Furthermore, little sample solvent is co-injected because the net electroosmotic velocity is typically much smaller than local electrophoretic transport. Using this approach, a 1000-fold sensitivity enhancement can easily be obtained.[63] Thus, detection limits in CE are not only dependent on the type of detector used, but also on the matrix of the sample and the injection procedure employed.

For drug determinations by CE, UV absorbance detection is currently the most popular method,

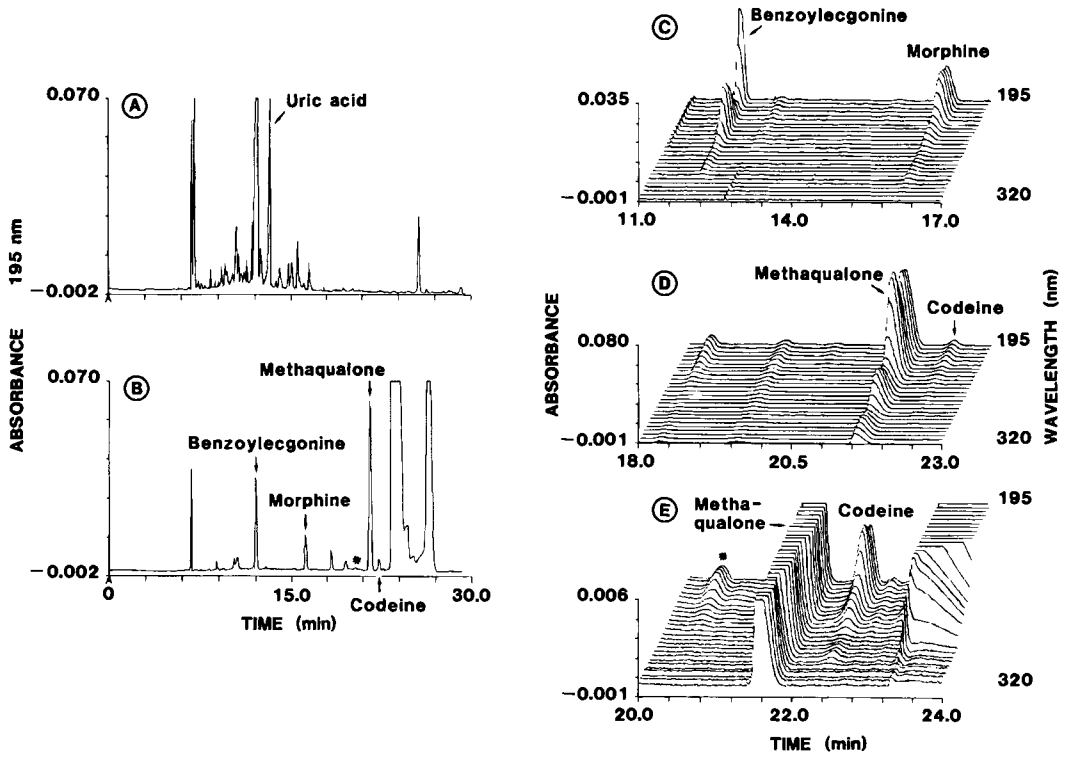


FIGURE 1–6. MECC analysis of an opiate and cocaine-positive urine specimen A) via direct injection, and B) after Bond Elut Certify solid-phase extraction using a homemade instrument with an on-column, fast scanning multiwavelength absorption detector. The electropherograms depicted in panels C to E represent sections of the three-dimensional data obtained with the cleaned-up sample. Data of panels B–E all stem from the same experiment. The detector was employed in the high speed polychrome mode by scanning from 195 to 320 nm at 5 nm intervals (26 wavelengths). Other instrumental configurations are the same as for Figure 1–3. The peak marked with an asterisk represents 6-acetylmorphine, a metabolite of heroin. *Source*: Adapted and reprinted from Wernly and Thormann, Analysis of illicit drugs in human urine by micellar electrokinetic capillary chromatography with on-column fast scanning polychrome absorption detection, *Anal. Chem.*, 63, 2878, 1991.

particularly in the approach of scanning multiwavelength monitoring, with which eluting zones are characterized by both retention and absorption behavior (Figure 1–6).[22,27,33] Because of the short optical pathlength within the detection cell, the lowest detectable concentration in CE (without preconcentration of solutes) with UV absorption detection is in the 1–10 μM range. This concentration sensitivity is 1–2 orders of magnitude worse than that encountered in HPLC.[64] By designing a Z-shaped or a multireflection detection cell to increase the pathlength, about a tenfold improvement is obtained. However, any path extension along the capillary axis is accompanied by a loss in column efficiency. Other detection techniques reported in the literature include fluorescence (F), laser induced fluorescense (LIF), MS and conductivity.[4,5] Compared to UV absorption detection, sensitivity enhancement when using F and LIF for drug detection are about 10 and up to 1000-fold, respectively. Furthermore, these two detection modes provide increased selectivity which is useful for identification of solutes (Figure 1–2). For the analysis of drugs in body fluids and using preconcentration via sample extraction and/or stacking, detection limits can be improved up to 1000-fold, this leading to a sub ng/mL solute detection capability. Thus, off-line or on-line sample concentration is an important aspect for drug monitoring by CE. Furthermore, hyphenation of CE with mass spectrometry (CE-MS) is an attractive approach for providing the solute's structural information,[42] this being particularly important for the assessment of drug metabolism and positive identification of illicit and banned drugs and/or their metabolites.

C. QUANTITATION, REPRODUCIBILITY, AND QUALITY CONTROL

In many cases of drug monitoring, the response has to be quantified after analysis. In CZE and MECC, peak height measurements and integration can be executed with a stand alone integrator or using integration software on a PC, both developed for the evaluation of chromatograms. Calibration graphs using bovine plasma, human serum, saliva or urine spiked with the drugs showed good linear correlations when assessed over a concentration range of not more than 2–3 orders of magnitude. Quantitation is typically performed by single or multi-level internal calibration using peak heights or peak areas,[11] and by running the samples only once.[11,15] For analysis by direct injection of the body fluid, no internal standard has to be included and single- or multi-level external calibration can be employed. Intraday and interday imprecisions are on the 1–5% and 2–8% levels, respectively.[43] Using peak heights as the basis for quantitation, RSD values are typically a bit lower compared to those obtained with peak areas. Moreover, high quality data obtained by CE has also been manifested via analysis of external quality control samples.[12,43]

III. ACHIEVEMENTS AND FUTURE OUTLOOK

A. VALIDATED ASSAYS

Monitoring drugs in body fluids using CZE or MECC has several areas of interest, the most important being 1) the determination of specific drugs or metabolites for therapeutic, diagnostic or research reasons and 2) the confirmation of drugs of abuse and/or their metabolites in a specimen which tested positively employing a routine screening procedure. Electrokinetic capillary technology is currently in the process of becoming applied to drug monitoring in routine laboratories. A survey of validated CE assays dealing with real samples is therefore presented. Papers describing the applicability of CE for drug monitoring employing blank body fluids spiked with drugs are mentioned only when necessary for comprehensiveness (for a more general review refer to Ref. 6). A list of selected assays for which drug levels measured by CZE or MECC have been compared to those obtained by non-isotopic immunoassays and/or a chromatographic method is presented in Table 1–1. Highly linear correlations of comparative drug levels ($r > 0.96$), small y-intercepts and slopes close to unity are noted. Furthermore, biases and RSD values were determined to be smaller than 7.4% in relation to a drug concentration in the center of the therapeutic or calibration ranges.[43] Thus, for clinical purposes drug levels obtained by CE agree sufficiently well with those of the reference assays and CE assays can therefore be employed instead of HPLC or non-isotopic immunoassays based

upon EMIT and FPIA technology.

CE protocols for screening and confirmation of abused drugs and their metabolites in human urine have been developed (Table 1–2). Data obtained after MECC analysis of a patient urine which was found to be markedly positive for cocaine and opiates using EMIT assays are presented in Figure 6. Direct urine injection (panel A) provided a complex electropherogram in which uric acid (convenient endogenous marker of a body fluid) could easily be identified. No peak, however, could be assigned to one of the illicit compounds of interest. In MECC with on-column UV absorption detection sample concentrations have to be at least on the μg/mL (μM) concentration level. For direct urine injection, this sensitivity limit is not as good compared to that of the commonly used immunological screening methods. Therefore, extraction of the drugs or their metabolites remains to be essential for their confirmation by MECC. Panel B of Figure 6 depicts single-wavelength data obtained after Bond Elut Certify extraction of the same patient urine using the procedure for cocaine and metabolites.[27] A much smaller number of peaks is detected despite that with this approach and 100% recovery a 50-fold concentration of the extracted compounds can be achieved. Having data between 195 and 320 nm, as shown in panels C to E, as well as reference spectra, permitted a quick and reliable confirmation of the presence of benzoylecgonine (cocaine metabolite), morphine, codeine, and methaqualone in that sample (Figure 1–7). The former three compounds were found to extract with efficiencies of about 50, 60 and 90%, respectively, and spiking of urine blank on the 200 ng/mL level still provided small peaks which could be unambiguously assigned. Using calibration graphs based on peak heights (linear, four-point calibration with standards between 0.25 and 10 μg/mL), the concentrations of benzoylecgonine, morphine and codeine in that patient urine were estimated to be 6.5, 4 and 1 μg/mL, respectively. It is important to add that these data were obtained without hydrolysis and derivatization of the sample. Furthermore, the peak marked with an asterisk (panels B and E of Figure 1–6) could be identified as 6-acetylmorphine, a specific metabolite of heroin. Thus, these MECC data confirm the presence of opiates and benzoylecgonine in this urine specimen as well as the heroin and cocaine consumption of this individual.

The most comprehensive protocol elucidated thus far for the screening and confirmation of illicit and abused drugs uses urine pretreatment via a two-step solid-phase extraction with a copolymeric sorbent which provides, from the very same aliquot of urine, barbiturates, some benzodiazepines, THC and methaqualone in the first eluate, as well as opioids, cocaine and benzoylecgonine, selected benzodiazepines or their metabolites, methadone and its primary metabolite, diphenhydramine and amphetamines in the second eluate.[28,29] With the exception of methadone and its metabolite which have to be determined by CZE (Figure 1–4),[25] all these extracted compounds can effectively be analyzed by MECC. Compared to GC-MS, electrokinetic capillary instrumentation is less expensive and somewhat simpler to operate. However, in addition to the structural information provided by MS, the detection sensitivity in the customarily used GC-MS is higher than that of MECC and CZE with on-column UV absorption detection. Nevertheless, for most compounds, the sensitivity of the MECC and CZE assays are equal or better than those of commercial immunoassays which are typically employed for rapid urine screening. Furthermore, CE assays were determined to be capable of recognizing false-negative and false-positive results from an immunological screening process.[26] Based upon these developments, a concept of an automated capillary analyzer featuring sample cleanup, multiple capillaries for simultaneous analysis of the fractions in one or several buffers and computer-aided peak identification has been proposed.[29]

CZE and MECC have also been shown to comprise rapid, inexpensive and highly efficient analytical methods for phenotyping in man via analysis of urinary dextromethorphan and dextrorphan,[37] urinary caffeine metabolites 5-acetylamino-6-formylamino-3-methyluracil and 1-methylxanthine,[35] four caffeine metabolites in urine,[34] urinary 4-hydroxymephenytoin and mephenytoin,[38] as well as for combined phenotyping with mephenytoin, dextromethorphan and caffeine via analysis of deglucuronidated urine by MECC.[36] These pharmacogenetic assays have been validated by customary HPLC methods. Furthermore, CE has been demonstrated to be a very attractive approach for the resolution and determination of enantiomers, including chiral separations in

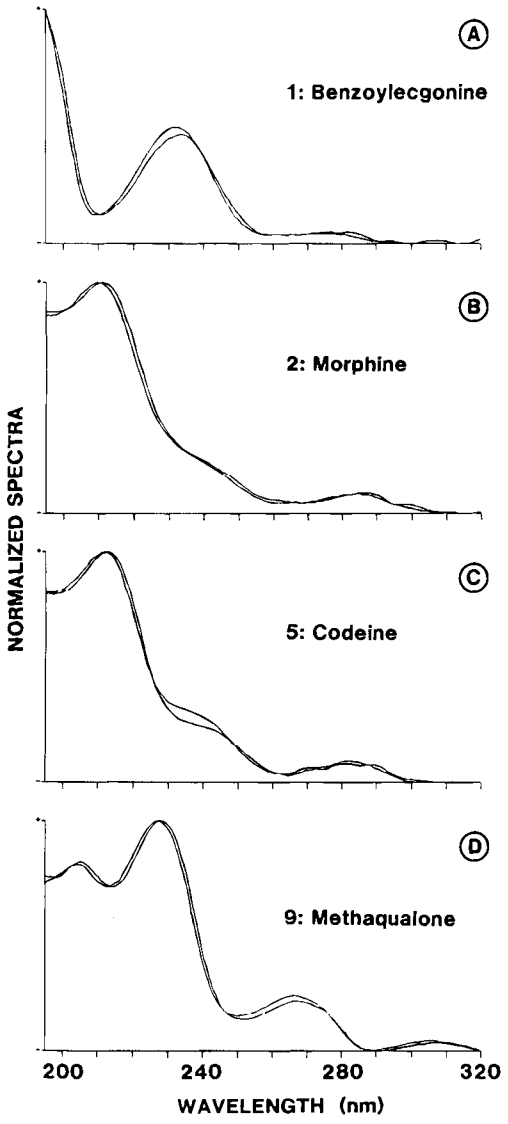


FIGURE 1–7. Normalized spectra (time slices through selected peaks) of A) benzoylecgonine, B) morphine, C) codeine, and D) methaqualone of the data of Figure 1–6 in comparison to those extracted from a run performed with standards. *Source*: Reprinted with permission from Wernly and Thormann, Analysis of illicit drugs in human urine by micellar electrokinetic capillary chromatography with on-column fast scanning polychrome absorption detection, *Anal. Chem.*, 63, 2878, 1991.

body fluids by cyclodextrin-modified CZE[40] or cyclodextrin-modified MECC.[38,41] It not only permits the quantitation of a single enantiomer, but also provides insight into the stereoselectivity of drug metabolism, such as the stereoselective hydroxylation of mephenytoin to S-4-hydroxymephenytoin.[38] How straightforward enantiomer separations by CE can be performed is illustrated with the three-dimensional data presented in Figure 1–8. Barbiturates have been shown to separate well using MECC.[22] Employing the borate-phosphate buffer at pH 9.2 (Figures 1–2, 1–3 and 1–6) and adding gamma-cyclodextrin and (+)-campher-10-sulfonic acid (40 mM each) for chiral discrimination, enantiomers of thiopental and its metabolite pentobarbital in serum could be separated after liquid-liquid extraction using acidified dichloromethane (Figures 1–8B and 1–8C). Because of significant differences in absorption characteristics of the two compounds of interest, multiwavelength solute detection is a very convenient approach for that case. The serum sample whose MECC data are presented in Figure 1–8C was from a patient under racemic thiopental pharmacotherapy. The total levels of thiopental and pentobarbital assessed by HPLC were determined to be 14.0 and 7.2 μg/mL, respectively, and equal amounts of the enantiomers of each compound were found by cyclodextrin MECC. Thus, the MECC data presented in Figure 1–8C suggest that there is no stere-

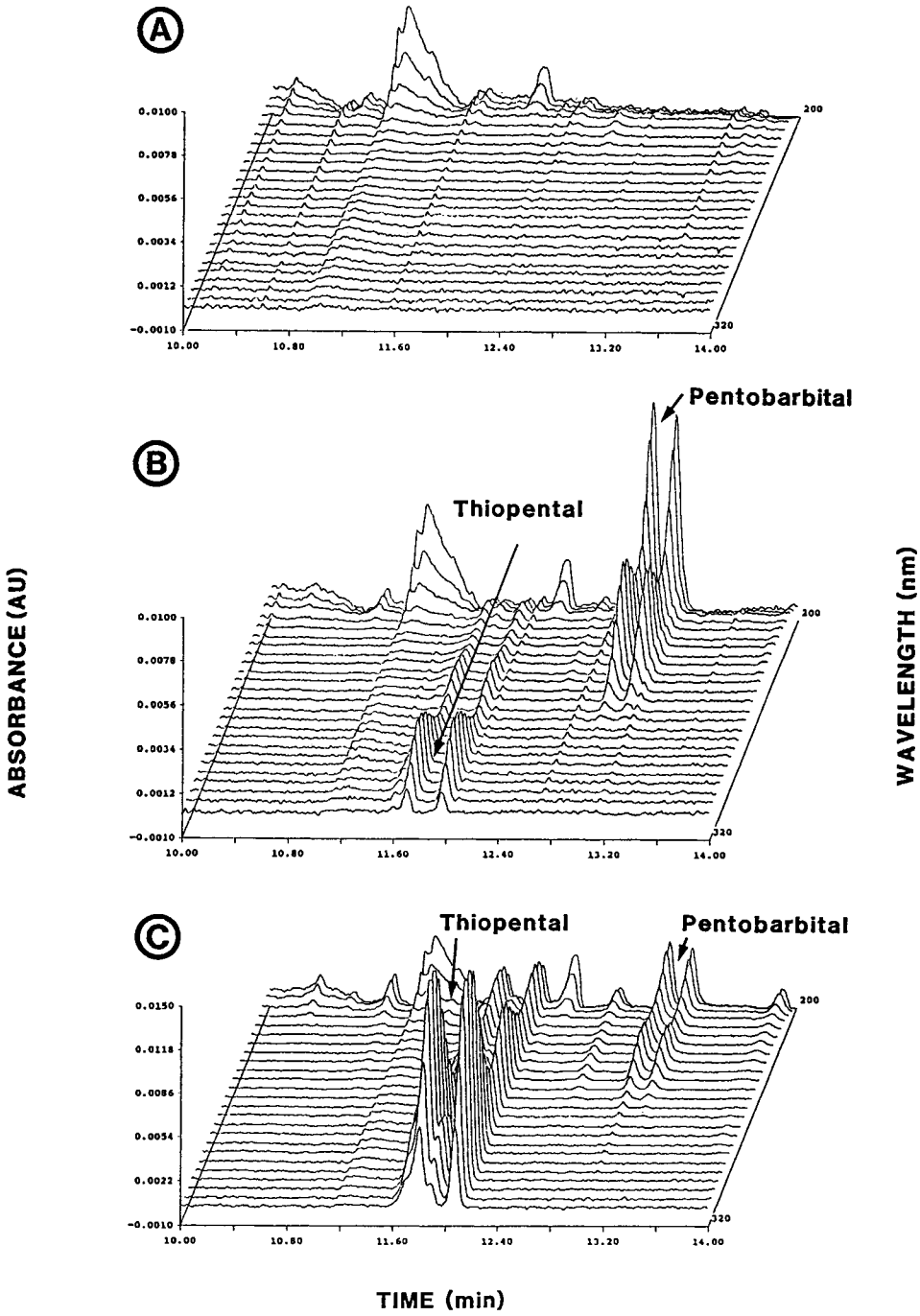


FIGURE 1–8. MECC separation of thiopental and pentobarbital enantiomers after liquid-liquid extraction with acidified dichloromethane from serum. Data shown were obtained with A) serum blank, B) serum blank spiked with racemic thiopental (25 µg/mL) and racemic pentobarbital (30 µg/ml) and C) a serum of a patient under thiopental pharmacotherapy. The Bio-Rad's BioFocus 3000 apparatus in the polychrome detection mode (200 to 320 nm at 5 nm interval) and an untreated 50 µm ID capillary of 50 cm total length (45 cm to the detector) were employed. The buffer was the same as for Figure 1–2 but complemented with 40 mM gamma-cyclodextrin and 40 mM (+)-campher-10-sulfonic acid (pH 9.2). Pressure sample injection was effected by application of 4 psi*s, the temperature of the cartridge was maintained at 20°C and the applied voltage was 15 kV (current about 41 µA). *Source*: From A. Schmutz, PharmD dissertation, University of Bern, Switzerland, 1994.

oselectivity involved in the hepatic thiopental to pentobarbital conversion. MECC has also been shown to be an effective method to explore the metabolism of dihydrocodeine via analysis of its urinary metabolites.[39] All this work on drug metabolism has been executed with UV absorption detection (mostly in multiwavelength fashion as employed for drug confirmation depicted in Figures 1–6 and 1–8). Fluorescence detection, on the other hand, has been used to selectively monitor metabolites.[58] Furthermore, capillary electrophoresis interfaced to mass spectrometry (CE-MS) has been demonstrated to be an attractive approach for the assessment of drug metabolism.[42] The structural information gained in CE-MS could also be used for confirmation of the presence of illicit, abused, and banned drugs in body fluids.

B. PROS AND CONS OF CE METHODS

All of the analytical techniques employed for drug monitoring have advantages and disadvantages. The reagents for many of the immunological assays are available in kit form, together with highly automated instrumentation. This permits such analyses to be performed easily and efficiently as well as with high sensitivity and precision. They provide the most rapid (high sample throughput) analytical procedures available to date. However, immunological techniques are prone to disturbances by molecules of similar structure (cross reactivity) and the availability of antibodies is limited to the most frequently measured drugs. Because of separation, the chromatographic assays provide specific results of multiple compounds but typically require extensive sample preparation and possibly also sample derivatization. The sample throughput is low because of sequential injection of the samples, and also complete automation of chromatographic protocols is rather difficult. Compared to HPLC, the advantages of electrokinetic capillary analyses are high resolution, efficiency and speed, minimal sample preparation, simple automation, small sample size, rapid and cost-effective method development, the use of small amounts of inexpensive and nonpolluting chemicals, and low cost of capillary columns. CE is complementary to automated immunoassays.

Electrokinetic capillary methods are nanoscale separation techniques performed in capillaries of 25–75 μm ID, the capillary and sample plug volumes being 0.1 to 5 μL and 1 to 10 nL, respectively. Furthermore, an interesting feature of MECC is the possibility of directly injecting serum or other proteinaceous fluids. On the other hand, the concentration sensitivity of CE is somewhat lower than in HPLC, this often calling for effective on-line or off-line preconcentration of analytes prior to analysis. Fortunately, electrophoretic techniques feature unique concentration effects (which are inherent to electrophoretic mass transport and very rarely seen in other separation techniques) providing formidable compensation for the low concentration sensisitivity.[63,64] Furthermore, solutes can be detected at 185 or 190 nm. Having absorption detection and no preconcentration, the limit of detection is in the low μg/mL (μM) range. Using preconcentration via stacking and/or extraction, ng/mL levels can be unambiguously monitored.[24,64] Moreover, having fluorescence detection, particularly laser induced fluorescence, a detection limit in the pg/mL is feasible. In CE, detection times can be dependent upon the sample matrix (Figure 1–3), thus making peak identification by detection times somewhat unreliable. Therefore, multiwavelength absorption detection (Figure 1–6 and 1–8), combined absorption/fluorescence detection (Figure 1–2), or for comprehensive structural information, hyphenation with mass spectrometry[42] should be employed for solute identification. In CZE and MECC, calibration graphs using blank body fluid spiked with the drugs show good linear correlations when assessed over a concentration range of not more than 2–3 orders of magnitude. Imprecisions for single determinations are on the 1–8% levels. Based on the data reported thus far, CE is shown to provide meaningful data of clinical and pharmacological interest. The quality of the data is assured such that this technology can be considered as suitable for therapeutic and diagnostic drug monitoring, screening and confirmation of illicit, abused and banned drugs, and exploration of drug metabolism. It is interesting to note, that drug analysis by CE is problably the most advanced application of CE in the clinical and forensic environment.[6]

CE is a low cost analytical methodology. Capillary columns (price range: $8 to about $100 per

meter with the plain fused-silica capillaries used in our laboratory being the cheapest) can be employed for hundreds to thousands of injections. Furthermore, buffer consumption for a specific assay is so low that, from an economical point of view, it does not add much to the expense of an assay. To the knowledge of the author, no ready made buffers for drug analysis are currently available through commercial sources. In our departmental drug assay laboratory, the determination of flucytosine serum levels is now routinely done by MECC with direct sample injection, an assay characterized with a throughput of up to 15 runs per hour.[14] Similar sample capacity was reported for the determination of naproxen.[57] However, with most other assays developed thus far, sample throughput is 3- to 5-fold lower.

C. NEW AND FUTURE HORIZONS

Not much explored and thus not discussed in this paper, but not less interesting, are new developments of the CE technology, including the performance of highly sensitive, homogeneous immunoassays for drugs,[65,66,67] the employment of on-line sample preparation,[49,64] and the design of fully automated multi-capillary analyzers.[29,68] For the widespread adoption of the electrokinetic capillary technology in routine laboratories, a number of improvements should be met. With the availability of instrumentation comprising multiple capillaries in parallel,[29,68] sample throughputs comparable to those obtained in automated immunoassays should be possible. Furthermore, the same goal will be reached with the availability of chip-based instrumentation, i.e., CE on a glass chip on which separation channels, a pL sample injector and solute detection are combined on an area of a few cm^2.[69,70] In that approach, fluid flow is driven electrokinetically through a network of intersecting small channels which have been fabricated on planar glass substrates by photolithographic masking and chemical etching techniques and have been formed by bonding the etched substrate to a plain glass plate. Capillaries of 30 to 70 μm width, about 10 μm height and a few cm length have been shown to provide analytical runs of a few seconds only. Thus, comprehensive, automated chip-based analyzers for high-throughput, low-cost monitoring of drugs in body fluids could be developed.

A praiseworthy aspect of capillary electrophoresis (CE) is its high mass sensitivity which is based upon the small (pL to nL) injection volume used. Although special injection procedures from 0.1 μL of sample[52] or from a single cell[53] have been developed in researchers laboratories, sample vials typically holding hundreds of μL sample are employed with both conventional and chip-based instrumentation. With current commercial systems, a minimum of 10 to 20 μL sample is required, thus permitting the analysis of samples which are already quite small to be pretreated, including serum samples of prematurely born infants,[33] droplets of tears, sweat, etc. Further commercial developments will lead to the application of even smaller sample volumes, such as those required for drug monitoring in submicroliter sample volumes, including those encountered in single cells and after collection of airway surface fluid.

All data gathered thus far are very encouraging and demonstrate the high potential of electrokinetic capillary methods for analysis of drugs in body fluids. Although first routine applications have been reported, further investigations and developments are needed for widespread adoption of CE as routine methodologies in a drug assay laboratory. Important areas include 1) the development of high-throughput, inexpensive instrumentation with reliable automated operation over long periods of time (overnight), 2) the establishment of automated data evaluation and recognition of unexpected interferences and capillary fouling, 3) the elucidation of optimized protocols and 4) the commercialization of protocols, reagent kits and clinical analyzers, such as that proposed for toxicological drug screening and confirmation.[29]

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Chapter 2

TOXICOLOGY THROUGH A LOOKING GLASS: STEREOCHEMICAL QUESTIONS AND SOME ANSWERS

Irving W. Wainer

CONTENTS

I. INTRODUCTION

The victim lay dead in the living room—the only clue, a bottle of cough medicine. It is up to Dr. Kay Scarpetta, Chief Medical Examiner of Richmond, Virginia, to solve the mystery, and the answer lies in a three-dimensional understanding of pharmacology and toxicology. Dr. Scarpetta reveals the solution to Police Lieutenant Marino in the following manner:

> "The active ingredient in the cough suppressant we found in Miss Harper's bathroom is dextromethorphan, an analogue of codeine. Dextromethorphan is benign unless you ingest a tremendous dose. It's the *d*-isomer of a compound, 3-methoxy-N-methylmorphinan.
>
> There's another drug that is the *l*-isomer of this same compound that dextromethorphan is the *d*-isomer of. The *l*-isomer compound is levomethorphan, a potent narcotic about five times stronger than morphine. And the only difference between the two drugs as far as detection goes is that, when viewed through an optical rotatory device called a polarimeter, dextromethorphan rotates (plane polarized) light to the right and levomethorphan rotates (plane polarized) light to the left."
>
> "In other words without this contraption you can't tell the difference between the two drugs," Marino concluded.

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> "Not in the tox tests routinely done. Levomethorphan comes up dextromethorphan because the compounds are the same. The only discernible difference is they bend (plane polarized) light in opposite directions. . . ."[1]

With knowledge of stereochemistry and a polarimeter, Dr. Scarpetta solved an apparently insolvable death; Miss Harper committed suicide using levomethorphan.

While Dr. Scarpetta's intellectual approach is cutting edge, her analytical methodology leaves a great deal to be desired. Over the past 15 years, analytical chemists have developed rapid and accurate methods for the identification and measurement of enantiomeric compounds. The key technological advance was the creation of commercially available chiral stationary phases (CSPs) for high-performance liquid chromatography (HPLC-CSPs) and gas chromatography (GC-CSPs). In the past few years, the techniques of enantioselective chromatography have also been extended to capillary electrophoresis (CE).

Today, every pharmacological and toxicological laboratory should be able to carry out enantioselective chromatographic separations. These are keys to solving many "unsolvable" questions and to unlocking many of the "mysteries" surrounding efficacious and toxic clinical responses. A brief overview of stereochemistry in pharmacology and some examples of the application of this point of view to clinical questions are presented below. They are offered with the hope that we may all become as sharp and as "three-dimensionally" aware as Dr. Scarpetta.

II. WHY PHARMACOLOGISTS AND TOXICOLOGISTS SHOULD KNOW THEIR RIGHT HAND FROM THEIR LEFT

With the separation of the enantiomorphic crystals of ammonium sodium tartrate, Louis Pasteur initiated the study of the physical and chemical properties of asymmetric molecules.[2] His observations led him to formulate a proposal which is the foundation of molecular stereochemistry: "the optical activity of organic solutions is determined by molecular asymmetry, which produces non-superimposable mirror image structures."[3]

Pasteur can also be credited with the initial observations of biological differences between enantiomorphs. In 1858, he reported that the *dextro* form of ammonium tartrate was more rapidly destroyed by the mold *Penicillium glaucum* than the *levo* isomer,[4] Pasteur's work led him to the recognition of the role stereochemistry plays in the basic mechanisms of life. He wrote:

> Most natural organic products, the essential products of life, are asymmetric and possess such asymmetry that they are not superimposable on their images . . . This establishes perhaps the only well-marked line of demarcation that can at present be drawn between the chemistry of dead matter and the chemistry of living matter.[3]

During the next 50 years, there were a number of additional reports of stereochemical differences in biochemical properties. The initial pharmacological observations are credited to Abderhalde and Müller, who, in 1908, described the differential pressor effects of (–)- and (+)-epinephrine.[4] With this report, chirality entered mainstream pharmacological research and by the 1930's, Cushny[5] and Easson and Stedman[6] had laid the basis for the initial theoretical understanding of stereochemical differences in pharmacological activity.

A. ENANTIOMERIC DIFFERENCES IN PHARMACOLOGICAL AND TOXICOLOGICAL PROPERTIES

Enantiomers are molecules which have the same chemical composition and only differ from one another in their three-dimensional structure. Enantiomers are related to each other as non-superimposable mirror images in the same manner as your right and left hands (thus the term "chirality" which is derived from the Greek word for hand "*chiros*").

Under normal conditions, enantiomers have the same physicochemical properties, e.g., melting point, solubility, and vapor pressure. However, when you place a pair of enantiomers in an asymmetric or chiral environment, each enantiomer interacts with this environment in a different way. This difference can be visualized by substituting right and left hands (which are nonsuperimposable mirror images) for the enantiomeric molecules and a left-handed glove for the chiral environment. The interaction (or fit) of the hand and glove is different for the two enantiomeric hands. Thus, the fact that enantiomers have different pharmacological and toxicological effects should not be surprising.

Living organisms contain asymmetric environments such as proteins, enzymes and receptors. These components are chiral compounds made from enantiomeric subunits such as L-amino acids, D-sugars, etc. Therefore, when a chiral compound interacts with the body, it is possible that the enantiomers will vary in their interaction with the various chiral environments producing differing responses. An example of this is the olfactory receptors in the nose which can differentiate between the enantiomers of the molecule carvone; (–)-carvone smells like spearmint, whereas (+)-carvone has the odor of caraway, Figure 2–1.

The different olfactory responses to the enantiomers of carvone are a reflection of the pharmacological differences between the stereoisomers of a chiral drug. Enantiomers usually differ in their pharmacokinetics, pharmacodynamics and toxicity, and these differences are often dramatic; vide supra Dr. Scarpetta's revelations regarding dextro- and levomethorphan. The possible efficacy and toxicity relationships between the enantiomers of a chiral drug are as follows:

1. The enantiomers have identical efficacy and toxicity.
2. The enantiomers have the same therapeutic and toxic effects but differ in the magnitude of these effects.
3. One enantiomer possesses all the pharmacological activity whereas the other is essentially biologically inactive.
4. Both enantiomers are pharmacologically active but with qualitatively different therapeutic and toxic effects.

The first possibility, where the enantiomers of the drug have the same efficacy and toxicity, is not the usual situation. However, several examples can be cited including the enantiomers of flecainide, an antiarrhythmic agent, which exert the similar electrophysiologic effects on the heart and the enantiomers of promethazine which have nearly equivalent antihistaminic properties and toxicity.[7]

It is more common for the enantiomers of a chiral drug to have the same therapeutic and toxic effects but to differ in the magnitude of these effects. An example of this phenomenon is the hypoprothrombinemic effect of (S)-warfarin, which is two to five times more potent than (R)-warfarin.[8] Some other examples are presented in Table 2–1.

(R)-CARVONE
OIL OF SPEARMINT

(S)-CARVONE
CARAWAY

FIGURE 2–1. The effect of stereochemistry on carvone fragrance. (The nose knows!)

TABLE 2–1
Enantiomeric Differences in Potency

Drug	Relative activity: Biological response	Relative activity: Enantiomer with greater activity	Ratio of enantiomer activities
Terbulatine	Trachea relaxation	(–)	3000:1
Propranolol	Isoprenaline blockade	(S)	100:1
Amosulalol	α_1-Adrenoreceptor blockade β_1-adrenoreceptor blockade	(–)	60:1
Warfarin	Anticoagulation	(S)	2–5:1
Verapamil	Negative dromotropic effect	(–)	11:1

Enantiomeric differences in potency often arise from the fact that while both isomers fit into the same receptor, one fits better than the other and elicits a stronger response. This situation has been generalized in Ariens' development of "Eudismic ratios".[9] This approach is based upon the observation that the more potent enantiomer (the eutomer) has a higher affinity for the target receptor than its less potent enantiomorph (the distomer). Using a series of structurally related compounds, one can construct eudismic ratios (affinity of the eutomer/affinity of the distomer), eudismic indices, and eudismic affinity quotients (EAQ). The EAQ reflects the relative binding stereospecificity within a series of chemically related chiral compounds and usually increases with the affinity on the eutomer. This effect is know as "Pfeiffer's rule".[9]

The situation which is most commonly found is when only one of the enantiomers possesses the desired pharmacologic activity and the other is considered essentially biologically inactive. However, as more research is done into the stereochemical aspects of pharmacology, it is becoming more apparent that both isomers are usually active with qualitatively and quantitatively different therapeutic and toxic effects, see Table 1–1. For example, (S)-propranolol possesses pharmacologic activity as a cardiovascular drug, the major therapeutic use of this compound, while (R)-propranolol is not effective in the treatment of hypertension.[10] However, (R)-propranolol is effective in the management of hypothyroidism and can be used in this therapeutic area without the fear of cardiotoxic side effects.[11]

B. ENANTIOMERIC DIFFERENCES IN PHARMACOKINETICS

The enantiomers of a chiral drug may also differ in their pharmacokinetics, metabolism and pharmacodynamic profiles. When this occurs, the administration of a racemic mixture creates potential problems particularly in the area of therapeutic drug monitoring. This is especially important when the desired pharmacological activity resides in only one of the stereoisomers.

An example of this situation is the monitoring of the plasma concentrations of leucovorin (LV), a reduced folate used in cancer chemotherapy. LV is administered as a mixture of two diastereomers, (6S)-LV and (6R)-LV. The (6S)-isomer is the active form[12] and is rapidly converted to an active metabolite, 5-methyltetrahydrofolate (5-MTH).[12] The plasma half-lives of (6S)-LV, 32 min, and 5-MTH, 227 min, are significantly shorter than that of (6R)-LV, 451 min, leading to plasma concentration versus time profiles such as the one presented in Figure 2–2. It is clear from these curves, that if one monitors the plasma levels of LV without separation and quantification of (6S)-LV and (6R)-LV, the data will only reflect the plasma concentration of the inactive isomer.

C. HIGHLY SOPHISTICATED SCIENTIFIC NONSENSE

The implications of stereochemical differences in pharmacokinetics and pharmacodynamics have been summarized by Ariéns.[13]

> Often only one isomer is therapeutically active, but this does not mean that the other is really inactive. It may very well contribute to the side effects. The therapeutically non-active isomer

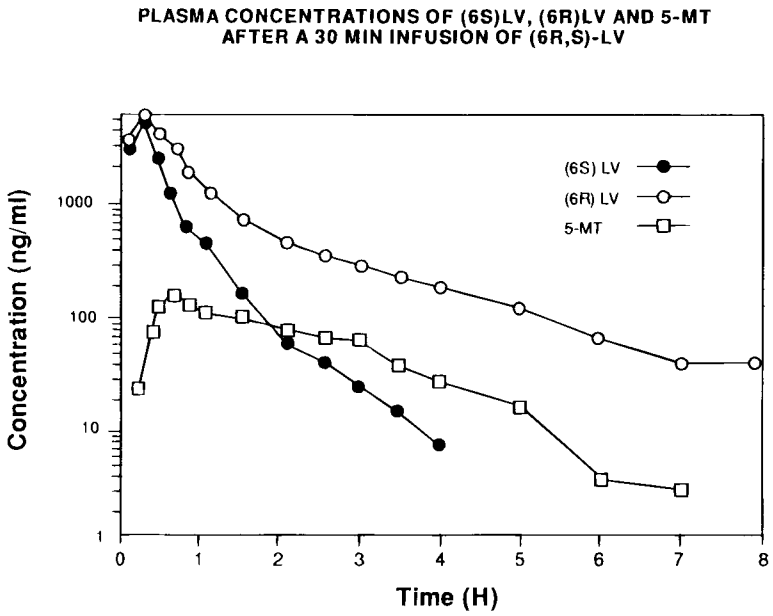

FIGURE 2–2. The serum concentration-time curves for (6S)-leucovorin, (6R)-leucovorin and 5-methyltetrahydrofolate following a 30-min infusion of 400 mg/m^2 of (6R,S)-leucovorin; where: ○ = (6R)-leucovorin, ● = (6S)-leucovorin, □ = 5-methyltetrahydrofolate.

in a racemate should be regarded as an impurity (50% or more). It is emphasized how in clinical pharmacology, and particularly in pharmacokinetics, neglect of stereoselectivity in action leads to the performance of expensive, highly sophisticated scientific nonsense.

To a great extent, "highly sophisticated scientific nonsense" has been produced because pharmacologists and toxicologists lacked adequate analytical techniques. This situation has dramatically changed over the past 15 years with the development of analytical methods capable of the rapid separation and accurate measurement of enantiomeric composition.

D. THE IMPACT OF NEW ENANTIOSELECTIVE CHROMATOGRAPHIC TECHNIQUES

The initial enantioselective resolution was Pasteur's magnifying glass and tweezers separation of the enantiomorphic crystals of ammonium sodium tartrate. Not satisfied with the tedious and tenuous nature of this separation, Pasteur searched for a chemical resolution of the tartaric enantiomers. The answer was the addition of a single-enantiomer natural occurring amine, quinicine or cinchonicine, to a solution of racemic tartaric acid.[2] The addition of either base resulted in the formation of a pair of distinct acid-base salts with unequal solubilities. The salts could be readily separated and when the free acid was regenerated, one salt yielded (R)-tartaric acid and the other (S)-tartaric acid.

Pasteur had converted the enantiomers of tartaric acid into diastereomeric salts through the addition of a second chiral center. In this case, the reaction of (R)- and (S)-tartaric acid with (S)-cinchonicine produced (R,S) and (S,S) salts. Diastereomers are stereoisomers which are not related to each other as non-superimposable mirror images and, unlike enantiomers, they have different physicochemical properties. The conversion of enantiomers to diastereomers is the basis for all current chemical separations of enantiomers.

Although the formation of diastereomeric salts remains one of the major routes for the large scale preparation of enantiomerically pure chemicals, it is not a viable analytical approach. Instead, chemists turned to the synthesis of diastereomeric derivatives which could then be separated by chromato-

graphic techniques. Thus, enantiomeric amines were converted to diastereomeric amides by reaction with a chirally pure acid chloride and vice versa, i.e., enantiomeric acids converted into diastereomeric amides. This is still an important analytical technique and has recently been reviewed by Gal.[14]

However, the key advance in this area came with the development of commercially available HPLC and gas chromatographic chiral stationary phases (CSPs). In this technique, transient diastereomeric complexes are formed between the analyte travelling through the chromatographic column and the chiral selector immobilized within the column. The CSP-containing columns can be used in the same manner as standard chromatographic columns. CSPs are now routinely incorporated in bioanalytical methods and these applications have recently been reviewed.[15]

The development of relatively cheap, reproducible and stable CSPs have produced an increased interest in the *in vivo* pharmacological fate and effect of the separate enantiomers of chiral substances. The response has been a sustained rise in the number of studies concerned with the pharmacokinetic and metabolic disposition of enantiomeric drugs. Some examples of these studies are presented below.

III. EFFECT OF ENANTIOMER-ENANTIOMER INTERACTIONS ON PHARMACOKINETICS, PHARMACODYNAMICS, AND TOXICOKINETICS

One of the important pharmacological aspects of enantiomers is that they have identical physicochemical properties, i.e., melting points, solubilities, hydrophobicities, etc. Thus, all passive pharmacological processes, such as absorption, will be the same for both isomers. The stereochemical differences between enantiomers is only expressed during an active process such as protein binding, enzymatic conversions, etc. Hence, there is an inherent duality of enantiomers: they are at the same time chemically identical and spatially different molecules. One can expect that these same/different molecules will bind to the same biopolymers and indeed compete with each other in the binding process. This situation does occur and has a direct effect on pharmacological parameters. This is illustrated below:

A. *IN VIVO* INTERACTION OF DISOPYRAMIDE (DP) ENANTIOMERS

In one of the initial observations of enantiomer-enantiomer interactions, Giacomini and coworkers[16] demonstrated that the co-administration of *d*-DP and *l*-DP affected the clearance of *d*-DP. When *d*-DP and *l*-DP were administered separately to healthy volunteers, the clearance, renal clearance and volume of distribution were the same for both isomers. However, when the racemate was administered, *d*-DP had a lower plasma clearance and renal clearance, longer half-life and smaller apparent volume of distribution than *l*-DP, Table 2–2. A study of the protein binding characteristics of the two isomers revealed that they both exhibit concentration-dependent plasma protein binding

TABLE 2–2
Pharmacokinetic parameters of *d*-disopyramide (*d*-DI) and *l*-disopyramide (*l*-DI) administered alone (single) or as a racemic mixture (racemate); where: CL_T = total clearance; $CL_{R\text{-}T}$ = renal clearance; $Vd_{SS\text{-}T}$ = volume of distribution; $t_{1/2}$ = half life.

	d-DI single	*d*-DI racemate	*l*-DI single	*l*-DI racemate
CL_T (ml/min)	111.0 ± 12.6	82.6 ± 13.0	111.0 ± 20.5	127.0 ± 13.4
$CL_{R\text{-}T}$ (ml/min)	56.3 ± 9.9	36.5 ± 7.6	55.3 ± 15.0	64.6 ± 13.0
$Vd_{SS\text{-}T}$ (L)	48.3 ± 5.7	32.8 ± 5.9	49.7 ± 8.7	59.8 ± 11.8
$t_{1/2}$ (min)	312.0 ± 40.7	338.0 ± 72.4	328 ± 70.5	283.0 ± 57.5

Source: From Giacomin; K. M. et al., *In vivo* interaction of enantiomers of disopuramide in human subjects. *J. Pharmacokin. Biopharm.*, 1986, 14, 335. With Permission.

with *d*-DP more avidly bound at lower concentrations than *l*-DP. Thus, when *d*-DP and *l*-DP are coadministered, *d*-DP displaces *l*-DP from plasma proteins and more of the *l*-isomer is available for metabolism and clearance.

B. *IN VITRO* METABOLIC INTERACTION OF WARFARIN (WAR) ENANTIOMERS

The metabolic interactions between (S)-WAR and (R)-WAR was investigated in microsomes obtained from three human livers.[17] In each microsomal preparation, (R)-WAR inhibited the production of (S)-6- and (S)-7-hydroxy-WAR with K_is which ranged from 7.0 to 8.4 μM and 6.0 to 6.9 μM, respectively. The K_ms for the 6- and 7-hydroxylation of (S)-WAR ranged from 3.6 to 3.8 μM and 3.3 to 3.9 μM, respectively. In contrast, except for the 4′-hydroxylation pathway, (S)-WAR was found to be a weak inhibitor of the metabolism of (R)-WAR. Since (S)-WAR is the more pharmacologically active enantiomer[9] and since WAR is administered as a racemic mixture, this interaction can have a direct effect on WAR clinical potency.

The authors of this study concluded that:

1. The kinetic parameters defining the interactions of two enantiomers of a racemic drug with the cytochrome P-450s or other macromolecular systems in the living organism can only be properly defined from experiments with the pure enantiomers;
2. an enantiomer of a racemic drug may contribute significantly to biological effect not by its inherent activity but by altering the pharmacokinetics of the eutomer;
3. enantiomeric interactions are not easily detected unless directly sought and may be relatively common.

C. TOXICOKINETICS OF RACEMIC-PROPRANOLOL (PRO) VERSUS (R)-PRO AND (S)-PRO

It has been reported that racemic-PRO is more toxic than the individual enantiomers in the male Wistar SPF Riv:TOX rat, when dosed i.v. at the same total mass.[18] The pharmacokinetic dispositions of (R)-PRO and (S)-PRO were also investigated after administration of the separate enantiomers and the racemate.[19] Between the two formulations (single isomer vs. racemate), there was no difference in the total plasma clearance for either enantiomer, but there were statistically significant decreases in (S)-PRO volume of distribution and half-life between the racemate and single isomer while the opposite was observed for (R)-PRO, Table 2–3.

The authors concluded that the effect was due to a protein binding interaction where (R)-PRO displaces (S)-PRO. This results in a higher systemic exposure of (S)-PRO when the drug is admin-

TABLE 2–3
Pharmacokinetic parameters of (S)-propranolol [(S)-PRO] and (R)-propranolol [(R)-PRO] adminstered alone (single) or as a racemic mixture (racemate); where: CL_T = total clearance; Vd_{SS} = volume of distribution; $t_{1/2}$ = half life.

	(S)-PRO single	(S)-PRO racemate	(R)-PRO single	(R)-PRO racemate
CL_T (ml/min)	29.5 ± 6.3	32.8 ± 4.1	16.3 ± 1.7	14.6 ± 2.3
Vd_{SS} (L)	3.46 ± 0.63	5.14 ± 0.65	2.06 ± 0.41	1.41 ± 0.30
$t_{1/2}$ (min)	90 ± 27	114 ± 22	93 ± 23	70 ± 19

Source: From Bode, W. et al., Toxiconetics of a single intrafenous dose of *rac*-propranolol versus optically pure propranolol in the rat. *Chirality*, 1995, 7, 626. With Permission.

istered as a racemate vs. the single isomer. Since (R)-PRO is less toxic than (S)-PRO, relative to β-blocking activity,[11] dosing of the racemate should result in higher toxicity than equivalent dosing of the separate enantiomers.

IV. IMPROVING CLINICAL EFFICACY AND REDUCING CLINICAL TOXICITY BY RACEMIC SWITCHES

One of the by-products of the recognition of the pharmacological differences between enantiomers is the possibility of accomplishing a "racemic switch". In this drug development strategy, currently marketed racemic drugs are reevaluated with the intention of developing a single-isomer formulation. The goal is a new chirally-pure drug with a better therapeutic index than the racemate and, perhaps, new clinical applications. These possibilities are illustrated by the use of verapamil in the treatment of adriamycin-resistant tumors and ifosfamide in general cancer chemotheraphy.

A. (R)-VERAPAMIL [R-VER] AS AN ANTICANCER AGENT

Verapamil (VER) is a calcium channel blocking drug and is widely used in the therapy of hypertension, supraventricular, arrhythmias, and angina pectora.[9] VER is a chiral compound which is administered as a racemic mixture of (R)-VER and (S)-VER. However , the enantiomers show different pharmacodynamic and pharmacokinetic properties. For example, (S)-VER is 10 to 20 times more potent than (R)-VER[21]; after i.v. administration, the plasma clearance and apparent volume of distribution of (S)-VER are almost twice as high as those of (R)-VER[22]; after p. o. administration, (S)-VER undergoes extensive first-pass metabolism, resulting in the predominance of (R)-VER in plasma[23]; and the protein binding is enantioselective with the free fraction of (S)-VER larger than that of (R)-VER.[24]

VER has also been shown to have another possible clinical application as a modifier of multidrug resistance (MDR). Initial *in vitro* experiments have demonstrated that the presence of VER in the incubation media increased the cytotoxity of vinca alkaloid and anthracycline derivatives in several resistant tumor cell lines; data for adriamycin sensitive and resistant cell lines are presented in Figure 2–3. One proposed source of MDR is a decrease in the accumulation of intracellular concentrations of the antineoplastic agents because of increased expression of a glycoprotein that acts as an efflux pump for cyctostatic drugs.[25]

Based upon the *in vitro* experiments, several clinical Phase I trials were carried out using VER in combination with adriamycin[26] and vinblastine.[27] However, these trials were not successful. Plasma levels which were comparable to the effective *in vitro* concentrations could not be achieved

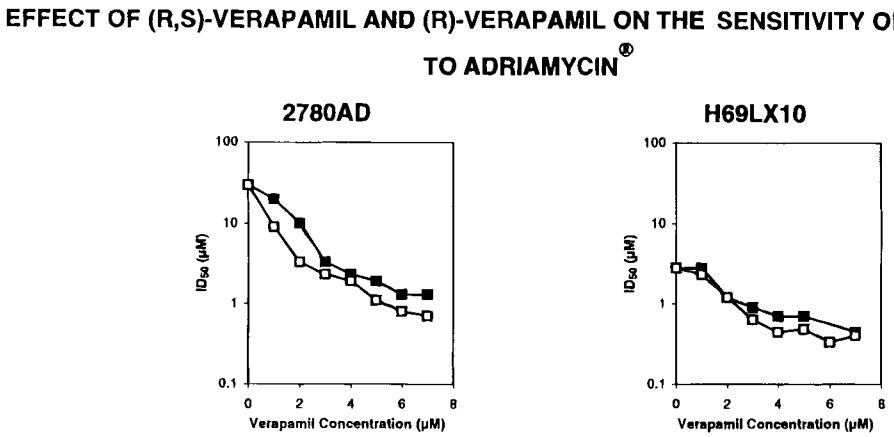

FIGURE 2–3. Effect of (R,S)-verapamil and (R)-verapamil on adriamycin resistant cell lines; where: ■ = (R,S)-verapamil; □ = (R,S)-verapamil.

due to the cardiotoxicity of VER. The results of one study involving the treatment of MDR ovarian cancer patients with VER and adriamycin were summarized as follows:

> However, the high infusion rates of verapamil (9 μg/kg/min) required to achieve these plasma levels produced an unacceptable degree of cardiac toxicity. Two patients developed transient atropine-responsive complete heart block and four patients developed transient congestive heart failure with increases in pulmonary capillary wedge pressure.... Future studies should use less cardiotoxic calcium channel blockers that can be safely administered to produce the plasma levels required for *in vitro* sensitization of drug resistant cells.[26]

One less cardiotoxic compound is the "inactive" isomer of VER, (R)-VER. While this isomer of VER has only 1/10 to 1/20 of the negative dromotropic, inotropic, and vasolidating activity of (S)-VER, it has equivalent activity in the modification of MDR, Figure 2–3.

The results with VER indicate that when stereochemistry is taken into account, it can lead to the discovery of new clinically active compounds, such as (R)-VER. Clinical Phase I trials of (R)-(+)-VER are currently underway.[28]

B. (R)-IFOSFAMIDE [(R)-IFF] AS AN ANTICANCER AGENT

Ifosfamide (IFF) is a structural isomer of cyclophosphamide, which is used in a wide range of malignancies, including adult soft-tissue sarcoma, osteosarcoma, small cell lung cancer, and pediatric tumors.[29,30] IFF, which contains a chiral center at the phosphorous atom, is clinically administered as a racemic mixture of its two isomers, (R)-IFF and (S)-IFF.

IFF is a prodrug, which is metabolized in the liver by two major pathways (Figure 2–1).[1] The first pathway involves ring oxidation through cytochrome P450 enzymes (CYP) and leads to the production of the cytotoxic isophosphoramide mustard,[31–33] Figure 2–4. In the second pathway, up to 50% of the administered dose can undergo CYP-mediated side-chain oxidation resulting in two dechloroethylated metabolites, 2-DCE-IFF and 3-DCE-IFF, Figure 2–4.[33] This pathway has been associated with a central nervous system (CNS) toxicity.[34,35]

Initial reports of IFF metabolism and disposition in humans utilized urinary excretion data only. These studies suggested stereospecific differences in the metabolism of IFF.[35–40] Urine samples from two patients who had received a racemic mixture of IFF [(R,S)-IFF] contained higher levels of (R)-IFF than (S)-IFF while the concentrations of dechloroethylated metabolites from (S)-IFF were higher than those coming from (R)-IFF.[38] These results have been confirmed by subsequent studies in humans[36,39,40] and animals.[41]

The complete pharmacokinetics of (R)-IFF and (S)-IFF as well as the enantiomers of 2-3-N-dechloroethylated metabolites (2-DCE-IFF and 3-DCE-IFF) have been recently described in 14 patients treated with a 3h infusion of (R,S)-IFF (3 g/m^2) with mesna uroprotection.[42] An enantioselective gas chromatographic/mass spectrometric (GC/MS) assay was used to determine the concentrations in plasma and urine.[43]

The mean plasma concentration-time curves for (R)-IFF and (S)-IFF are presented in Figure 2–5. The AUCs of (R)-IFF were significantly larger than those of (S)-IFF (2480 ± 200 vs. 1960 ± 150 μM • h). The terminal half-lives (7.57 ± 0.99 h) and mean residence times (11.17 ± 1.10 h) of (R)-IFF were significantly longer than those of (S)-IFF, 6.03 ± 0.82 h and 9.37 ± 0.88 h, respectively. The mean volume of distribution at steady state of (R)-IFF (25.68 ± 0.80 l/m^2) was slightly smaller than that of (S)-IFF (27.35 ± 0.89 l/m^2). While the renal clearances of (R)-IFF and (S)-IFF were similar, the nonrenal clearance was significantly lower for (R)-IFF (30.20 ± 2.70 vs. 41.40 ± 3.55 ml/m^2/min) as was total clearance (41.52 ± 2.90 [(R)-IFF] vs. 52.37 ± 3.75 [(S)-IFF] ml/m^2/min).

The mean plasma concentration-time curves for the N-dechloroethylated metabolites are presented in Figure 2–6. The AUC values for all of the DCE metabolites from (S)-IFF were significantly greater than those from (R)-IFF with 47% of the measured AUC accounted for by DCE from

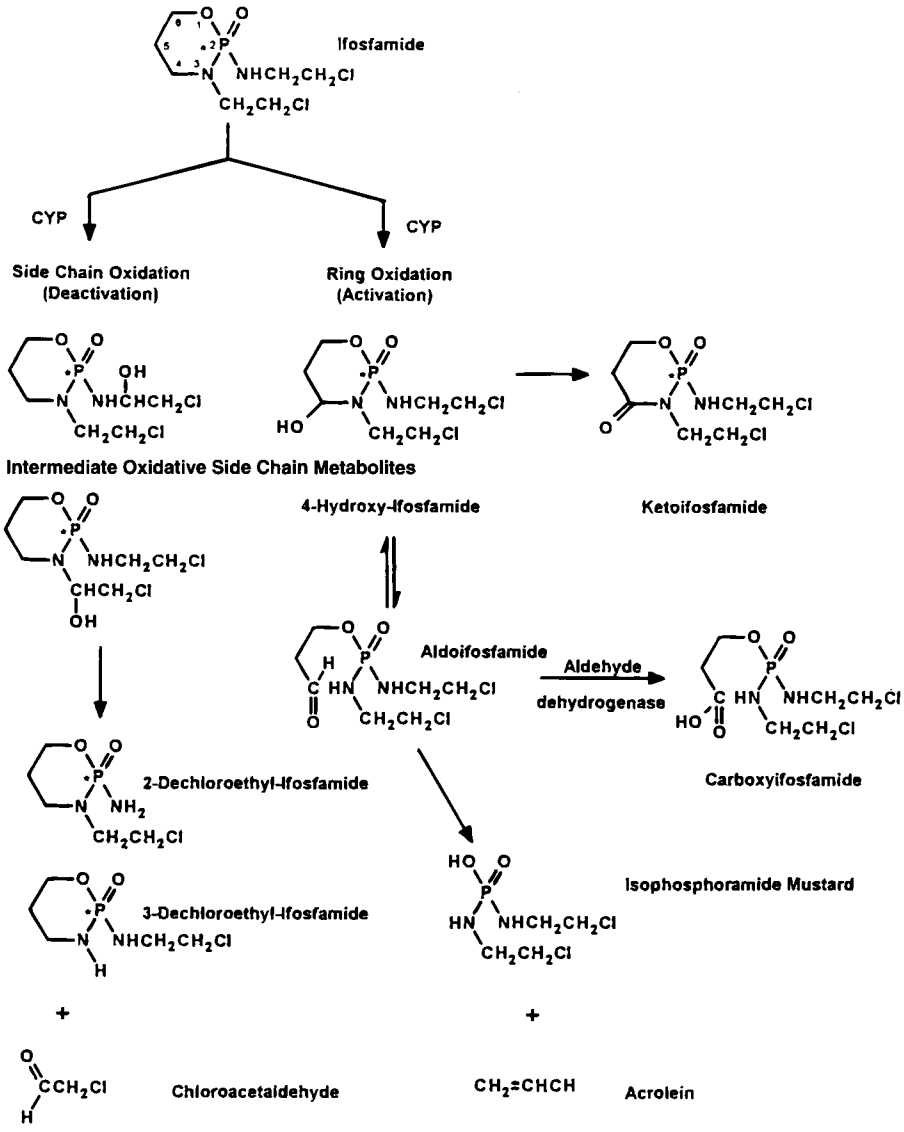

FIGURE 2–4. The metabolic pathways of ifosfamide.

(S)-IFF compared to only 20% for (R)-IFF. Therefore, the enantioselective difference in IFF elimination can be partially explained by differences in N-dechloroethylation.

The cumulative urinary excretions of (R)-IFF and (S)-IFF and the 2-DCE-IFF and 3-DCE-IFF metabolites were also determined in these patients.[40] The results indicated an enantioselective excretion of the parent and N-dechloroethylated metabolites: the urinary recovery of (R)-IFF was significantly greater than that of (S)-IFF (1.73 ± 0.45 vs. 1.43 ± 0.41 mmol, $p < 0.0001$); the excretion of (S)-2-DCE-IFF (0.75 ± 0.53 mmol) was greater than that of (R)-2-DCE-IFF (0.42 ± 0.22 mmol, $p = 0.071$) while the excretion of (R)-3-DCE-IFF (1.64 ± 0.76 mmol) was greater than that of (S)-3-DCE-IFF (0.77 ± 0.59 mmol, $p = 0.012$). The study also revealed two distinct metabolic patterns in which the urinary recoveries of (R)-2-DCE-IFF and (R)-3-DCE-IFF were linked as were those of (S)-2-DCE-IFF and (S)-3-DCE-IFF.

The clinical relevance of interindividual differences in IFF N-dechloroethylation have been indicated by a study which identified a relationship between the occurrence of CNS toxicity and the urinary excretion of (R)-3-DCE-IFF, Figure 2–7.[36] The one patient who experienced severe neuro-

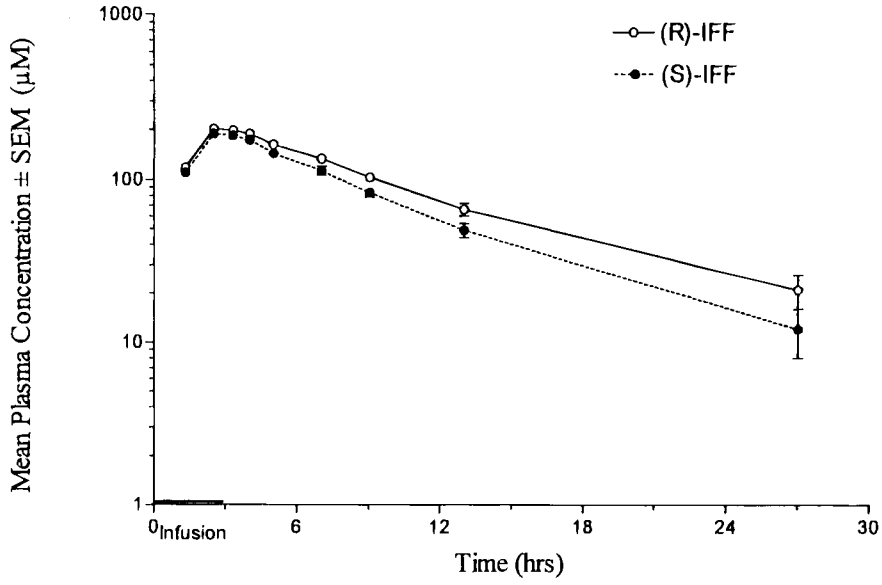

FIGURE 2–5. Mean plasma concentration vs. time profiles of (R)-IFF and (S)-IFF following a 3h infusion of 3 g/m^2 (R,S)-IFF in 14 cancer patients.

toxicity excreted elevated levels of (R)-3-DCE-IFF (28% of the administered dose vs. an average of 13%) and (R)-2-DCE-IFF (8% vs. 4%) while no significant changes were detected in the urinary excretion of (S)-2-DCE-IFF and (S)-3-DCE-IFF.

Since the (R)-3-DCE-IFF metabolite arises from (S)-IFF, the conclusion drawn from these studies is that the treatment associated neurotoxicity of IFF could be reduced or avoided by administration of only (R)-IFF. Clinical trials of single (R)-IFF are currently underway in this laboratory.

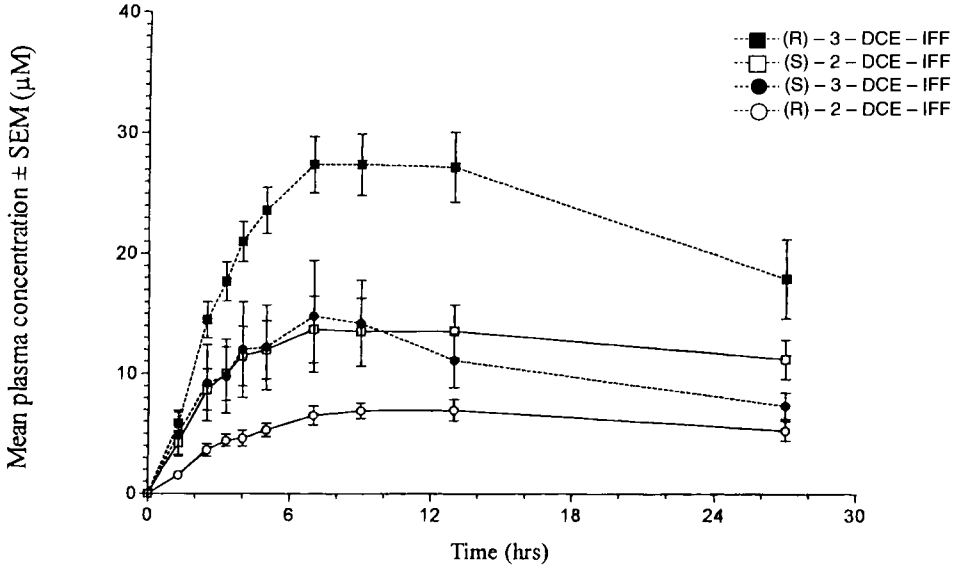

FIGURE 2–6. Mean plasma concentration vs. time curves of the enantiomers of 2-DCE-IFF and 3-DCE-IFF following a 3h infusion of 3 g/m^2 (R,S)-IFF in 14 cancer patients; where: ■ = (R)-3-DCE-IFF; □ = (S)-2-DCE-IFF; ● = (S)-3-DCE-IFF; ○ = (R)-2-DCE-IFF.

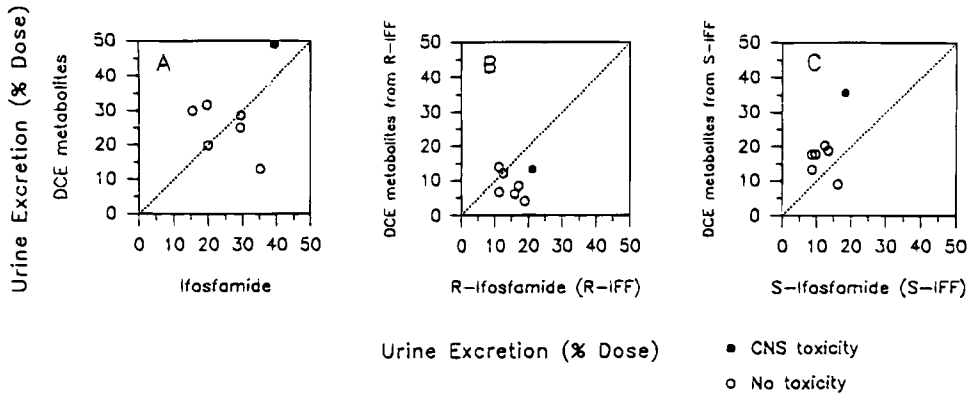

FIGURE 2–7. The urinary excretion of ifosfamide enantiomers and their corresponding N-dechloroethylated metabolites after a 3h infusion of racemic ifosfamide (3 g/m^2) to seven cancer patients. Where: ○ patients with CNS toxicity; ● = patient without CNS toxicity. The excretion is expressed as per cent total dose.
Source: Derived from Wainer, I. W. et al., Ifosfamide Stereoselective dechloroethylation and neurotoxcity. *Lancet*, 383, 982, 1994.

V. CONCLUSION: THREE DIMENSIONAL TOXICOLOGY FOR A THREE DIMENSIONAL WORLD

On February 3, 1860, Louis Pasteur delivered his second lecture to the Conseil de la Societé Chimique de Paris reporting the results of his 1848–1850 studies on molecular asymmetry. In the conclusion of this lecture, he stated:

> You have understood, as we proceeded, why I entitled my exposition, "On the Molecular Asymmetry of Natural Organic Products." It is, in fact, the theory of molecular asymmetry that we have just established, one of the most exalted chapters of the science. It was completely unforeseen, and opens to physiology new horizons, distant, but sure.[3]

It has taken almost 150 years to reach the "new horizons", but we are surely there. With the analytical and chemical tools in hand, it is now up to the pharmacologists and toxicologists to explore this new dimension. It can no longer be acceptable to ignore stereochemistry. We live in a three-dimensional world, and we should insist on three-dimensional science.

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Chapter 3

DNA ANALYSIS

Victor W. Weedn, Demris A. Lee, Rhonda K. Roby, and Mitchell M. Holland

CONTENTS

I. INTRODUCTION

In a time of great advances in the analysis of biologic specimens, perhaps the greatest is in the area of deoxyribonucleic acid (DNA). Biological and medical textbooks are being rewritten to include the vast amounts of information flowing from these discoveries. DNA, the blueprint for all heritable traits, is present in bodily tissues and fluids. The polymorphic nature of DNA provides sufficient discriminatory power to identify individuals. As a result, DNA analysis has moved to the forefront of forensic investigations.

The sheer impact DNA testing is having on the forensic sciences is reason enough for toxicologists to be aware of DNA technologies.[1,2] Traditionally, DNA analysis has been beyond the purview of American forensic toxicologists as it originally developed in the biological rather than chemical or even biochemical disciplines. Since biologic specimens used by toxicologists are amenable to DNA analysis, there is an increased demand for toxicologists to become aware of DNA testing. With increasing cost containment, laboratory staff are directed to cross-train and diversify into these

* The views expressed are those of the authors and do not necessarily reflect those of the Department of the Army or the Department of Defense.

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areas. The forensic DNA analyses are performed for the same clientele as traditional toxicologic analyses, e.g., medical examiners and law enforcement officials. Indeed, toxicology laboratories in the United States and particulary in Europe are increasingly performing forensic DNA analyses.

DNA testing is superseding traditional serologic testing in crime labs because of its inherent advantages of higher sensitivity, greater discriminatory power, wider application to biologic materials, and its resistance to environmental insults.[3,4] DNA is the primary determinant of all traditional serologic markers and HLA typing. Furthermore, since the informational content of DNA is present in its primary chemical structure, this information is remarkably well preserved after death.[5,6] In contrast, protein-based markers must have intact secondary and tertiary structure to provide information. Such markers represent expressed phenotypes; whereas, DNA defines the genotype of the individual. Traditional serologic markers are present in full complement only in blood specimens; whereas all biological tissues and fluids contain DNA. The size and structure of the DNA genome is such that all individuals, barring identical twins, can readily be shown to have a unique genetic profile.[7]

DNA typing represents a paradigm shift from traditional concepts of laboratory testing. The difference can be likened to the difference between analog and digital data. Most clinical and toxicologic laboratory testing methods determine the presence or amount of a given analyte. Such laboratory tests involve mass action, chemical equilibria, enzymatic rates, and qualitative or quantitative assessment of a product level. DNA testing depends upon the genetic code, specifically the base sequence, and not on the determination of relative amounts of any given reaction product. The information is written into the DNA molecule itself. This digital approach permits greater tolerance in the testing methods and yet can achieve greater accuracy and precision in the laboratory results.

Ultimately, DNA will be used to help interpret toxicology results themselves. Urine drug specimens can now be tested by these new DNA technologies to indicate if the samples have been switched.[8–12] Many of the DNA elements necessary to metabolize drugs are known. Thus, DNA testing could provide information on the metabolic system inherited by an individual. For instance, DNA analysis could determine whether an individual is a fast or slow acetylator and thus assist in analytic interpretation. It may be that the optimal future test for the state of induction of an enzyme system will be the measured levels of messenger RNA (mRNA) in whole blood. In turn, genetic tests will be able to establish susceptibility to toxicants. Already, the determination of glucose-6-phosphatase deficiency (G6PD) is an important basis for hemolysis upon exposure to reducing agents. Therefore, DNA testing may have a profound impact on the direction of modern forensic toxicology in the coming years.

II. HISTORICAL PERSPECTIVE

The birth of forensic DNA typing is generally attributed to Alec Jeffreys, who described the "DNA fingerprint" in a 1985 article in the journal *Nature*.[13] Jeffreys first applied his method to an immigration case that resulted in the verification of kinship for a Ghanian boy and his admittance into Britain. Shortly thereafter, he applied his technique to a double rape homicide in Leicestershire County. In this case, the first suspect was exonerated by the DNA tests. Later, 5500 men of the community submitted samples for DNA analysis which eventually lead to a conviction in 1988 of Colin Pitchfork.[14]

In the United States, forensic DNA testing efforts began with commercial laboratories, in 1986 by Forensic Science Associates and Lifecodes, followed by Cellmark in 1987. The Federal Bureau of Investigations (FBI) began casework in December 1988. The first state crime laboratory in operation was Virginia in March 1989. Even today, many crime laboratories have not yet begun to accept DNA casework.

The first criminal conviction in the United States based on DNA testing occurred in the case *Florida vs. Andrews* in 1987.[15] DNA analysis is currently used in approximately 10,000 new criminal investigations each year; and undoubtedly, this number will increase.

III. NUCLEIC ACID BIOCHEMISTRY AND MOLECULAR GENETICS

The central dogma of biology is summarized in four key concepts: 1) the genetic code is contained in DNA molecules; 2) these DNA molecules can be self-replicated; 3) the genetic code in the DNA is transcribed into mRNA in a controlled process; and 4) the messenger RNA is translated into proteins, which are the constituent building blocks of living organisms.[16,17] Thus, DNA has been described as the "blueprint of life."

The genetic basis for all inheritance is found encoded in sets of DNA molecules packaged as chromosomes comprising the genome.[18] Nucleotide bases (adenine, cytosine, guanine, and thymine; abbreviated A, C, G, and T, respectively), the building blocks of DNA, are arranged in a specific sequence. These four bases, A, C, G, and T, make up the genetic alphabet that produces the words, sentences, paragraphs, and chapters which are eventually read into proteins that comprise biological organisms.

Human DNA contains approximately 100,000 genes which code for approximately 100,000 protein products. One of the extraordinary features of mammalian DNA, including human DNA, is the relatively small fraction of the genome that contains genetic coding information. A much greater proportion has no coding function, or no known function. A substantial portion of this noncoding DNA is repetitive DNA sequence. In fact, 20 to 30% of the human genome consists of DNA that is moderately or highly repetitive. Repeated sequences may be short or long, from a few bases to thousands of bases. They can also be interspersed throughout the genome or repeated in tandem (head-to-tail fashion).

DNA is characterized by four traits that make it useful for forensic identity testing: 1) DNA is present in all cells (except mature red cells); 2) DNA is the same throughout the body (sex cells contain only half sets of DNA, e.g., haploid); 3) DNA is the same throughout life (excepting mutational events); and 4) DNA is different in all individuals (except identical twins).

The human genome consists of roughly six billion base pairs per diploid human cell (5–6 picograms), divided into 23 pairs of chromosomes within the nucleus. Additionally, DNA is also found within mitochondria in the cytoplasm. While mitochondrial DNA is only 16.5 kilobases in length, there are many copies per cell, and thus it accounts for roughly 0.5% of the total DNA.

DNA is a negatively-charged linear, double-stranded molecule. The two strands are attached by hydrogen bonds and twisted to form a double helix. Each strand of DNA is a polymer of nucleotide subunits, consisting of a deoxyribose sugar, a phosphate group, and a nitrogenous base. The sugar and phosphate groups form the side rails of the ladder-like structure and the planar nitrogenous bases from each strand point inward to form hydrogen-bonded pairs which make up the rungs of the ladder.

The nitrogenous bases may be a purine (adenine or guanine) or a pyrimidine (cytosine or thymine). Genetic information in the DNA is encoded in the linear sequence of the bases. DNA, and ultimately mRNA, determines the amino acid sequences of proteins or functional RNA molecules such as ribosomal and transfer RNAs using a triplet base code.

The nucleotide monomers are linked in succession by phosphodiester bonds between the 3′-carbon of one sugar residue and the 5′-carbon of the next, so that the linear chain of nucleotide subunits has a polarity, i.e., a 5′ and a 3′ end. The two strands of the helix run with opposite polarity.

The nitrogenous bases are present in pairs in a complementary fashion in which an adenine from one strand is always paired with a thymine on the opposing strand and a cytosine is always paired with a guanine. G-C base pairs have three hydrogen bonds and therefore more strongly linked than A-T base pairs which have only two hydrogen bonds. The consequence of this base pairing is that the base sequence of one single strand can be used to determine the sequence of the opposite or complementary strand.

During DNA replication in the cell, the two strands of DNA are transiently separated, so that each may serve as a template for replication of a new opposite strand, complementary to the original. An unincorporated nucleotide is added to the 3′ end of the strand being synthesized which is

mated to the opposing complementary base of the template strand. *In vitro* enzymatic replication of DNA also occurs in this manner, and is the basis for the polymerase chain reaction (PCR, discussed below).

During laboratory testing, the two strands of the double helix can be separated in a process called denaturation. This is accomplished by conditions that destabilize the hydrogen bonding between base pairs, such as increased temperature, low ionic strength, very high pH, or the presence of chemical denaturants such as urea or formamide. In solution, single-stranded DNA will diffuse and hybridize to its complementary opposite strand. Once denatured, the single strands of DNA will reanneal if conditions permitting hydrogen bonding are restored. This sequence-specific reassociation is the basis for techniques that utilize hybridization probes which recognize specific genetic sequences. During probe hybridization, the degree of specificity required for annealing, so-called "stringency", can be modified by adjusting temperature and ionic strength. This is the basis of restriction fragment length polymorphism testing (RFLP, discussed below).

DNA is relatively inert and surprisingly tolerant of extremes of temperature, pH, salt and other factors that destroy proteins. Indeed, this chemical robustness has allowed for the emergence of molecular archaeology, to include the DNA typing of 7000 year old brain matter, the 5500 year old Tyrolean Ice Man, and from insects preserved in amber after 30 million years.[19–22] Validation studies in forensic science laboratories have shown that DNA typing results are not adversely effected when biologic specimens are exposed to bleach, detergents, oil, gasoline, and other adulterants.[23,24] In addition, DNA can sometimes be extracted and typed after relatively extensive incineration of the tissue.[25]

IV. DNA TESTING METHODS AND APPLICATIONS

DNA analysis can be used to link a suspect to a crime. The blood on a knife found in the possession of the suspect may match that of the victim or the suspect's blood may be found at the homicide scene. DNA extracted from an envelope seal or a stamp may be the clue to solve an extortion case. A variety of biological specimens have been found useful for a surprising variety of scenarios.[5,24–29] Although DNA may link a suspect to a crime, it does not, by itself, provide proof of criminal guilt. For instance, the presence of a suspect's DNA type from a rape case may later be rendered moot by showing that the sexual contact was consensual.

Not only can DNA testing of evidentiary specimens incriminate suspects, it can exonerate falsely accused suspects as well. In approximately one third of the cases, DNA tests prove to be exculpatory.

Conceptually, determination of certain genetic characteristics may be useful for criminal investigators to significantly narrow the field of possible suspects or provide an important lead. For example, many laboratories have implemented gender determination. Although not currently being considered, such traits as hair and eye color could ultimately be determined by a DNA test.

DNA may permit the recognition of serial crimes; for instance, multiple rape victims may be associated by a common semen DNA profile. Recidivist crimes will be linked by computer matches across jurisdictional bounds. The Federal Bureau of Investigation has implemented the Combined DNA Index System (CODIS), a national database for DNA typing results.[1,30] Conversely, DNA evidence may betray the perpetrator of a copycat crime.

DNA testing may be used to reconstruct a crime or accident scene. The identification of bloodstains at a crime scene may permit the interpretation of the actions which took place. Blood on a windshield may be used to identify the driver in an automobile crash involving the fatal ejection of two occupants.

DNA can be used to identify victims. The identification of a corpse recovered from a crime scene is very important to the prosection of the crime. Identification of bodies is important in routine medical examiner and coroner casework and particularly in mass disasters. DNA typing overcomes many of the limitations of traditional fingerprint and dental identification methods.

Fingerprints become unusable with advanced decomposition. Water fluoridation is creating a new generation with fewer or even no restorations. Antemortem fingerprint and up-to-date dental records are necessary for traditional identification, but are often not available. On the other hand, other family members are almost always readily available as sources of reference DNA material. Neither fingerprints nor dentition will permit identification of all parts in cases of fragmentation. DNA is generally useful despite severe decomposition, partial incineration, and fragmentation. Some pathologists are now saving a small DNA specimen from autopsies should any medicolegal issue subsequently arise. The Department of Defense collects DNA specimens from all service members to this end. Furthermore, the confirmation of the identification of several prominent historical figures has hinged on DNA testing, such as Czar Nicholas II of Russia and Joseph Mengele, the Nazi Angel of Death.[31–34]

Parentage testing, usually paternity testing for child support, is now largely performed by DNA testing.[25–34] Perhaps, DNA testing is being performed for parentage on an order of magnitude more than criminalistic investigations. Criminalistic, or forensic; parentage testing may arise in the case of a conception arising from a rape or in the case of a baby thrown in the dumpster.

DNA can be used to determine species and genus, as well as group and individual identification.[1–37] Plant and animal DNA testing has been central to the resolution of several crimes. Species identification is often central to poaching cases. In a few cases, plant debris left as trace evidence has been traced back to a certain location and thus a key criminalistic clue.

DNA tests can be used to resolve specimen mix-ups.[38–40] Pathology laboratories will find application of DNA identity testing when samples are inadvertently switched or pathologic material floats onto a histologic or cytologic slide. Blood tests for clinical chemistry analyses may demonstrate switching. Urine drug tests which have been challenged and alleged to have been switched may sometimes be laid to rest by DNA testing. Testing may be used to confirm or refute mislabeling of biopsy specimens. A small fragment of cancer ("floater") on a glass microscope slide can be determined to be from someone other than the patient.

DNA will ultimately be used for a variety of clinical genetic testing. Indeed, a revolution in biomedical testing is being created through the information being generated by the Human Genome Project. DNA testing, even screening, will eventually become routine and commonplace, if it hasn't already. However, because so much information is contained in the DNA of an individual, ethical concerns have been raised as to the storage and testing of DNA specimens. Alleged concerns that DNA will enable predictions of such things as criminal behavior are overblown, as environmental factors generally outweigh genetic influences. As long as the forensic application of genetic testing avoids the traits which confer disease or predisposition to disease, the ethical issues will be somewhat benign.

A. POLYMORPHISMS

Forensic identification, in general, is based on finding differences, so-called polymorphisms, between different individuals. These differences can take many forms, such as differences in facial appearance, differences in hair color, differences in height, etc.; some variations are unique and some are not. Individual variation is a tenet of biology. Sometimes the polymorphism is acquired, such as a surgical scar. Some differences change with time.

Polymorphisms within the DNA molecule are the basis for all individual characteristics. Each of us has two sets of chromosomes, each of which is made up of 3 billion bases. On average, only one base in a thousand may differ between individuals. Approximately 97% of the human genome is the same as that of a chimp. Nevertheless, a sufficient amount of the DNA is different, allowing for genetic-based identification.

There are two major types of polymorphisms in human DNA; length polymorphisms and sequence polymorphisms. Perhaps most DNA polymorphisms arise from mutations. Mutations are errors made in DNA replication. These can be point mutations, where an incorrect nucleotide is incorporated, or insertions or deletions of one, several, or many bases.

Tandemly repeated sequences account for length polymorphisms.[41,42] This variability, usually called variable number of tandem repeats, or VNTR polymorphisms, is stable in individuals, and is inherited according to normal Mendelian rules. VNTR polymorphisms can be typed by both RFLP and by PCR methods (see below). Regions with core repeat sequences greater than 6 bp have been called "minisatellite" or long tandem repeat (LTR) regions. Those with core repeat sequences of 2 to 6 bp are called "microsatellite" or short tandem repeat (STR) regions.

Sequence polymorphisms consist of changes in one or more bases in a DNA sequence at a particular location in the genome. Sequence variations can be manifested as base substitutions, additions or deletions. However, most sequence polymorphisms are mere point mutations.

Analysis of a few sequence or length-based polymorphic regions can provide significant information about the origin of a biologic specimen.

B. RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)

Of the DNA typing methods, the first widespread forensic DNA test was restriction fragment length polymorphism (RFLP) analysis. This test is still the best known and the one that is the most commonly associated with forensic laboratory testing.

DNA fragments containing VNTRs are cut from chromosomal DNA with restriction enzymes. Restriction fragments differ in size between individuals. These fragments are large, typically varying from 0.5 to 20 kb in length; core repeats of RFLP VNTR loci are typically approximately 35 bp in length. A given fragment length is known as an "allele". The key to the application of RFLP analysis to forensics was the development of DNA probes to hypervariable VNTR loci. Originally, RFLP was used to detect the presence or absence of a sequence mutation, thus dividing the population into two sets. VNTR loci demonstrate far more than a mere biallelic profile, in fact they demonstrate extraordinary length polymorphisms. Hence these loci are forensically powerful tools. No two individuals, other than identical twins, have ever been found to have matching DNA types, if five or more RFLP loci have been analyzed.

Characteristics of tandem arrays cause them to experience mutation rates that are higher than normal. Point mutations, insertions and deletions can occur within the array through higher frequency processes such as replication error and slippage, and unequal crossing-over. These events could generate new copies of the VNTR that differ in the sequence or number of repeat elements.

The six steps in a complete RFLP analysis include:

1. DNA extraction from the specimen;
2. cutting the DNA into relatively small fragments at specific sites with special enzymes called "restriction enzymes";
3. separating the fragments by size using agarose gel electrophoresis;
4. denaturation of the DNA into single strands;
5. transferring and immobilizing the separated DNA fragments onto a nylon membrane by the Southern blot technique;[42]
6. hybridization to radioisotopically-labeled probes (themselves small fragments of single-stranded DNA) or probes which allow for chemiluminescent detection; and
7. autoradiography, in which an x-ray film is directly placed on the membrane, resulting in exposure of the x-ray film at the point of the probe.

DNA probes are highly specific. In fact, they typically have higher affinity and specificity than antibodies for antigens.

The first applications of DNA "fingerprinting" used probes that hybridize loosely to many different minisatellite loci simultaneously, so-called multilocus probes, which yielded very powerful multi-banded patterns resembling bar codes.[13] Subsequently, batteries of single-locus probes have been developed to permit identification based on discrete loci with well-defined population statistics.[43]

RFLP analysis is a robust and powerful DNA typing technology, often yielding discriminatory values of one in many millions; however, it is also labor-intensive, costly, and time consuming.[4] Typically, it takes 4–6 weeks to perform RFLP analysis due to the incubation period required to expose the x-ray film. The autoradiography of traditional RFLP analysis which uses radio-labelled probes is, in fact, giving way to chemiluminescent techniques.[44]

Moreover, RFLP is subject to limited sensitivity and susceptibility to degradation of the evidentiary specimen. Biologic specimens are susceptible to decompositional changes and bacterial digestion. Specifically, nucleases (DNases) will enzymatically cut the DNA into small fragments upon exposure. The average fragment size in highly degraded DNA may be less than a few hundred bases in length. RFLP testing is precluded in the absence of "high molecular weight DNA", where significant DNA degradation has occurred, as random fragmentation thwarts detection of a specific large uncut fragment population. In an effort to circumvent the disadvantages of RFLP analysis, newer methods have been developed and successfully employed in cases where increased degradation of the specimen has taken place. The polymerase chain reaction (PCR) is a technical breakthrough which overcomes the limitations of RFLP testing and may replace RFLP testing.

C. POLYMERASE CHAIN REACTIONS (PCR)

The polymerase chain reaction (PCR) has revolutionized DNA testing. Although PCR is simply a method of copying a small targeted region of DNA, Kary Mullis was awarded the Nobel Prize in 1993 for its discovery.[45–47]

PCR amplification, as with restriction digestion, can be considered a sample preparation technique which enables further testing to detect various polymorphisms. With PCR, however, non-amplified DNA becomes undetectable background against the amplified target sequence. Furthermore, labels such as fluorescent tags, radioactive isotopes, biotin, or chemiluminescent tags can be added to the amplified fragments during their synthesis. PCR testing is not only very sensitive, but it is quicker, less labor-intensive, and less demanding than RFLP testing.

The target sequence is amplified in an exponential fashion to generate billions of new DNA fragments ("amplicons"). The PCR process is often analogized to a photocopier, wherein new copies are produced from an original. However, it is an exponential process in which the photocopies themselves are photocopied. Even a single DNA fragment may be used to generate a sufficient number of copies to yield a typeable result. This sensitivity is paramount to forensics since certain types of evidence would otherwise not have sufficient DNA to be analyzed by RFLP such as the very tiny droplets of blood found in a blood spatter pattern or a single hair root.

The PCR amplification process involves repeated cycles of three steps: 1) *denaturation*—splitting of the double-stranded DNA into single-stranded DNA by literally melting the strands apart; 2) *annealing*—the binding of two small single-stranded pieces of DNA, primers, to the opposite strands bracketing the area of interest; and 3) *extension*—replication of the strands being created from the extension of the two primers by a DNA polymerase.[48] The result is a doubling of target DNA for each three-step cycle. The newly synthesized DNA strand serves as a template for the next cycle. The Taq polymerase from the *Thermus aquaticus* bacteria which grow in thermal geysers permits the use of high temperatures without significant denaturation of the enzyme. This enzyme has made PCR amenable to automation. The different steps proceed at different temperatures with the same reaction mixture; and therefore, PCR can be carried out in an instrument called a "thermal cycler".

PCR-based methods are often successful even though the specimen is degraded because only a few copies of relatively short segments need remain intact. Because of its exquisite sensitivity, PCR testing is susceptible to the potential for cross-contamination, however, PCR testing can nevertheless be performed reliably when adequate precautions are taken.

The PCR methods including reverse dot-blot systems, amplified fragment length polymorphism (AmpFLPs), short tandem repeats (STRs), and direct mitochondrial DNA (mtDNA) sequencing promise to revolutionize the area of forensic DNA testing.

D. REVERSE DOT BLOTS

Sequence information can be obtained by direct sequencing of the DNA locus or more quickly, easily, and inexpensively by probe hybridization. The PCR-based typing technique which is most commonly used at present, is referred to as the reverse dot blot method.[49–51] Dot blots involve a series of DNA probes to detect specific target sequences in a given region of DNA. A DNA probe is a small piece of single-stranded DNA (oligonucleotide) which will bind to other single-stranded DNA with the complementary sequence. A sequence-specific oligonucleotide (SSO) probe, also known as an allele-specific oligonucleotide (ASO) probe, is generally composed of 20 to 35 nucleotides. These probes are long enough to confer great specificity, and yet sufficiently short to be destabilized by a single base mismatch, so that it binds only to the exact complement sequence. SSO probes are used to detect the presence or absence of alternative sequence types.

Technically, reverse dot blot systems involve probes transfixed to a solid support medium. The target sequence is labeled during PCR amplification. Subsequent to PCR, the target analyte is added to the solid support medium. The analyte will only hybridize to the probe if the sequence is complementary.

Commercial kits are now available which produce a pattern of colored dots on a strip that represent a specific DNA genotype (DNA type of both alleles). The first dot blot strip kit available involved the histocompatibility locus, AmpliType® HLA DQ-alpha PCR Amplification and Typing Kit developed by the Cetus Corporation (now Perkin-Elmer/Roche Molecular Systems).[49,50] This system originally detected 6 alleles dividing the population into 21 genotypes and had a discriminatory value of roughly one in twenty. The newer kits contain subtyping capabilities for one of the alleles creating seven additional genotypes for a total of 28. The genotypes are very well documented in a variety of populations. The kits have been subjected to extensive validation studies in numerous laboratories. The test kits have proven to be robust assays that are not difficult to perform and testing can be performed by one analyst in less than one day.

A second commercial dot blot kit, known as the AmpliType® PM PCR Amplification and Typing Kit, involves as additional five genetic loci and collectively raises the discriminatory power to one in a few thousand.[51] The five different loci simultaneously tested are: glycophorin A (GYPA), hemoglobin gamma-globin chain (HBGG), group specific component (GC), D7S8, and low density lipoprotein receptor (LDLR). Glycophorin A is the protein that carries the classical MN blood-group polymorphism. The GC (group specific component) is a human serum protein polymorphism that has been typed for years using protein chemistry methods. The low density lipoprotein receptor (LDLR), D7S8, and HB γ-globin (HBGG) loci have not previously been utilized in forensic work. The single amino acid difference polymorphism in the γ-chains of human hemoglobin F, resulting from apparently duplicated γ-chain genes, was described long ago. AmpliType® PM detects the basis for this difference at the DNA level. Each of these systems exhibits two or three alleles, resulting in three to six genotypes.

These two systems have recently been marketed as a single kit; the AmpliType PM + DQA1. The fragment lengths amplified in these two commercial dot-blot systems vary between 138 and 242 base pairs (bp) long. The two systems are amplified simultaneously and therefore, less sample is consumed.

E. AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AmpFLPs)

Amplified fragment length polymorphism (AmpFLP) analysis is similar to RFLP testing, but is PCR-based, and involves the electrophoretic separation of DNA fragments polymorphic in size produced by amplification rather than excision. Since the VNTR loci typed by PCR methods are smaller, they are usually analyzed on polyacrylamide rather than agarose gels. In addition, since the amplified DNA so overwhelmingly predominates, the resultant gels may be stained directly with non-specific DNA stains, such as silver; hence hybridization by probes and autoradiography become unnecessary. Consequently, AmpFLP analysis can be performed in one or two days instead of the weeks necessary for tranitional RFLP analysis, employing autoradiography.

The first AmpFLPs described involved VNTRs with core repeats greater than 6 bp long, which have been called long tandem repeats or LTRs. Several loci, including D1S80, D17S30 (also called D17S5), collagen A (ColA) and APOB (a repeat sequence polymorphism 3′ to the apolipoprotein B locus), have been analyzed using PCR methods.[52–54] The best developed among them appears to be D1S80, for which a typing kit is now commercially available from Roche Molecular Systems.[55,56] The D1S80 locus has a core repeat element of 16 bp and its amplified fragments vary between 369 and greater than 801 bp.

Another class of AmpFLPs currently being developed for identification applications are the so-called short tandem repeats or STRs with core repeats of 2 to 6 bp.[57,58] For technical reasons, the dinucleotide repeats are not generally used in forensic science laboratories. Several systems have been described and some are commercially available.[59–62]

STR tandem arrays are generally much smaller than LTRs, with a mean length on the order of 200 bp. There are more than 30,000 separate STR loci in the human genome. STRs have many of the same properties as RFLP VNTR loci, including high heterozygosity. However, because they are shorter, they can be discretely analyzed on acrylamide gels to the resolution of a single repeat unit, or single nucleotide base. Their small size makes them less susceptible to allelic dropout or preferential amplification. This greatly enhances the utility of STR analysis for degraded samples. STR analysis is easier to perform in combined sets and on automated instrumentation. With sufficient numbers of STR systems, discriminatory powers similar to current RFLP testing can be achieved.

F. MITOCHONDRIAL DNA

Mitochondria are subcellular organelles contained in the cytoplasm of human cells involved in oxidative energy metabolism. They contain their own complement of DNA, consistent with their presumed endosymbiotic origin (they are thought to have originally arisen from intracellular parasites upon which modern aerobic cells have become dependent).[63,64]

Mitochondrial DNA (mtDNA) has very different properties than nuclear DNA reflecting this endosymbiotic origin; it is present in high copy number, non-recombinatorial, and maternally inherited.[65,66] Somatic cells of the body may contain from hundreds to thousands of mtDNA within the cytoplasm depending on the tissue type, but only one set of paired chromosomes. Because of the increased number of mtDNA genomes in a single cell, mtDNA may be recovered from specimens when nuclear DNA cannot.

Mitochondrial DNA is inherited strictly from mother to child, without contribution from the father. Therefore, an exact mtDNA sequence match can be followed in a family through maternal lineages for many generations. This is vital when considering that nuclear DNA testing will not be useful when only distant references are available.

In humans, the mtDNA genome is 16,569 bp in a closed circular loop. MtDNA codes for 13 proteins, 2 ribosomal RNAs and 22 transfer RNAs. In addition, the genome contains little repetitive or non-coding DNA. The only substantial section of non-coding DNA occurs in one region, of approximately 1000 bp, known as the "control region" or "displacement loop" (or more simply the "D-loop"). The D-loop does, however, contain the origin of replication for one of the two DNA strands, as well as regulatory sites for gene transcription. It is in this generally non-coding region that the greatest variation among people is found and forensic identity testing has focused.[26,29,67,72] The polymorphisms consist of scattered single base differences concentrated within two hypervariable regions, each roughly 300 bp in length.

Sequence analysis of mtDNA is an evolving technology, with greatest application when extremely small quantities of sample DNA are present, e.g., saliva stains, shed hairs (which have virtually no nuclear DNA), or where the DNA is extremely degraded as found in old or ancient skeletal remains.[26,27] Mitochondrial DNA analysis will find particular application to the analysis of hair. Hairs are commonly found at crime scenes. Nuclear DNA analysis requires the hair root, but most shed hairs do not have typable amounts of nuclear DNA. However, hair shafts do have sufficient mtDNA to perform mtDNA typing.

Mitochondrial DNA exhibits a roughly ten-fold faster rate of evolution (base substitution over time) than does nuclear DNA, possibly due to differences in DNA replication fidelity or repair efficiency.[73,74] This high evolutionary rate has caused mtDNA to be an extremely important tool in population genetics and phylogenetic analysis within and between many species, including humans. The discriminatory power of mtDNA sequencing is not as high as that of nuclear DNA testing, as it is only a single genetic system. Nuclear DNA testing involves multiple genetic systems which permit the multiplication of statistical chances. Nevertheless, the average chance that unrelated individuals will have the same sequence over both hypervariable regions is less than one percent.[75]

Sequencing is at present very expensive to perform and currently is being performed in approximately five laboratories. However, a dot blot assay, which captures most of the information obtained from sequencing is under development. Regardless of the method employed, mtDNA sequence analysis is far more sensitive than other PCR methods and enhanced precautions are critical.

V. SAMPLE COLLECTION

Sample collection is the first line to successful DNA analysis.[76] Without good sample collection techniques and chain of custody practices, DNA test results may prove to be worthless. Forensic samples are often present in trace amounts, they may be mixed with other biological specimens or evidence, and/or they may have been exposed to harsh environmental insults. Therefore, handling of the specimens during the collection process should be performed with the utmost care.

DNA tests have proven to be surprisingly robust. One important aspect of DNA testing is that the outcome from environmental damage oftentimes results in an inability to obtain a test result rather than a "wrong" result. DNA can be isolated and tested from virtually any postmortem tissue, although after death the tissues will undergo progressive degeneration. DNA is generally broken down into degraded fragments through autolytic and bacterial enzymes, specifically DNases. DNA will be quickly degraded due to cellular or bacterial enzymes when damp, but can be remarkably well-preserved by cold storage or desiccation. The information within the DNA is still encoded in the DNA fragments and, therefore, is not completely lost despite extensive fragmentation.

However, not all DNA testing is appropriate or possible when the DNA is degraded. Traditional RFLP testing will require nondegraded high molecular weight DNA, whereas PCR-based analysis can be performed on degraded samples. DNA that has been heavily damaged or degraded will not permit RFLP testing, but may allow PCR-based testing. Smaller DNA fragments are more likely to be recovered from samples in which the DNA is degraded, hence STR systems are more robust than LTR systems. In general, PCR-based systems are more sensitive and are less subject to degradation. Finally, the most damaged and DNA limited specimens will require mitochondrial DNA testing.

Due to the degree of sensitivity of modern DNA technologies, great care should be taken to prevent contamination of one specimen by other sources of DNA. Immaculately clean utensils should be used for collection. These utensils should be changed or cleaned between subjects if working on many fragmented body parts. If it is possible that more than one individual may be present, sterile utensils must be used between samples when obtaining a specimen for DNA analysis.

In relatively fresh cadavers and cadaveric tissues, unclotted blood preserved in EDTA or ACD (purple top and yellow top tubes, respectively) is the preferable source of DNA. Although heme is an inhibitor of PCR, laboratories are accustomed to blood as a DNA specimen and whereas only white blood cells carry the DNA, ample DNA is present for testing. Due to the settling out of white blood cells, clotted blood may not be as good a source of DNA. Furthermore, blood is a good culture medium and bacterial growth may render blood samples useless. Virtually any tissue can be used successfully for DNA typing purposes. If available, bright red muscle samples are good sources of DNA. Brain tissue is said to be a particularly good source when isolated from human remains of an intermediate postmortem time period. Hard tissues such as bone and teeth are the best source of DNA in cases of advanced decomposition. Teeth tend to better preserve DNA than skeletal bones, which are better than soft tissues.

The specimens should be kept cold or preferably frozen (although repeated freezing and thawing is not good). Sample air drying is critical for bloodstains and swabs prior to cold storage. Tissues in formalin are not optimal, but can often be used for PCR-based DNA testing. Any tissue or biologic fluid should not be discarded as inadequate without first attempting DNA testing.

Reference samples can generally be obtained from suspects or victims themselves, but reference samples may come from other sources as well. Specimens from family members may serve as reference specimens. Primary DNA specimens of the individual may sometimes be available from biopsies or tissue slides archived in a hospital's pathology department, from stored blood donations, from licked envelopes and stamps, or in the case of mitochondrial DNA, from locks of baby hair. All states require that infants provide a blood sample (such as a bloodstain card) for phenylketonuria (PKU) testing; some state health departments store these cards for significant periods of time and may be a source of DNA reference material.

VI. QUALITY ASSURANCE

Standards and guidelines in the forensic DNA typing community are derived from a variety of sources. The FBI's Technical Working Group on DNA Analysis Methods (TWGDAM) has promulgated guidelines that detail quality assurance measures.[77] Quality assurance and quality control programs are essential for any laboratory.

The American Society of Crime Laboratory Directors (ASCLD) has established accreditation standards for forensic laboratories.[78] The American Board of Criminalists (ABC) certifies criminalists and has a subspecialty category for DNA analysts. The DNA Identification Act, a component of the Crime Bill passed into law in 1994, includes a mandate for standards to be issued by the Director of the FBI upon the advice of the DNA Advisory Board.

Proficiency testing is an important aspect of quality assurance adopted by the forensic DNA typing community.[78,79] TWGDAM guidelines call for two proficiency tests per DNA analyst per annum. One proficiency test must be completed in each 6 month period with at least one as an external proficiency test. The ABC requires that ABC certified criminalists participate in proficiency testing. Furthermore, ASCLD has incorporated this requirement into their accreditation standards. ASCLD has established a Proficiency Review Committee (PRC) which is charged with oversight of the proficiency testing of crime laboratories. The DNA Identification Act mandates proficiency testing. Proficiency tests for forensic DNA identity testing are currently available from multiple commercial vendors.

Interlaboratory comparisons have consistently demonstrated the great precision of RFLP and PCR testing by the forensic DNA typing community. Proficiency surveys for RFLP testing report mean fragment size measurements that differ by only a few bases, and the range of results are well within the plus or minus 2.5% matching window used by most crime laboratories. This precision is achieved despite different protocols and procedures. This is probably reflective of the fact that there is general agreement as to the proper laboratory procedures that should be used in forensic DNA testing.

VII. JUDICIAL ACCEPTANCE

Forensic DNA evidence has generally been accepted by courts. According to FBI statistics, as of September 1995, the DNA test results were admitted in 175 of 226 reported federal and state cases involving RFLP and PCR DNA tests. In 25 cases the results were excluded and/or remanded and in another 26 the results were admitted with little or no statistics. However, the furor involved in a few cases has created a perception of concern over forensic uses of DNA testing.

The defense arguments have evolved over time. Originally, the defense challenged the validity of the technology itself, but this was never successful. The defense strategy which met with greatest success was in the challenge to statistical interpretation of a DNA match. The argument involved the

appropriate population frequency statistics, given that our population is composed of subgroups. It has been repeatedly demonstrated that individual variation is far greater than intergroup variation and that a rare DNA typing result is rare regardless of the population. The defense challenge merely suggests a dilution of the statistical power of discrimination, but courts often ruled the statistic interpretation as inadmissible due to the lack of consensus agreement. It makes little difference that the evidence points to the accused with a statistic of one in a few hundred million or one in a billion. It can be said that there is now general agreement among population geneticists and statisticians as to the validity of the statistics. Consequently, the defense challenge is decreasing. The most common areas of challenge today are in the potential for contamination of PCR tests, quality assurance and error rates, and the possibility of planting or tampering with the evidence.

VIII. DNA DATABANKS

Policymakers have embraced this new technology more completely than the courts. The Office of Technology Assessment and the National Academy of Sciences have issued reports very supportive of the forensic uses of DNA identity testing. State legislatures have promulgated legislation creating DNA databanks from convicted offenders, the federal government has passed the DNA Identification Act, and the military too has established a DNA databanking program to identify the remains of victims through DNA testing.

At the time of this writing, 43 states have received legislation mandating the creation of computerized databases of DNA typing information from convicted sex and felony offenders. The DNA Identification Act authorizes funding to further support the establishment of these state DNA databanks. The FBI's Combined DNA Index System or CODIS (also known as the National DNA Identification Index) will permit cross checks of state DNA databanks. Most states will keep samples of the DNA in addition to the typing information.

IX. CONCLUSIONS

Only recently has law enforcement begun to use DNA tests, but it has already transformed the field of criminalistics. DNA technologies continue to evolve, with ever greater sensitivity and decreasing cost, turn-around-time, and manual manipulation. Gel electrophoresis and paper blots will eventually give way to capillary electrophoresis, microchips, and possibly mass spectrometry. DNA tests will become routine in ways not currently imaginable. Courts and juries will come to accept such testing. Finally, DNA testing will increasingly become a familiar tool to toxicologists.

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Chapter 4

SUPERCRITICAL FLUID CHROMATOGRAPHY

Steven H. Y. Wong

CONTENTS

I. INTRODUCTION

Supercritical fluid chromatography (SFC) and supercritical fluid extraction (SFE) are two complementary techniques of potential, and in selected settings of practical interest to analysts in toxicology and therapeutic drug monitoring (TDM).[1–12] As compared with the more established and widely used gas chromatography (GC) and high-performance liquid chromatography (HPLC), SFC may be regarded as a more recent entry in the early 1980s, and SFE in the mid-1980s. Although SFC is relatively "older" than SFE, the more recent applications in SFE may have actually enhanced the further application of SFC.[8–10] Currently, SFC may be characterized as a complementary methodology for analysis not readily performed by GC and HPLC, whereas SFE offers unique advantages, compared with traditional extraction methods. For their applications in TDM and toxicology, SFC may be characterized as a supplementary instrumentation, primarily limited to clinical research laboratories,[6] whereas promising applications of SFE are being demonstrated for toxicological analyses, especially in sample preparations for environmental toxicology.[10]

Recent reviews updated, up to 1993, the major developments of SFC and SFE.[2,5,6,10–13] Chester et al.[12] suggested that despite the lack of theoretical boundaries, pressure control over the entire length of the SFC column is unique, in comparison with GC and HPLC. There may be some "blurring" in the distinction between open-tubular SFC and GC, and packed-column SFC and high-temperature HPLC. This is readily demonstrated by the proposed concept of unified chromatography. According to Schurig et al.,[14] unified chromatography for the enantioselective capillary analysis of hexobarbital was achievable by using the same microcolumn (1 m × 50 μm i.d.) packed with β-

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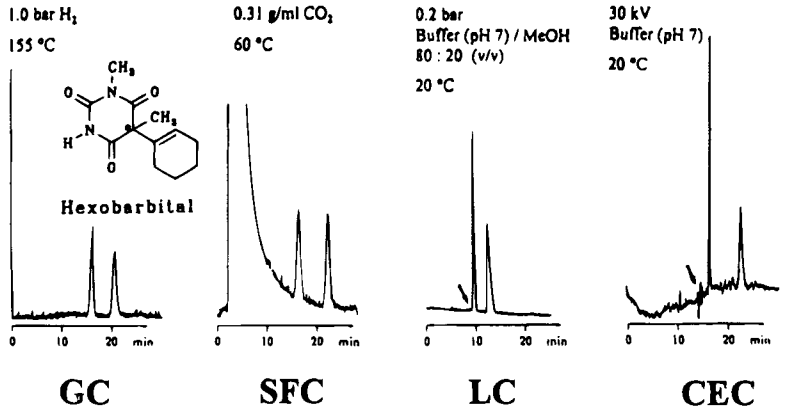

FIGURE 4–1. Enantiomer separation of hexobarbital on a 1 m × 50 μm i.d. fused-silica column coated with Chirasil-DEX (film thickness of 250 nm) by GC, SFC, LC, and CEC in approximately 20 min. Effective column length in LC and CEC is 85 cm. Buffer is borate phosphate (pH 7) (arrow indicates the dead volume). (From Schurig, V., et al., *J. Chromatogr. A*, 694, 119–128, 1995. With permission.)

cyclodextrin for analyses by GC, HPLC, SFC, and capillary electrochromatography (CEC), as shown in Figure 4–1. Furthermore, SFC has been proposed to include applications in near-critical fluid chromatography, subcritical fluid chromatography, enhanced-fluidity liquid chromatography (LC), and others.[12] Recent SFC developments include increasing applications of packed-column SFC, and newly introduced instrumentations offering binary, ternary, and composition gradients of mobile phase for enhanced chromatographic selectivity; advances in SFE were attributed to its being a viable alternative to the traditional extraction, with the high costs associated with the purchase of solvent and disposal of waste in compliance with regulatory guidelines, and the availability of automated and low-cost instrumentation. These recent developments merit a renewed consideration of potential applications of both SFC and SFE for TDM and toxicology. This chapter assesses the current and future roles of SFC by describing the basic principles of SFC, updating the currently available instrumentations and technologies, and providing the readers with selected applications that may be of clinical and/or research interest to toxicology. The next chapter addresses the principle, roles, and applications of SFE.[10] Thus, the focus would be different from several recently published reviews by the author[2,5,6] and others.[1,9–12]

II. SUPERCRITICAL FLUIDS (SFs)

SF provides the medium for the partition processes of the analytes in SFC and SFE. A SF is defined as a fluid maintained above its critical pressure and temperature. Consequently, a low-density fluid (gas) can be compressed to a high-density fluid (liquid-like) without discontinuity in density and without gas-to-liquid condensation.[15] For performing SFC and SFE, carbon dioxide is the most popular SF, with corresponding critical pressure and temperature of 72 Pa and 31.3°C. Figure 4–2 shows the reduced isothermal plot of reduced density and pressure for carbon dioxide. Reduced temperature, *Tr*, is defined as: $Tr = T/Tc$, wherein *Tc* and *T* are the critical and experimental temperatures, respectively. Area A represents the one-phase, SF region, whereas liquid carbon dioxide exists in area B. In area C, carbon dioxide exists as both liquid and gas. In performing SFC and SFE, the pressure and temperature are maintained above critical values and within area A. As a SFC mobile phase, diffusion coefficients of SFs range from 0.3 to 1.0 ($\times 10^{-3}$) cm^2/s, between that of a gas (0.01 to 1.0 [$\times 10^{-3}$] cm^2/s) and a liquid (0.5 to 2.0 [$\times 10^{-5}$] cm^2/s), resulting in high-column efficiency, appreciable solute solubility, and applicable molecular mass range from 1 to 1000

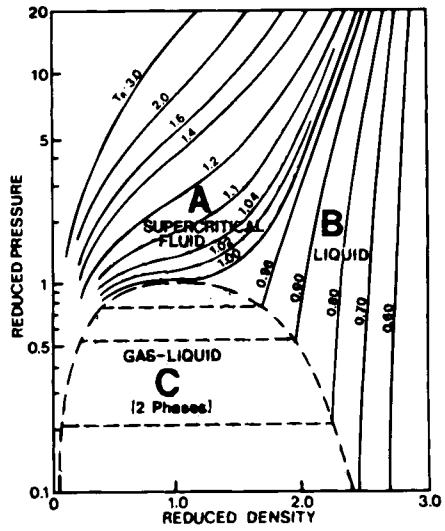

FIGURE 4–2. Reduced isothermal plots of the reduced variables, pressure, and density for carbon dioxide. *Area A* is part of the one-phase carbon dioxide supercritical fluid experimental region of this study. *Area B* is a one-phase carbon dioxide liquid region. *Area C* is a two-phase region of carbon dioxide gas and liquid. T_R is reduced temperature (isotherms). (From Gere, D. R., et al., *Anal. Chem.*, 54, 736–740, 1982. With permission.)

Da.[1] Additives to the SF could substantially change the analyte diffusion, thus offering added selectivity.

Phase behavior and strength of SF fluid/mixture are critical to the selectivity and separation in SFC, and to the partitioning of analytes in SFE. Phase behavior may be investigated by modeling using equation-of-state for the SF fluid/mixtures and by empirical testing of phase equilibra. A recent review by Chester et al.[12] emphasized the need for further understanding of complex phase behavior. For SFC, injection of sample solution into a pure SF carbon dioxide may result in temporary formation of a mixture. Furthermore, the difference in the temperature of the injector and column may result in phase separation, with unpredictable outcome for the analysis. On the contrary, phase separation is essential for SFE. Mixing SF with additives, such as acetone or methanol, can enhance the polarity or strength. Further compilation of these basic informations is needed to enhance future SFC and SFE applications. For example, only five solvent/carbon dioxide mixtures may be successfully used for SFC applications.[16]

III. SUPERCRITICAL FLUID CHROMATOGRAPHY (SFC)

Klesper et al.[17] demonstrated the first SFC analysis of porphyrins by using SF freon. From the investigation of the separation modes of SFC and pressure programming, Sie et al.[18] proposed the term *supercritical fluid chromatography*. Giddings et al.[19,20] and Gouw and Jentoft[21] studied the fundamentals of the SFC processes. Gere et al.[13] were credited with the modification of an existing HPLC to perform SFC in the early 1980s, whereas Novotny et al.[22] and Lee and Markides[23] demonstrated the initial application of capillary SFC.

In the early development of SFC,[1] proposed desirable separation characteristics included solvating mobile phase, lower separation temperature than that of GC (advantageous for thermally labile analytes), higher separation temperature in most application compared with that of HPLC, high efficiency, and universal detection possibly achieved with flame ionization and other GC detectors. For open-tubular capillary SFC, precise pressure control offers instantaneous density programming.[1,22–25] This latter characteristic enhances the separation of complex mixture and increases the molecular range of analytes. Thus, SFC offers complementary selectivity and separation characteristics to GC, HPLC, and CEC. However, retention mechanisms of SFC remain not well established.

A. GENERAL CONSIDERATION FOR INSTRUMENTATION

Early investigations demonstrated that SFC might be constructed from combinations and modifications of components from existing GC and HPLC,[25–27] whereas commercial SF chromatographs were introduced by several companies in the late 1980s. Currently, the number of commercially available SFCs is about five, less than that from the last review.[5] However, the recent models are more versatile, offering mobile phase programming capabilities and accommodating both packed and open-tubular capillary columns. Figure 4–3 shows the schematics of a SFC.[26] The main components are SF carbon dioxide source with accompanying refrigeration unit, a high pressure pump, injector, column, restrictor for the decompression of SF before its entry into a detector such as the flame ionization detector (FID). The pump may be an HPLC reciprocating pump or a pulseless syringe pump. In modifying existing chromatographs, Sanagi and Smith[27] and Lee and Markides[26] detailed the technical considerations. Current instrumentation follows the development of other chromatographs with modular design, computer control, and data processing for precise mobile phase mixing. The following section describes the various parts of the SFC downstream from the SF source to the detector.

B. SFs AND MODIFIERS

Carbon dioxide is the most popular mobile phase because of its low critical temperature, easy purification, inertness, safety consideration, and compatibility with FID. Other mobile phases include: xenon, *n*-pentane, SF_6, and ammonia. Because of several accidents in SFE using nitrous oxide, its use for SFE and SFC is being discouraged.[28] In packed-column application, selectivity of the stationary phase is changed by a low concentration of modifiers, such as methanol, water, formic acid, formamide, acetonitrile, methylene chloride, tetrahydrofuran, and citric acid. They may be combined with SF carbon dioxide in a binary, ternary, or gradient elution as in HPLC. The modifiers may be mixed with carbon dioxide by the suppliers in a cylinder with a dip tube. However, the phase behavior may not be persistent as the content is depleted. Alternatively, a secondary pump or source, such as a saturator column, may be used to introduce the modifiers, with added flexibility and convenience in programming gradient elution.[12]

C. PUMPS

Conventional HPLC pump head, such as that of a Waters Model 6000 A HPLC pump, may be modified for introducing coolant to maintain the desired temperature of the mobile phase.[29] Modern pumps may be cooled by Peltier electrothermally, with feedback control for compression compensation and minimizing pressure fluctuation. A second pump may be used for delivery of a single or mixed modifier. It may be used for both packed and open-tubular capillary columns.

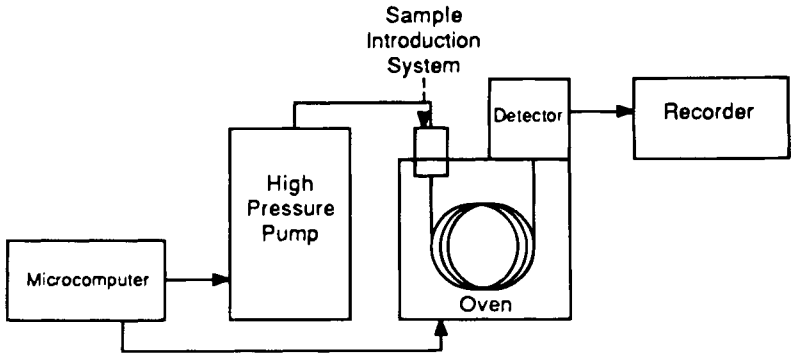

FIGURE 4–3. Schematic diagram of an SFC instrument. (From Lee, M. L. and Markides, K. E., *Analytical Supercritical Fluid Chromatography and Extraction*, Chromatography Conferences, Inc., Provo, 1990, 145–312. With permission.)

Alternatively, a syringe pump may be used for open-tubular capillary SFC for low volume and pulse-free delivery.

D. INJECTION AND COLUMNS

Kirschner and Taylor[30] reviewed the injection processes. For packed column, injection is HPLC-like. For open-capillary column, the small column volume limits the injection volume. Typically, split injection supplemented by a retention gap similar to those in capillary GC is used to avoid flooding the column and band-broadening. Further understanding of the injection process, as indicated by Chester et al.,[12] would enhance SFC applications to achieve the comparable precision achieved by GC and HPLC procedures.

As in HPLC and GC, the columns for SFC may be readily divided into two categories: packed and open-tubular capillary.[6,11,12,31] The basic principles of microcolumn, according to Scott,[32] are included in the chapter on "Automation, Direct-Sample-Analysis, and Microcolumn Liquid Chromatography."[33] Briefly, in SFC, packed columns would essentially include the conventional HPLC column ≥ 1 mm i.d., whereas open-tubular capillary column is similar to the capillary GC column, with an i.d. of 25 to 50 μm.[34] Packed columns may be readily coupled to SFC modified from existing HPLC as described in the previous section. They are easy to use with fast separation, are less susceptible to band-broadening, and are able to accept high injection volume. Capillary column offers high efficiency and sensitivity, low flow rate, a wide range of detectors, and density programming but accepts limited injection volume. With some of the currently available SFC, both columns may be used with a wide range of detectors.

The selectivity of both packed and open-tubular capillary column is determined by the column stationary phase, temperature, and SF. Partition of the analyte may be because of its vapor pressure, or the strength of SF, or both. Rapid analysis is achievable by using short (2 to 5 m) and narrow bore columns (25 to 50 μm), and rapid pressure programming with correspondingly high linear velocity. Alternatively, high-efficiency separation may be achieved by low-density programming and high-separation temperature.

For the open-tubular capillary, the polysiloxane stationary phase is coated onto the inside wall of a typically 50-μm i.d. column, with film thickness ranging from 0.25 to 0.50 μm. Larger load volume and enhanced delectability may be achieved by coating with ≥1 μm. Polarizability and selectivity are affected by the functional groups, such as phenyl, methyl, cyanoproyl, and octyl. A novel liquid crystal polysilane column offers selectivity based on the size and shape of the analytes.[35] Chiral analysis by SFC has also been successfully demonstrated by Gasparrini et al.,[36] and Schleimer and Schurig.[37] More recently, Schmalzing et al.[38] and Jung and Schurig[39] used carbohydrate chiral stationary phase, such as immobilized permethyl-β-cyclodextrin, to separate drugs like hexobarbitol, ketoprofen, ibuprofen, and norgestrel. Armstrong et al.[40] demonstrated its use in GC, CEC and SFC.

E. DETECTORS

With commercially available SFC, both GC and HPLC detectors may be readily incorporated to offer the added versatility. A restrictor is connected to the end of column before entry into GC detectors, and at the outlet of the high pressure and temperature-regulated flow cell of HPLC detectors to maintain the critical pressure of the SF. A variable restrictor offers the flexibility of both flow and pressure programming. Popular GC detectors used in SFC include FID, electron capture detector, alkaline flame detector, chemiluminescence detector, Fourier transform infrared detector, microwave-induced plasma or inductively coupled plasma (ICP), atomic emission detectors, and ion mobility detector. Published studies demonstrated the feasibility of interfacing SFC with mass spectrometers,[41] such as a Paul-type ion trap,[42] off-line plasma desorption mass spectrometry (MS), and atmospheric pressure chemical ionization.[43] Sample introduction into plasma MS was reviewed by Carey et al.[44] Perkins et al.[45] demonstrated SFC-MS analysis of 10 sulfonamides using both moving-belt and modified thermospray interfaces.

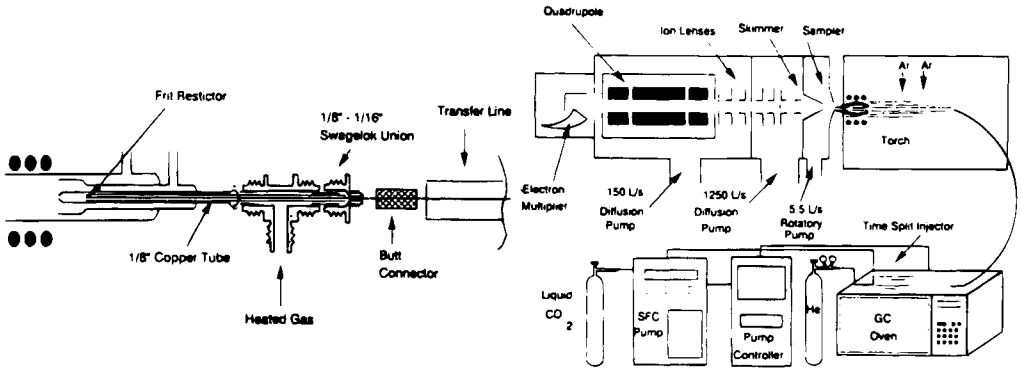

FIGURE 4–4. SFC-ICP-MS instrument diagram showing the SFC–ICP interface. (From Shen, W. L., et al., *Anal. Chem.*, 63, 1491–1496, 1991. With permission.)

As a detector, plasma MS offers sensitive detection for toxic elements of interest in environmental toxicology. Chromium SFC analysis was performed with FID and ICP-MS detection.[46] Shen et al.[47] detailed the interfacing of SFC and ICP-MS for the analysis of tetraalkyltin compounds by using a three-way union for connecting to the restrictor, column, and make-up gas as shown by Figure 4–4. Tomlinson et al.[48] reviewed the ready interfacing of ICP-MS to both SFC and capillary zone electrophoresis for the speciation of tin, chromium, and arensic. Temperature control of the interface was important in minimizing peak-broadening. Additional advantages of the interface include detection of multielements, ready interfacing with LC, high sensitivity, isotopic dilution analysis, and a wide dynamic range. Table 4–1 shows that this detection is more sensitive than FID.

HPLC detectors would include ultraviolet (UV), equipped with high-pressure and temperature-regulated flow cells, refractive index-detector, and fluorescence detector with postcolumn SF mixing with another solvent detected in a low-pressure flow cell. Other detectors of future interest include the nuclear magnetic resonance detector and the electrolytic conductivity detector.

TABLE 4–1
Detection Limits in Picograms for Various Organometals with Both SFC-ICP-MS and FID-Detection[48]

Compound	ICP-MS	FID
Tetrabutyltin (TBT)	0.26	10.3
Tributyltin chloride (TrBT-CI)	0.80	12.5
Triphenyltin chloride (TrPT-CI)	0.57	12.0
Tetraphenyltin (TPT)	0.20	9.0
Trimethylarsine (TMAs)	4.8	NR[a]
Triphenylarsine (TPAs)	3.1	138
Triphenylarsenic oxide (TPAsO)	0.43	3×10^3
Triphenylantimony (TPSb)	0.01	NR
Diphenylmercury (DPHg)	0.05	NR
Chromium(III) 2,2,6,5-tetramethylheptane-3,5-dionate (MHDC)	0.9	NR
Chromium(III) pentane-2,4-dionate (PDC)	3.0	NR

[a] NR, no reading.

IV. SFC APPLICATIONS

Currently, routine clinical applications by SFC had not been demonstrated for toxicology and TDM,[5] whereas potentially useful analysis has been published for environmental toxicology. Packed-column analysis was initially shown by Gere et al.[15] SFC-MS drug detection was demonstrated by Crowther and Henion.[49] More recently, preliminary studies demonstrated limited applications of SFC for drug analysis in both toxicology and TDM.[3,4,7] Later et al.[24] proposed that SFC offered drug analysis with high efficiency and without derivatization of polar drugs and metabolites. It was ideal for analyzing thermally labile drugs, and for drugs not readily detected by HPLC-UV and fluorescence detectors. Pinkston et al.[50] investigated the efficacy of open-capillary SFC-MS for the determination of drugs in biological matrix, using a model drug (mebeverine) in dog plasma. They observed that column performance varied as in GC and HPLC, and there was need to increase allowable injection volume for increasing detection limit. Potential analysis of mycotoxin by SFC-MS may complement other instrumental techniques, such as LC-MS, MS-MS, and immunological methods.[51]

A. TOXICOLOGY

Crowther and Henion[49] demonstrated analysis of polar drugs using packed-column SFC-MS. The drugs included caffeine, codeine, cocaine, phenylbutazone, and methocarbamol. The column, packed with 5-μm particles, was connected to the MS with a direct liquid–introduction interface. SF carbon dioxide was mixed with various amount of methanol as a modifier. A restrictor was placed into the exit tube into the MS. For the analysis of caffeine, a silica column was used with a mobile phase of carbon dioxide/methanol (8:2). Retention time was 2.3 min. A single-ion chromatogram was obtained with on m/z of 195. An amino column with a mobile phase of carbon dioxide/ethanol (9:1) was used to analyze codeine and cocaine, with retention times of 2.3 and 1 min, respectively. Total ion chromatograms were obtained for m/z = 300 and 282. SFC-MS (chemical ionization) detected a few fragments of 282 and 300 (M + 1)+ for codeine, and 182 and 304 (M + 1)+ for cocaine. Detection limit for cocaine was 20 ng per injection. For analysis of phenylbutazone and metabolites, the negative chemical ionization showed enhanced detection. By using a silica column and a low ion-source temperature of 140°C to minimize decomposition, thermally labile methocarbamol was analyzed by carbon dioxide/methanol (85:15). Later et al.[24] used an open-capillary column and SF carbon dioxide for analyzing dexamethasone and betamethasone, predisolone, and cortisone and hydrocortisone. With the low analysis temperature of 130°C, the two epimers, betamethasone and dexamethasone, were totally resolved as a result of minimized on-column epimerization. Another application showed the presence of 9-carboxyl tetrahydrocannabinol in a human urine extract, confirmed by immunoassay. Figure 4–5 shows the SFC chromatogram.[24]

Berger and Wilson[52] investigated SFC analysis of stimulants. They were strongly retained, requiring a ternary SF mobile phase with modifier, such as carbon dioxide/methanol/isopropylamine. Figure 4–6 shows the SFC chromatogram of stimulants: amphetamine, nylidrine, phenmetrazine, methamphetamine, phenylpropanolamine, ephedrine, naphazoline, phenylephrine, and hydroxyamphetamine. Berger and Wilson concluded that modifier concentration changed significantly the retention and selectivity of stimulants, and that the injection of a low volume of an aqueous solution of tetrahydrozoline was compatible with SFC.

B. THERAPEUTIC DRUG MONITORING

Wong and DellaFera[3] demonstrated in a preliminary study of phenobarbital (as shown by Figure 4–7), that solid-phase extraction minimized contamination that would result in column selectivity loss. Based on this study, the selectivity of SFC was proposed to be "HPLC normal-phase like." This characteristic is important in the design of subsequent sample preparation protocols involving biological matrices and is helpful in establishing selectivity and elution order.

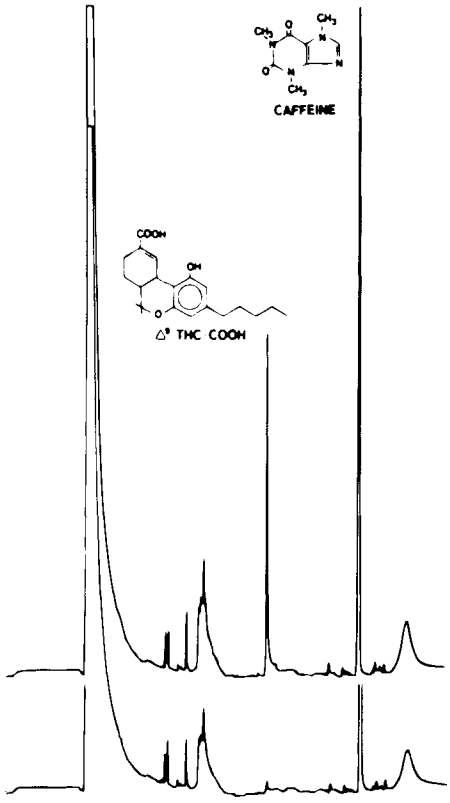

FIGURE 4–5. Capillary supercritical fluid chromatograms of human urine extracts containing 9-carboxy-tetrahydrocannabinol (top) and control urine (bottom). Conditions: supercritical carbon dioxide at 120°C; 15 m × 50 μm (i.d.); SE-33 column; density programmed from 0.25 g/ml after a 15-min hold to 0.4 g/ml at a rate of 0.015 g/ml/min, then to 0.65/ml at 0.01 g/ml/min; and FID at 280°C. (From Later, D. W., et al., *J. Chromatogr.*, 24, 249–253, 1986. With permission.)

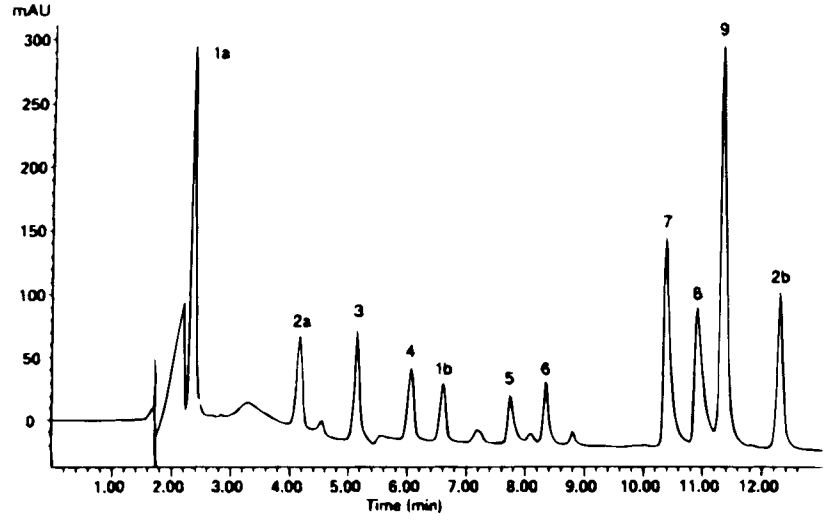

FIGURE 4–6. Composition program of the test mix. Conditions were as follows: 2 ml/min of methanol plus 0.5% isopropylamine in carbon dioxide, 40°C, 200-bar outlet pressure, and 5-μl injection. Initial modifier concentration was 5% and then programmed at 1.5%/min to 20%, where it was held for the remainder of the procedure. Solute concentrations were 110 ppm each, except naphazoline, which was 11 ppm. Detection by UV spectrophotometry was at 220 nm (4-nm bandwidth). Solutes were as follows: 1a and 1b, amphetamine; 2a and 2b, nylidrine; 3, phenmetrazine; 4, methamphetamine; 5, phenylpropanolamine; 6, ephedrine; 7, naphazoline; 8, phenylephrine; and 9, hydroxyamphetamine. (From Berger, T. A. and Wilson, W. H., *J. Pharm. Sci.*, 84, 489–492, 1995. With permission.)

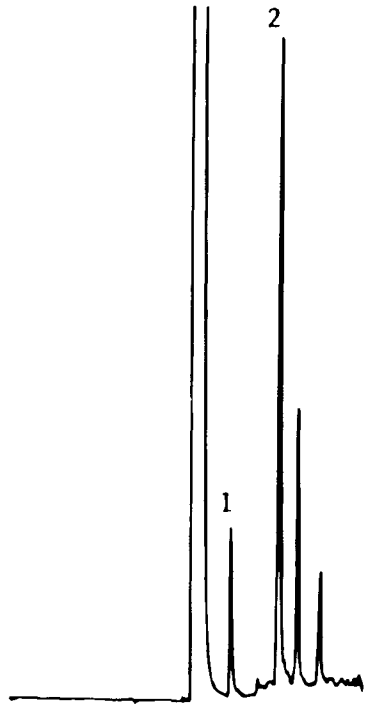

FIGURE 4–7. SFC chromatogram of a patient serum extract by solid-phase extraction. Peak identification: 1, internal standard—barbital, 5.6 min; and 2, phenobarbital (20 mg/L), 6.8 min. (From Wong, S. H. Y. and DellaFera, S. S., *J. Liq. Chromatogr. Clin. Anal.*, 13, 1105–1124, 1990. With permission.)

Cyclosporine A (CsA) and tacrolimus (FK 506), a macrolide, are potent immunosuppressants clinically used for treatment of liver transplant patients.[4,6,7] CsA is also clinically used for kidney, heart, lung, bone marrow, pancreas, and other transplants, whereas a multicenter FK 506 trial is in progress for kidney transplant. Rapamycin is currently evaluated for liver transplant and others.[53,54] SFC parameters for their analyses were SB-biphenyl column, 50 μm i.d., 10 m length, carbon dioxide as the mobile phase, 70°C, 100 to 300 atoms at 10 atoms/min, injection volume of 20 nl, and FID as the detector. Figure 4–8 shows the analysis of whole-blood extract of CsA. FK 506 and its tautomer (as shown by Figure 4–9A FK 506) eluted with retention times of 17.5 and 17.8 min, respectively, whereas an HPLC chromatogram (Figure 4–9B) shows the "more polar" tautomer peak at about 4 min before the FK 506 at 5 min. This HPLC elution order confirmed the observation previously established by Friob et al.[55] The "normal-phase" selectivity of SFC, with the HPLC selectivity as a reference, was thus reconfirmed as in our previous observation.[3] Figure 4–10 (A and B) shows the retention times of rapamycin by SFC and HPLC to be 18.4 and 5 min, respectively.[7]

The preliminary SFC analyses of FK 506 whole-blood extracts were not successful. This was caused by the low concentration of FK 506 in whole blood in the range of 5 to 30 ng/ml, limited injection volume of the open-capillary SFC, and the relative "low" sensitivity of FID. Although trough concentrations of 10 to 30 ng/ml were proposed for rapamycin by Yatscoff et al.[53,54] and based on the unsuccessful FK 506 studies, no attempt was made to perform SFC analysis of rapamycin in whole blood. In the future, a mass spectrometer interfaced with SFC for higher sensitivity detection may be used to overcome some of these limitations.

Berger and Wilson[56,57] systematically investigated the SFC retention and selectivity of antidepressants and phenothiazine antipsychotics by using a packed cyanopropyl column at various tem-

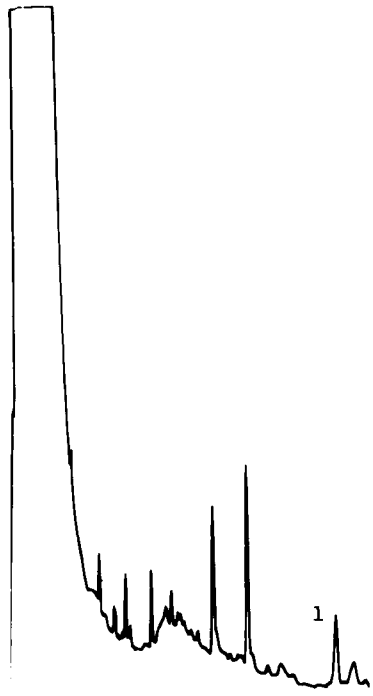

FIGURE 4–8. SFC chromatogram of a whole-blood extract of a bone marrow transplant patient with about 228 μg/l of CsA at 18.4 min. Pressure programming from 100 to 300 atm at 10 atm/min. Peak identification: 1, CsA. (From Wong, S. H. Y., et al., *J. Liq. Chromatogr. Clin. Anal.*, 17, 2093–2109, 1994. With permission.)

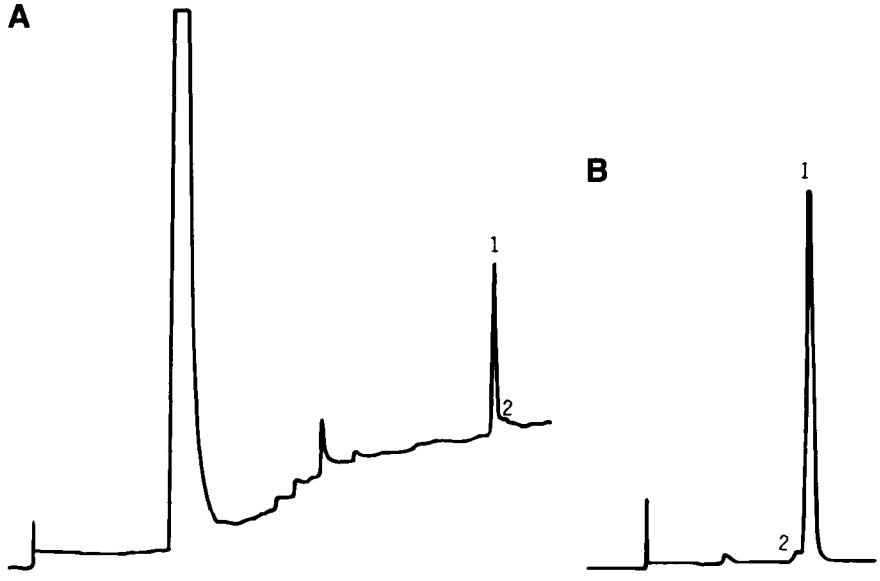

FIGURE 4–9. (A) SFC and (B) HPLC chromatograms of FK 506. Peak identification: 1, FK 506 and 2, FK 506 tautomer. (From Wong, S. H. Y., et al., *J. Liq. Chromatogr. Clin. Anal.*, 17, 2093–2109, 1994. With permission.)

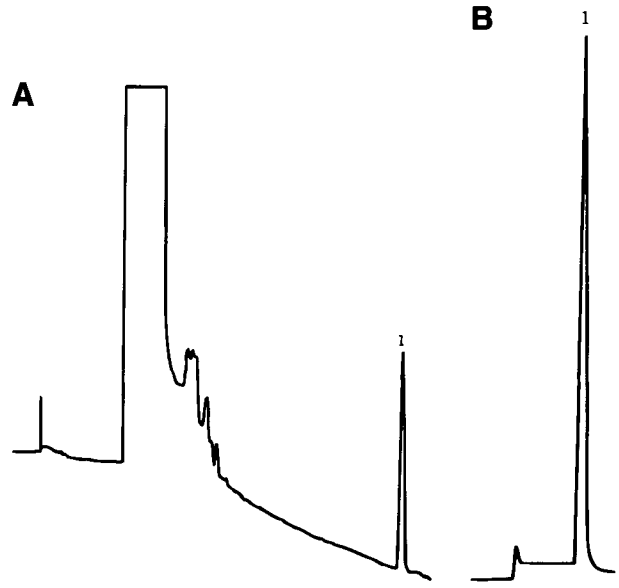

FIGURE 4–10. (A) SFC and (B) HPLC chromatograms of rapamycin. (From Wong, S. H. Y., et al., *J. Liq. Chromatogr. Clin. Anal.*, 17, 2093–2109, 1994. With permission.)

peratures. The tertiary mobile phase consisted of carbon dioxide, methanol, and isopropylamine. For a mixture of ten antidepressants—amitriptyline, imipramine, nortriptyline, desipramine, protriptyline, buclizine, benactyzine, hydroxyzine, perphenazine, and thioridazine—analysis for five selected antidepressants was completed in less than 6 min (as shown by Figure 4–11), with a detection limit of 88 ppb (ng/ml), and for ten phenothiazines—trifluprомazine, carphenazine, methotrimeprazine, promazine, molindone, perphenazine, chloroprothixine, deserpidine, thiothixene, and reserpine—11 min at 88 ppb (ng/ml) (as shown by Figure 4–12). Changing modifier concentration significantly affected the selectivity for antidepressants, but not for phenothiazines. Temperature programming offered the best resolution of phenothiazines.

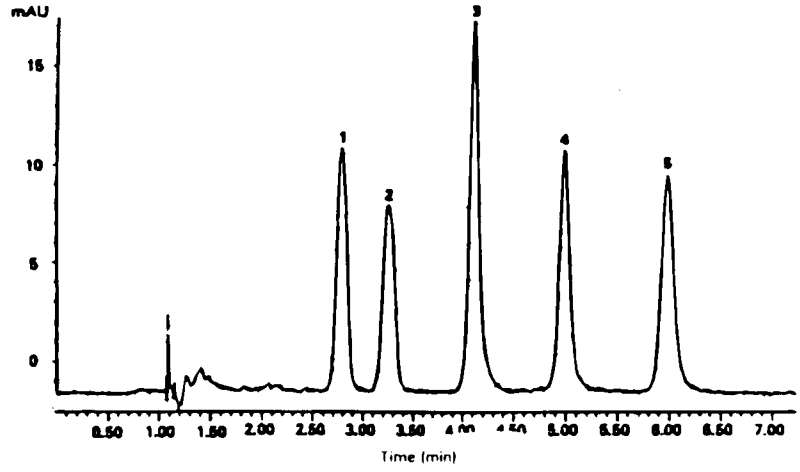

FIGURE 4–11. Greater resolution of mix 1. Conditions were as follows: 10% modifier (MeOH + 0.5% IPAm); 200-bar outlet pressure; and 50°C. Test mix 1: 1, amitriptyline; 2, imipramine; 3, nortriptyline; 4, desipramine; and 5, protriptyline. (From Berger, T. A. and Wilson, W. H., *J. Pharm. Sci.*, 83, 287–290, 1994. With permission.)

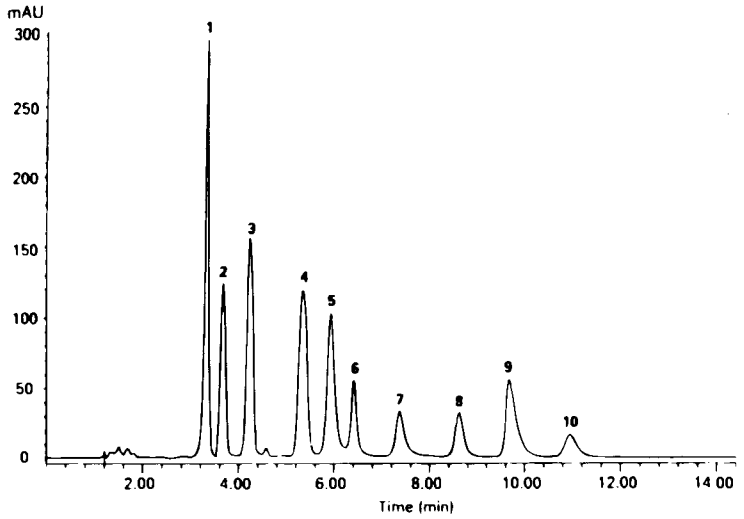

FIGURE 4–12. Chromatogram of a temperature program showing essentially baseline resolution. Program: initial temperature, 30°C; then 6°C/min to 60°C; and hold 5 min. SFC chromatogram of antipsychotics. Peak identification: 1, triflupromazine; 2, carphenazine; 3, methotrimeprazine; 4, promazine; 5, molindone; 6, perphenazine; 7, chloroprothixine; 8, deserpidine; 9, thiothixene; and 10, reserpine. (From Berger, T. A. and Wilson, W. H., *J. Pharm. Sci.*, 83, 281–286, 1994. With permission.)

C. MISCELLANEOUS DRUGS

By interfacing SFC directly to HPLC, Nair and Huber[58] analyzed caffeine, ibuprofen, and an impurity by using a commercial SF extractor loaded with Celite. SF carbon dioxide was used to elute the drugs, followed by direct injection into the HPLC for qualitative analysis.

By using an open-capillary column coating mono-6-*O*-octamethylenepermethyl-β-cyclodextrin to dimethylpolysiloxane, Schurig et al.[14] demonstrated unified enantioselective capillary chromatography—GC, SFC, LC, and CEC analysis of hexobarbital—as shown by Figure 4–1. SFC analysis was performed by using carbon dioxide at 60°C. Miniaturization offered the following advantages: low flow rate enhancing LC-MS interface, low-pressure drop, minimized packing and mobile phase utilization, enhanced temperature programming, and mass sensitivity.[14] In comparison, SFC, along with GC and LC, were not as favorable as CEC in chiral separation, resolution, and efficiency.

Nonsteroidal anti-inflammatory agents were analyzed using SB-biphenyl-30 or SB-cyanopropyl-50 columns open-capillary columns and carbon dioxide as the mobile phase.[59] Using pressure gradients, a mixture of flufenamic acid, mefenamic acid, fenbufen, and indomethacin was separated by the SB-biphenyl-30, whereas two mixtures ([1] flufenamic acid, mefenamic acid, fenbufen, ketoprofen and acetylsalicylic acid; and [2] ibuprofen, naproxen, fenoprofen, ketoprofen and tolmetin) were analyzed by the SB-cyanopropyl-50 column. Total analysis time ranged from 20 to 25 min. This procedure was used for dosage formulation analysis.

Pinkston et al.[50] investigated the efficacy of open-capillary SFC-MS for the determination of drugs in biological matrix, using a model drug, mebeverine, in dog plasma. After solid-phase extraction with a C-18 cartridge, the extract was injected into an open-capillary column, SB-methyl-100 with carbon dioxide as the mobile phase. For a calibration curve of 6 to 60 ng/ml, the accuracy was about ±15%, with a relative standard deviation of 5 to 12.5%. Pinkston et al. observed variance in column performance and suggested increasing the allowable injection volume for enhanced detection.

Perkins et al.[43] demonstrated SFC-MS analysis of 10 sulfonamides using both moving-belt and modified thermospray interfaces. Packed-column SFC analysis was performed by using either a silica or amino-bonded column with carbon dioxide mixed with 10 to 25% methanol as modifier.

Using a 5-μm amino-bonded phase column with carbon dioxide and 15% methanol as the SF, and a moving belt-interface, about 26 ng was identified in a spiked porcine kidney extract.

Panaxadiol and panaxatriol in ginseng and Chinese herbal medicine were analyzed by using open-capillary SFC, following liquid–liquid extraction, partition, and then adsorption column sample purification.[60]

Felodipine in sustained-release tablet, a calcium channel blocker used for the treatment of hypertension, was extracted by a carbon dioxide/methanol (8%) mixture, followed by packed-column SFC analysis, electron capture, and multiwavelength detections.[61] Felodipine was separated from its potential oxidative product, H152/37, in less than 6 min. The advantages included faster analysis time, compared with LC, and lowered cost in solvent utilization and disposal.

After solid-phase extraction with C-18 cartridges of serum spiked with phenylbutazone and its major metabolite, oxyphenylbutazone, SFC analysis was performed with a C-18 packed column with carbon dioxide/methanol (5%) as the mobile phase.[62] Detection limits for phenylbutazone and oxyphenylbutazone were 0.1 and 1 mg/l. This procedure offered similar performance as in HPLC and was also used for dosage form analysis with high recovery.

Artemisinin (qinghaosu), a sesquiterpene lactone endoperoxide antimalarial drug, was isolated from *Artemesia annua* L. in China.[63] It was extracted in whole blood by hexane, followed by packed-column SFC analysis using a Deltabond CN column with carbon dioxide as the mobile phase and electron-capture detection. Detection was possibly because of the endoperoxide moiety, with a limit of 20 μg/l.

Packed column subcritical fluid chromatography and SFC for enantiomeric analysis of basic and acidic drugs were investigated by Kot et al.[64] The drugs included β-blockers (clenbuterol, metaprolol, Org-β-A, propranolol, and salbutamol), nonsteroidal anti-inflammatory drugs (ibuprofen, fenoprofen, ketoprofen, flurbiprofen, tlaprofenic acid, and naproxen), and benzodiazepines (oxazepam, lorazepam, and others [mianserin and Org-B]). Figure 4–13 shows the four chiral stationary phases. Analysis of mianserin by subcritical fluid chromatography was shown in Figure 4–14. When compared with the capillary column, packed-column analysis was generally faster. Semipreparative separations were demonstrated for 250 μg of mianserin and 1 mg of flurbiprofen in less than 20 min. For optimized selectivity, three columns (Chiralpak AD, Chiralcel CD, and Chirex 3022) were combined with carbon dioxide/methanol (4 to 30%, and 0.5% of triethylamine and 0.5% of trifluoroacetic acid) as the mobile phase to resolve all of the aforementioned drugs (as shown in Figure 4–15).

SFC analysis of β-blockers—atenolol, alprenolol, labetolol, metoprolol, practolol, pindolol, propranolol, and xamoterol—were satisfactorily performed by using a column, 250 × 4.6 mm i.d.,

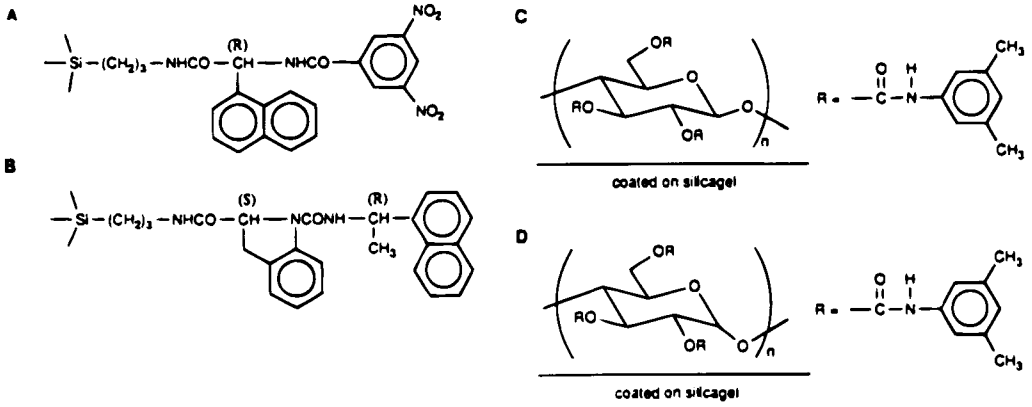

FIGURE 4–13. Structures of the chiral stationary phases. (From Kot, A., et al., *J. Chromatogr. Sci.*, 32, 439–448, 1994. With permission.)

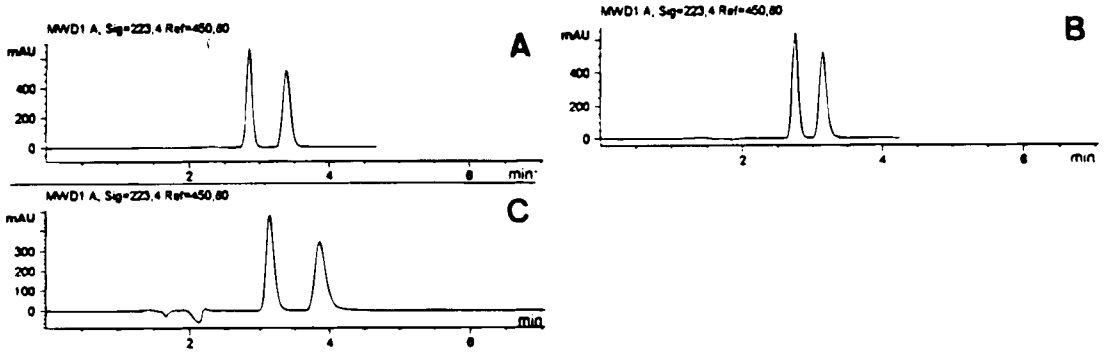

FIGURE 4–14. Methanol (A), ethanol (B), and isopropanol (C) are the modifier. Conditions: column, Chiralcel OD; mobile phase, 30% modifier with 0.5% isopropylamine in carbon dioxide; flow rate, 2 ml/min; pressure, 200 bar; temperature, 30°C; and detection, 223 nm. (From Kot, A., et al., *J. Chromatogr. Sci.*, 32, 439–448, 1994. With permission.)

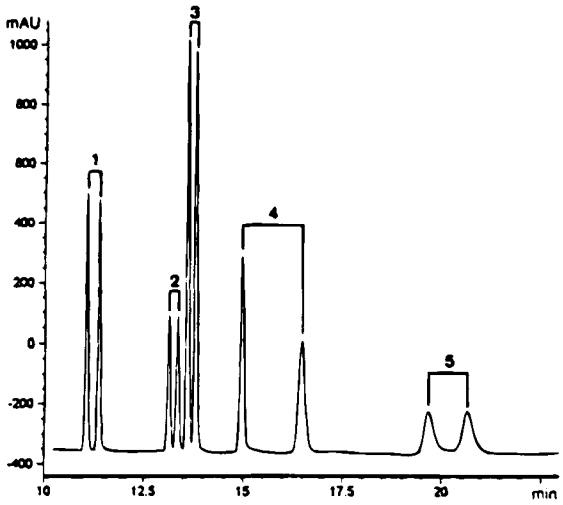

FIGURE 4–15. Separation on different racemates on a triplet column. Conditions: columns, Chiralpak AD-Chiralcel OD-Chirex 3022; mobile phase, carbon dioxide/methanol (0.5% triethylamine + 0.5% trifluoroacetic acid) with methanol programmed from 4% (5 min) to 30% at 5%/min; flow rate, 2 ml/min; pressure, 200 bar; temperature, 25°C. Peak identification: 1, ibuprofen; 2, fenoprofen; 3, clenbuterol; 4, propanolol; and 5, lorazepam. (From Kot, A., et al., *J. Chromatogr. Sci.*, 32, 439–448, 1994. With permission.)

packed with aminopropyl or diol-bonded phases and carbon dioxide/methanol/triethylamine (80:20:0.2) as the mobile phase.[65] Triethylamine was used to mask the silanol groups of the packing. Most of these drugs were well resolved with a symmetrical peak (as shown in Figure 4–16). Kot et al. also attributed the retention of diol column analysis showing normal-phase selectivity. However, no serum extracts analysis was included in this study.

Siret et al.[66] investigated the chiral discrimination mechanism of SFC chiral analysis of β-blockers by using two stationary phases: 3,4-dinitrobenzoyl tyrosine and ChyRoSine-A. The mobile phase consisted of carbon dioxide/methanol/propylamine. Using either nuclear magnetic resonance or the molecular model, the authors suggested carbon dioxide bridging with the hydroxyl and amine protons of the solute. This offered lower acid–basic properties and a more rigid conformity, resulting in the desired chiral discrimination. Potential analysis of mycotoxin by SFC-MS may complement other instrumental techniques, such as LC-MS, MS-MS, and immunological methods.[51]

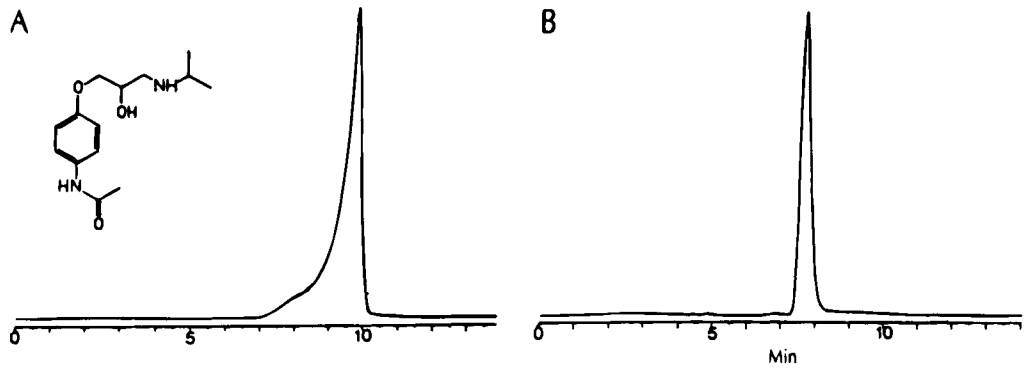

FIGURE 4–16. SFC of practolol (structure in inset) on an aminopropyl-bonded silica column with methanol (A) and carbon dioxide/methanol/triethylamine (B) as the mobile phase. Poor peak shape is clearly apparent in the absence of triethylamine. (From Bailey, C. J., et al., *J. Chromatogr. Sci.*, 32, 426–429, 1994. With permission.)

D. ENVIRONMENTAL TOXICOLOGY

Laintz et al.[67] investigated SFC analysis of arsenic and antimony species in water and biological samples for environmental toxicology. Initial extractions were performed with lithium bis(trifluoroethyl) dithiocarbamate for arsenic(III) and antimony(III). For arsenic(V) and antimony(V), the extraction was preceded by a reduction with potassium and sodium thiosulfate. Detection limits for arsenic and antimony were 7 and 11 pg, respectively.

Chromium SFC analysis was performed by using a SB-biphenyl-30 column and nitrous oxide or carbon dioxide as the mobile phase,[46] with FID and an ICP-MS detector. The compounds included a pair of β-ketonate chromium compounds—chromium(III) 2,4-pentanedionate and chromium(III) 2,2,6,6-tetramethyl-3,5-heptane-dionate—and a thermally labile organochromium-pentamethylcyclopentadienyl chromium dicarbonyl dimer. Because using carbon dioxide as the mobile phase generated an isobaric interference at *m/z* 52 from $^{40}Ar\ ^{12}C^{+}$, nitrous oxide was used instead. Because of thermal decomposition at the interface, organochromium-pentamethylcyclopentadienyl chromium dicarbonyl dimer was nondetectable by ICP-MS (as shown in Figure 4–17). Calibration was linear from 0.1 to 100 ng for this procedure. Detection limit by ICP-MS ranged from 0.9 to 3 pg, and detection limit by FID ranged from 10 to 250 pg. Laintz et al. suggested this as a potentially useful application in environmental toxicology.

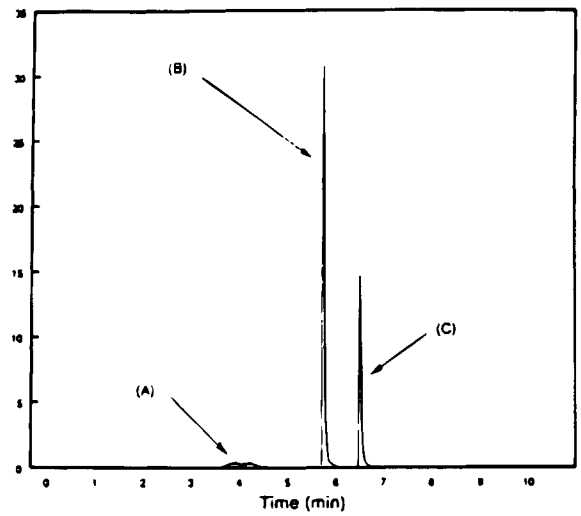

FIGURE 4–17. SFC-ICP-MS chromatogram at *m/z* 52 using the optimal conditions and a 10-ng mixture. A, Solvent; B, chromium(III) 2,2,6,5-tetramethylheptane-3,5-dionate; C, chromium(III) pentane-2,4-dionate. (From Carey, J. M., et al., *J. Chromatogr. A*, 662, 329–340, 1994. With permission.)

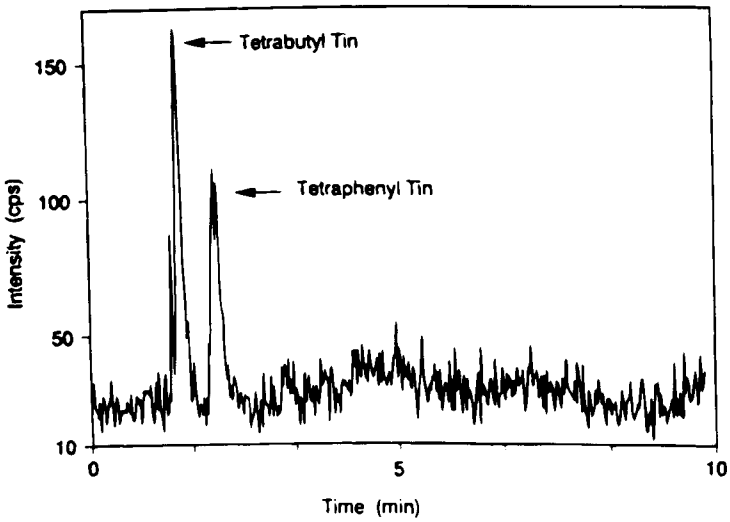

FIGURE 4–18. SPC-ICP-MS chromatogram for a 1-pg injection of tetrabutyltin and tetraphenyltin. (From Shen, W. L., et al., *Anal. Chem.*, 63, 1491–1496, 1991. With permission.)

Shen et al.[47] analyzed tetraalkyltin compounds—tetramethyltin (TMT), tetrabutyltin (TBT), tetraphenyltin (TPT), tributyltin acetate (TBTA), and dibutyltin diacetate (DBTDA)—by SFC using an open-capillary SB-Octyl-50 column and carbon dioxide as the mobile phase, with detection by ICP-MS. Organotin compounds may be used as antifouling agents in paint, polymer stabilizer, and pesticides. Organotin pollution is caused by their industrial applications and subsequent release into the environments. Calibration ranged from 1 to 1000 pg, with a detection limit of 0.034 pg for TBT and 0.047 pg for TPT. Figure 4–18 shows the chromatogram of 1 pg each of TBT and TPT. However, SFC was not optimized for the determination of TMT, TBTA, and DBTDA.

Tomlinson et al.[48] reviewed interfacing ICP-MS to both SFC and capillary zone electrophoresis. SCF-ICP-MS was used for speciation of organotin, arensic, and chromium compounds. With optimized interface temperature, sensitivity for tin compounds was established at a low picogram level. For arsenic compounds, methanol was used as a modifier to avoid an interference at *m/z* 75. Review of chromium analysis was similar to that previously described. Table 4–1 shows the higher sensitivity of ICP-MS to FID for the various metals.

V. CONCLUSIONS

The application of SFC for toxicology and TDM in particular has been stagnant. This is probably because of the established efficacy of competing chromatographic techniques, such as GC and HPLC, in fulfilling the needs, as well as the use of immunoassays for the majority of the drug assays. There is currently a lack of published studies demonstrating the uniqueness of SFC for performing routine clinical drug analysis not possible by the aforementioned competing methods. Furthermore, some of the published studies were performed with drug standard solutions. Thus, their clinical efficacy remains unsubstantiated. With the emergence of CEC, the enhanced sensitivity offered by this newer microcolumn methodology would also fulfill some of the previously established advantages offered by open-tubular capillary SFC.

However, recent studies may point to the potential revitalization of SFC by using packed column. From these studies, there is increasing evidence of the normal-phase selectivity of SF carbon dioxide. Potential applications in environmental toxicology using ICP-MS as a multielements detector would certainly strengthen future consideration for SFC. Along with interfacing to other MS and the advent of SFE, SFC may yet provide a unique complementary alternative for toxicology.

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Chapter 5

SUPERCRITICAL FLUID EXTRACTION AS A SAMPLE PREPARATION TOOL IN ANALYTICAL TOXICOLOGY

Robert J. Maxwell and Janet F. Morrison

CONTENTS

I. INTRODUCTION

One of the key challenges faced by analytical toxicologists is the development of highly sensitive, reliable techniques that can be performed in a rapid and economical fashion, preferably through automation. The accuracy of the analytical techniques used for trace analyte detection depends on the adequacy of the sample preparation method used to isolate the target species from the sample matrix. Biological samples pose unique challenges to the analytical chemist from the standpoint of sample preparation and extraction. Compounds of interest must be released and recovered from complex matrices, such as tissue, blood, urine, and hair before the application of high-sensitivity

analytical measurements. Sample preparation methods must be designed to recover quantitatively the target species from a complex background of potentially interfering matrix co-extractables in a manner that also preserves analyte integrity.

The possession of unique properties intermediate between those of gases and liquids makes supercritical fluids (SFs) attractive alternatives to conventional liquid solvents for the extraction of trace analytes from complex matrices. The "gas-like" mass transport properties (i.e., low viscosities and high diffusivities) of SFs impart excellent matrix-penetrating power, thereby permitting more rapid and efficient extraction from difficult-to-access sample types when compared with liquid extractants. Densities (and, hence, solvating power) of SFs approach those of liquid solvents, and this solvating power can be easily varied by changing extraction temperature, pressure, or fluid composition. The existence of a greater number of parameters for method optimization and the ease of variation of these parameters allows a degree of selectivity "tuning" with supercritical fluid extraction (SFE) not readily available with traditional liquid–liquid extraction (LLE) or solid-phase extraction (SPE) methods. The present availability of fully automated commercial SFE instrumentation offers the potential for significant reductions in analysis time because of improvements in sample throughput.

In addition to time and selectivity advantages, SFE is also attractive from an environmental standpoint. Carbon dioxide (CO_2) is currently the most widely used fluid for SFE, primarily because of its easily attainable critical parameters (31°C and 7.4 MPa), low toxicity, chemical inertness, low cost, and availability. Because CO_2 is a gas at ambient conditions, the generation of hazardous solvent waste is virtually eliminated, and postextraction concentration steps are greatly simplified. Many laboratories are currently evaluating SFs as "environmentally friendly" alternatives to liquid solvents for sample preparation and extraction, spurred by the high costs associated with solvent purchase and disposal and regulatory measures, such as the Environmental Protection Agency's hazardous waste reduction program and the Montreal Protocol calling for production phaseout of ozone-depleting chlorofluorocarbons.[1–3] With the advent of automated commercial instrumentation, analytical SFE technology is now finding routine use in many of these laboratories. The literature is replete with demonstrations of SFE for the recovery of a wide range of analytes, including environmental pollutants, agrochemicals, essential oils, fats, polymer components and additives, natural products, drugs, and metal-containing compounds, from diverse sample matrices. The vast majority of these applications have involved the isolation of environmentally relevant compounds (e.g., hydrocarbons, polycyclic aromatic hydrocarbons [PAHs], polychlorinated biphenyls [PCBs], and agrochemicals) from environmental solids (e.g., soils, sediments, and air particulate matter). For a survey of SFE applications, the reader is referred to a recent literature review.[4]

Despite demonstrated successes in other disciplines, acceptance and application of SFE in the field of analytical toxicology have been much slower, and the potential benefits of this sample preparation technology for biological samples have remained largely unexplored. The prospects of increased analysis efficiency, lower solvent consumption, and reduced hazardous waste generation, coupled with the selectivity advantage afforded by SFE, suggest the suitability of this technology for sample preparation and extraction in toxicological applications. However, unlike many of the volatile and semivolatile analytes of interest in environmental SFE applications, target analytes in toxicological applications are often highly polar in nature (e.g., drugs), and the matrices in which these analytes are entrained often have high moisture and/or co-extractable lipid content. Recent analytical developments in strategies for improving selectivity, sensitivity, and extractability from biological matrices (such as the incorporation of selective sorbents, dispersants, and/or solid supports in the extraction vessel; the use of selective fluid and matrix modifiers; the direct coupling of SFE with gas chromatography [GC] or GC-mass spectrometry [GC-MS]; and the combination of SPE with SF elution) have demonstrated the ability of SFE to meet successfully the challenges

* 1 MPa = 9.87 atm = 145.04 psi = 10 bar = 10.20 kg/cm^2

posed by such matrices. Indeed, quantitative SFE recoveries of toxicologically relevant analytes, such as drugs-of-abuse, therapeutic drugs, veterinary drug residues, pesticides, herbicides, and PCBs have recently been reported from tissues, hair, urine, and blood (see "Applications of SFE in Analytical Toxicology"). These developments have coincided with advances in our fundamental understanding of analyte–SF–matrix interactions and the role of modifiers and additives in SFE.

The following sections provide the reader with a basic introduction to SFE, with particular emphasis on the practical and applied aspects, as well as the advantages and limitations of the technology as they relate to biological samples. It is the authors' goal to provide sufficient background and examples to establish a sound foundation of knowledge, so that potential and current users can make informed decisions regarding SFE method development and implementation in the analytical toxicology laboratory. For a more detailed treatment of the fundamental principles and theoretical aspects of SFE, the reader is referred to several recent reviews.[5–11]

II. SOLUBILITY MEASUREMENTS OF ANALYTES IN PURE SUPERCRITICAL FLUIDS (SFs)

Knowledge of the solubility of analytes in SFs is helpful in assessing whether a particular compound has the potential for extraction by this technology. Such measurements have been of interest to investigators for over a century. The solubility of a compound can be described as the concentration of that compound in the supercritical phase at equilibrium with the pure fluid. The first report of critical point phenomena was by Hannay and Hogarth[12] in 1879. They measured the solubilities of certain inorganic salts in supercritical ethanol. Since that time, other investigators have measured the SF solubilities of many compounds, both organic and inorganic in nature. The most extensive study to date was reported by Francis[13] in 1954. His pioneering investigation included the solubilities of 261 organic compounds in near-critical CO_2 (6.2 MPa, 25°C). His data is still useful to investigators, because it attempts to classify the solubilities of compounds according to functional group, such as esters, alcohols, carboxylic acids, amines, heterocyclics, amides, nitriles, and phenols.

Stahl and Glatz[14] investigated the solubilities of 35 steroids in SF-CO_2 at 40°C over the pressure range of 8 to 20 MPa. Using the results of this study as a basis, they constructed a table describing the relationship between substituents on the steroid structures and their solubilities in SF-CO_2. The results demonstrated that increasing the number of free hydroxyl groups on the basic backbone structure increased the minimum pressure at which the steroid could be extracted. Similarly, when a carboxyl group is added to the structure, as in the case of the bile acid desoxycholic acid, an even higher minimum pressure was required to begin extraction. This concept was elucidated earlier by Giddings et al.[15] as the "threshold density," which is defined as that density (or pressure) at which detectable solubilization of the analyte commences.

Stahl and Willing[16] extended their earlier investigation of threshold densities to a series of alkaloids extracted at 18 to 23°C and 8 to 10 MPa, and, in a later investigation,[17] they studied this alkaloid series at higher temperatures and pressures. The results of the latter study are listed in Table 5–1. Among the opium alkaloids tested, codeine had a threshold pressure of 9 MPa, whereas morphine displayed only slight solubility at 20 MPa, because of its increased polarity. In addition to SF-CO_2, these investigators examined the solubilities of the alkaloids in SF nitrous oxide (SF-N_2O) and SF trifluoromethane (SF-CHF_3 or fluoroform). This is one of the few investigations in the literature that reports the solubilities of organic compounds in fluids other than CO_2. With the exception of codeine, all of the drugs that they examined were more soluble in SF-CHF_3 than in CO_2 or N_2O. The solubility of morphine was also determined by these workers; however, they did not include the results for this compound in the table, because it exhibited little solubility in SF-CO_2 (5 μg/g at 20 MPa, 40°C). Although this value may be low compared with other opium alkaloids, its solubility may be sufficient for its extraction as a trace level residue in a biological matrix. From these investigations of the solubilities of natural products in SFs, Stahl[18] conceived of what he termed the solubility "rules of thumb" that are summarized below:

TABLE 5–1
Solubilities[a] of Opium Alkaloids in Three Dense Gases[b]

	CO_2	CHF_3	N_2O
Codeine	900	560	1000
Thebaine	200	660	
Papaverine	40	710	
Noscapine	90	620	200

[a] Solubilities in μg/g.
[b] Conditions: 15 MPa, 40°C.

Source: Adapted from Stahl, E., et al., *Dense Gases for Extraction and Refining*, Springer-Verlag, New York, 1988. With permission.

1. Hydrocarbons and other typically lipophilic organic compounds of relatively low polarity, such as esters, ethers, lactones, and oxides, can be extracted at low pressures (i.e., between 7 and 10 MPa).
2. Compounds with strongly polar functional groups such as—OH and—COOH can be extracted, but with more difficulty. For example, substances with up to 3 phenolic hydroxyl groups or with 1 carboxyl and 2 hydroxyl groups are still extractable. Aromatic compounds with 1 carboxyl and 3 or more hydroxyl groups cannot be extracted.
3. More strongly polar compounds, such as sugars, amino acids, and similar compounds, cannot be extracted by SF-CO_2 in the pressure range up to 50 MPa. (Remember that these guidelines are "rules of thumb." Stahl based these concepts on the extraction of pure substances from matrices such as quartz wool. Different results may be obtained when trace levels of substances are to be isolated from matrices such as hair, blood, tissue, and urine using pure or modified SFs.)

The SF-CO_2 solubilities of some individual and classes of compounds of interest to toxicologists have been determined by a number of investigators. Among these compounds are the steroids. In 1986, Wong and Johnston[19] obtained the solubilities of cholesterol, stigmasterol, and ergosterol in SF-CO_2 with and without co-solvents over a range of 10 to 35 MPa. The solubility curves for these compounds measured at 35°C are shown in Figure 5–1. Although these sterols are similar in structure, large differences were observed in their solubilities. For instance, cholesterol exhibited three times greater solubility in SF-CO_2 than stigmasterol and 50 times greater solubility than ergosterol. These differences in sterol solubility were attributed to corresponding wide differences in vapor pressures, with the vapor pressure of cholesterol about two times greater than stigmasterol and 35 times greater than ergosterol. This example illustrates an important difference between SFE and conventional liquid-liquid extraction (LLE), namely, that interactions or physical properties that are not important in organic solvent-based extractions may assume a larger significance in solute-CO_2 extractions. Another study by Lee et al.[20] determined the solubilities of cholesterol, progesterone, and testosterone in SF-CO_2 and mixtures of SF-CO_2 and N_2O between 8 and 25 MPa. This study demonstrated that these compounds were soluble in the SFs at concentrations 10^4 to 10^6 greater than predicted only by the ideal gas law.

Aside from the steroids listed herein, there are few reports in the literature describing solubility measurements of other pharmaceuticals. Ko et al.[21] measured the solubility of penicillin V in SF-CO_2, and they compared their results with those obtained by other investigators for several other biological compounds, such as cholesterol,[19] monocrotaline,[22] and naphthalene[23] (Figure 5–2). Unlike many polar pharmaceuticals, they found that penicillin V exhibited appreciable solubility in SF-CO_2 considering its large molecular size and high polarity. In fact, penicillin V was found to be more soluble in this fluid than cholesterol, a natural constituent of fats.

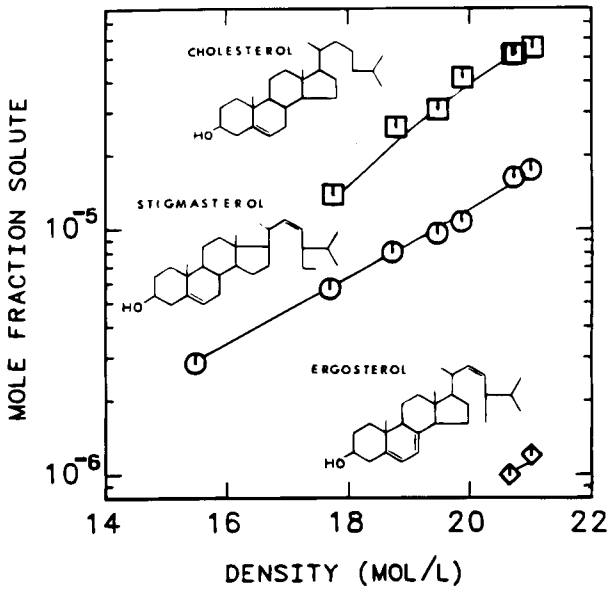

FIGURE 5–1. Solubility vs. density isotherms of cholesterol (□), stigmasterol (○), and ergosterol (◊) in pure CO_2 at 35°C. (From Wong, J. M. and Johnston, K. P., *Biotechnol. Progr.*, 2, 29, 1986. With permission.)

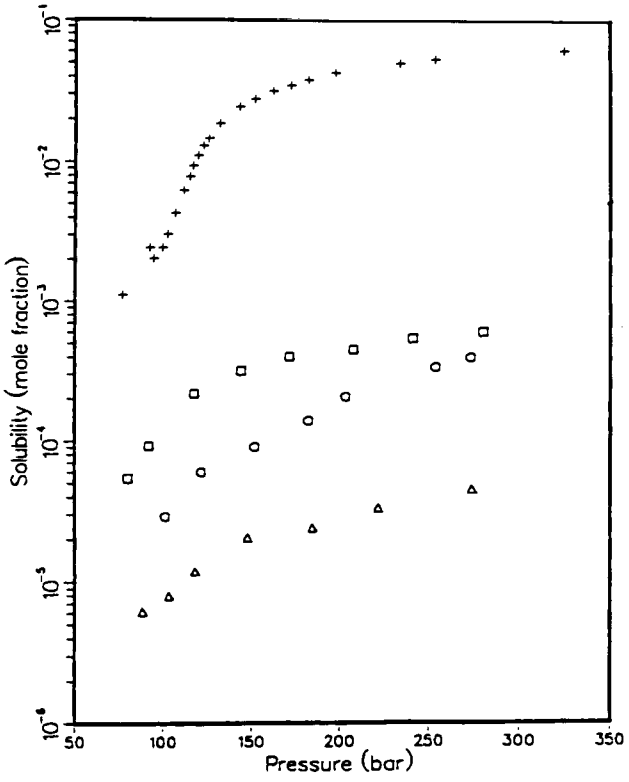

Figure 5–2. Comparison of solubility vs. pressure for penicillin V, cholesterol, monocrotaline, and naphthalene in pure CO_2. □, Penicillin V, 334.85 K (Ref. 21); ○, cholesterol, 333.15 K (Ref. 19); △, monocrotaline, 328.15 K (Ref. 22); and +, naphthalene, 328.15 K (Ref. 23). 1 bar = 0.1 MPa (From Ko, M., et al., *J. Supercrit. Fluids*, 4, 32, 1991. With permission.)

Finally, Maxwell et al.[24] studied the solubility of a class of compounds known as the polycyclic ether antibiotics in SF-CO_2 and in CO_2 modified with water and methanol (MeOH). The compounds (lasalocid, monensin, narasin, and salinomysin) are complex cations under physiological conditions and were therefore studied as their sodium salts. The polycyclic ethers are characterized by a number of cyclic ether functionalities within their structures that accounts for their cyclic conformations in solutions and their ability to complex metal cations. Because of their unusual solution properties, this antibiotic class afforded the opportunity to examine conformation effects on solubility behavior in SFs. The observed solubility behavior of these antibiotic salts led to the postulation that the polyethers assume conformations in SFs similar to those reported in liquids.

At present, not enough SF solubility data are available on complex compounds, such as drugs-of-abuse and pharmaceuticals, to predict whether they can be extracted as trace residues from biological matrices. In lieu of these data, investigators should first perform determinations on compounds of unknown solubility by conducting extraction and recovery studies from inert supports before attempting to isolate the substances from complex biological matrices (see "Pre-SFE Sample Preparation").

III. INCREASING THE SOLVATING POWER OF SFs

Although CO_2 is by far the most commonly used fluid for SFE, an obvious limitation is its unpredictable and/or insufficient solvating power for very polar or high molecular weight analytes. One possible solution to this problem is to use alternate SFs with greater polarities (Table 5–2); however, candidate fluids that are attractive from a solvent strength standpoint are often not practical because of extreme critical parameters (e.g., MeOH); corrosive properties, reactivity, and toxicity (e.g., ammonia); explosive potential (e.g., N_2O); or environmental ozone-depletion impact (e.g., chlorodifluoromethane). For example, N_2O, with critical parameters nearly identical to CO_2, has a small dipole moment and has demonstrated selectivity and higher recoveries for some analytes;[25–28] however, recent reports strongly caution against its use because of its explosive potential when readily oxidizable organics are present.[29,30] Chlorodifluoromethane (or freon-22) has excellent solvent strength properties, reasonable critical parameters, and has demonstrated dramatic improvements in recoveries of a variety of analytes compared with CO_2;[31] however, the Montreal Protocol calling for production phaseout of ozone-depleting chlorofluorocarbons will severely limit its use. Fluoroform (CHF_3), with its mild critical parameters and high dipole moment, may provide a useful alternative to freon-22, because it is not currently scheduled for phaseout under the Montreal Protocol.

TABLE 5–2
Critical Parameters and Dipole Moments of Some Representative SFs

Name	Formula	T_c (°C)	P_c (MPa)	Dipole Moment[a,b] (coulomb-meter)
Ammonia	NH_3	132.5	11.40	4.90×10^{-30}
Carbon dioxide	CO_2	31	7.39	0
Chlorodifluoromethane (freon-22)	$CHClF_2$	96	4.91	4.74×10^{-30}
Methanol	CH_3OH	240	7.95	5.67×10^{-30}
Nitrous oxide	N_2O	36.5	7.27	0.57×10^{-30}
Trifluoromethane (fluoroform)	CHF_3	25.9	4.75	5.50×10^{-30}
Water	H_2O	374.1	22.12	6.17×10^{-30}

[a] Gas phase.
[b] 1 coulomb-meter = 2.9979×10^{29} debye

Source: From Weast, R. C. and Astle, M. J., Eds., *CRC Handbook of Chemistry and Physics*, 61st ed., CRC Press, Boca Raton, FL, 1980.

A more common and practical approach to increasing the solvent strength of the SF extractant has been to incorporate small amounts of polar organic solvents, called modifiers or co-solvents, in the primary fluid.[32,33] A wide variety of solvents have been used as modifiers in SFE, including MeOH and other alcohols, water, acetonitrile, methylene chloride, toluene, organic acids (e.g., acetic acid), organic bases (e.g., aniline, diethylamine, and triethylamine), and others. The selection of modifiers has been largely empirical in the past; however, recent SFE studies[26,33–36] have significantly advanced our fundamental understanding of the role of modifiers and the interactions that occur among analytes, SFs, modifiers, and matrix binding sites. This improved understanding has resulted in the potential for more rational method optimization choices and some predictive capability with respect to modifier selection. These studies have likewise demonstrated that, in many cases, the modifier has a more important role in facilitating analyte release from matrix binding sites (rather than improving bulk solubility of the analyte); this idea will be explored in more detail in the following section. Nonetheless, when the goal is to increase the solvent strength of the extraction fluid, a useful starting point is to employ a modifier that is capable of selective interactions with the target analyte and that is a good solvent in the liquid state for for the analyte.[7] Langenfeld et al.[33] recently reported a detailed evaluation of nine CO_2-modifier mixtures for a variety of analyte–matrix combinations and discussed their results in terms of selective interactions of the modifiers with the target analytes (e.g., hydrogen bonding, dispersion, and induced dipole interactions). In an earlier study, Page et al.[37] provided a detailed summary of modifiers that have been used in SFC and their potential interactions with solutes; this information can provide a useful starting point for method development in SFE.

A unique approach for enabling the extraction of very polar or even ionic compounds is *in situ* derivatization, complexation, or ion-pairing of the analyte to form a less polar and, therefore, CO_2-extractable species. This approach is potentially very useful for toxicological applications, especially for the extraction of polar drugs from biological samples. The derivatization, complexation, or ion-pairing reaction can take place directly in the SFE vessel during the extraction and is often referred to as "on-line," "in-line," or "simultaneous" derivatization (complexation and ion pair)/SFE. Quaternary ammonium salts, such as trimethylphenylammonium hydroxide and tetrabutylammonium hydroxide, have been used as ion-pairing reagents in the SFE of acid herbicides, microbial phospholipid fatty acids, wastewater phenolics,[38] and sulfonated aliphatic and aromatic surfactants in sewage sludge.[39] The analytes were extracted as their quaternary ammonium ion pairs, with subsequent methylation in the injection port of the GC to form esters or ethers (i.e., here the quaternary ammonium salt acts as both an ion-pairing and injection port derivatization reagent). Hills et al.[40] have added commercially available silylation reagents directly to the sample before SFE to facilitate the extraction of polar analytes from coffee beans, roasted tea, and marine sediment. In addition to forming less polar analyte derivatives, the silylation reagent is thought to aid the extraction process by displacing analytes from active matrix sites. Metals and organometallics have been extracted from solids and aqueous media using a variety of *in situ* chelation, derivatization, and ion-pairing methods.[41–44] An ion pair/SFE method has been reported for the recovery of the β-adrenergic agonist clenbuterol from food matrices[45] using a salt of camphorsulfonic acid as the ion-pairing reagent. Recently, Chatfield et al.[46] described the simultaneous SFE and methylation of acidic analytes, including drug compounds, from aqueous media after adsorption of the anionic forms of the analytes onto ion-exchange resins. Methyl iodide was used as the methylation reagent. Such an approach may have potential usefulness for the recovery of drugs and metabolites from urine samples.

In a remarkable recent development, Johnston and colleagues[47] have demonstrated the solubilization of a protein by SF-CO_2 containing a fluoroether surfactant which, together with water, forms micelles in the CO_2 phase. The surfactant was observed to dissolve in CO_2, forming aggregates containing water droplets with properties approaching those of bulk water. The micelles enabled the solubilization of bovine serum albumin, a protein whose solubility in neat CO_2 is essentially zero. This development has important and far-reaching implications for the isolation of highly polar compounds previously thought to be unextractable using CO_2.

IV. THE SUPERCRITICAL FLUID EXTRACTION (SFE) PROCESS

Although solubilization of an analyte by SFs is certainly a necessary condition for extraction, it represents only one aspect of the SFE process. A growing body of research in the environmental SFE literature has demonstrated that the nature of the matrix and the manner in which analytes are incorporated in that matrix have a profound influence on both extraction kinetics and the conditions required for optimum extraction with SFs.[25,26,33–36,48,49] SFE of trace analytes from adsorbing matrices proceeds through several distinct steps, which have been described by Pawliszyn[50] for porous environmental solids as: 1) removal (desorption) of the analyte from the matrix binding site; 2) diffusion of the analyte through the matrix to the matrix–fluid interface; 3) solvation of the analyte by the SF; and 4) diffusion of the solubilized analyte through the porous matrix to the flowing bulk SF for its subsequent removal and collection from the extraction system. Which of these processes constitutes the rate-determining step will depend on the specific analyte, the matrix, and the type and strength of interactions that exist between the matrix and the analyte. The ability of the SF or SF-modifier mixture to overcome analyte–matrix bonding is in many cases of greater importance than high analyte solubility for obtaining efficient SFE recoveries.[7,11] Consequently, knowledge of the physical and chemical nature of the matrix and the chemical nature of analyte–matrix interactions must be considered, along with analyte solubility for successful method development using SFE.

In situations wherein matrix effects are thought to account for low analyte recoveries by SFE, two approaches have been used to help overcome analyte–matrix interactions and release the analytes for partitioning into the SF. Recent research has demonstrated that increasing the extraction temperature can improve recoveries by making desorption kinetics more favorable.[36,48,49] The increased temperature provides the necessary thermal energy to overcome the kinetic energy barrier to desorption.[26] Higher temperatures also aid the extraction process by increasing analyte diffusivity and volatility.[5,6,8] Temperature must be used cautiously, however, for analytes that exhibit thermal instability. A second, more aggressive, approach for overcoming matrix effects involves using a more polar fluid or incorporating a modifier (co-solvent) or additive in the primary extraction fluid that can compete with the target analyte for sorptive matrix sites[11,26,33–36] and/or interact with the analyte–matrix complex to lower the activation energy barrier to desorption.[26,33] For example, in a study of the SFE of aromatic amines from soils and sludges, Oostdyk et al.[34] used an amine with a pK_a greater than the analytes of interest as a modifier to block active matrix sites and release the adsorbed analytes. The mechanism was postulated to be one of competitive displacement.

Although the SFE models and theories on analyte–SF–matrix interactions have been developed based largely on studies involving environmental analytes in environmental samples, the principles should likewise be applicable to biological matrices. Analytes of interest in biological samples may bind to receptors, plasma proteins, or other tissue components; partition into specific compartments, such as fat or lipid bilayers; or become conjugated with other endogenous substrates to facilitate storage, transport, or excretion. Further complicating the situation is the fact that analytes may interact at several different sites with different binding strengths in a heterogeneous matrix.[7] Analytes present at different discrete matrix sites may thus exhibit different SFE behavior, depending on both their physical location within the sample matrix and the chemical nature of analyte–matrix interactions (e.g., the physical adsorption of cocaine on the surface of hair caused by passive [environmental] exposure vs. its chemisorption within the keratin matrix; see "Applications of SFE in Analytical Toxicology"). Thus, the biological matrix significantly influences the availability of the analytes for extraction. To recover analytes successfully from biological matrices using SFE, the physical and chemical nature of the matrix and potential analyte–matrix interactions should be considered.

An example of a forensic toxicology application of SFE that illustrates the influence of the matrix on analyte extractability involves the extraction of cocaine from human hair.[51,52] Although cocaine exhibits reasonable SF-CO_2 solubility, poor recoveries of cocaine were obtained from drug users' hair using the pure SF. Upon addition of a modifier containing triethylamine and water to the

sample, however, quantitative recoveries were achieved. This dramatic improvement in extractability is postulated to result from a displacement mechanism by which triethylamine (TEA) competes for binding sites in the hair matrix, effecting release of cocaine (see "Applications of SFE in Analytical Toxicology").

V. THE PRACTICE OF SFE

A. SFE INSTRUMENTATION

Conceptually, SFE instruments are simple in design. Modern commercial or laboratory-assembled instruments range from very basic systems operated manually to highly sophisticated automated devices. However, in principal, they are all designed using five basic components: a pumping system, a constant temperature oven or bath, an extraction vessel to contain the sample matrix, a restrictor to decompress the SF, and a trapping device for collecting extracted analytes.

Figure 5–3a depicts a highly simplified schematic of such an SFE instrument. The SF (e.g., CO_2, freon, or a modified SF) is directed to a pump where the fluid is compressed to the appropriate pressure, which is indicated on the gauge shown connected in-line with the pump. Then, the compressed fluid is directed to the constant temperature oven or bath, where it passes first through a heat exchanger before flowing into the extraction vessel containing the sample matrix. The compressed fluid, now in its supercritical state, diffuses through the sample matrix and exits the vessel laden with the extracted analyte. Finally, the SF passes through the restrictor, where it is decompressed at its tip. The SF, now in the gaseous state, bubbles through the solvent in the cold trap collector, deposits the extracted analytes in the collection solvent, and vents as a harmless gas.

With that overview of the SFE process, we will next examine in some detail the role of the individual system components shown in Figure 5–3 (a to c).

1. Pumps

A wide variety of pumps are used in both laboratory-assembled and commercially produced SF extractors. Two of the most commonly used types are syringe and reciprocating pumps. These pumps are familiar to any analyst who has operated a high-performance liquid chromatograph. SFE pumps should be capable of maintaining constant pressures up to levels of 68 MPa (10,000 psi). For many extraction applications, an upper pressure limit of 35 to 40 MPa is sufficient; however, many analytes of interest to toxicologists are polar in nature and may require an instrument having the 68

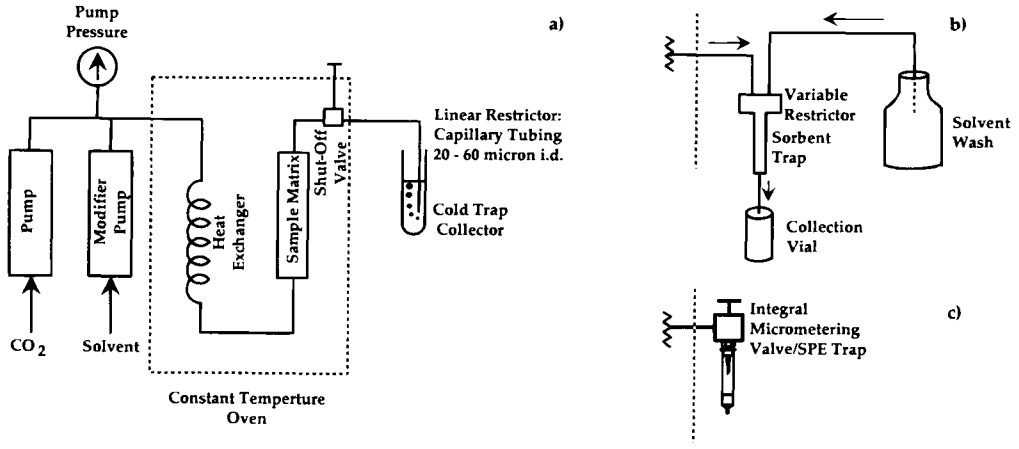

FIGURE 5–3. Schematic illustration of a SFE apparatus displaying three types of restrictor/analyte collector designs. a) Linear restrictor/cold trap collector (off-line solvent trap); b) variable restrictor/sorbent assembly; and c) integral micrometering valve/SPE assembly.

MPa upper pressure limit. Most reciprocating pumps are limited to pressures of 35 to 40 MPa and, for that reason, may not be suitable for forensic or clinical applications. Syringe pumps, on the other hand, are capable of providing pressures up to 68 MPa. In addition, they deliver a pulseless fluid flow to the extractor. A problem with the use of syringe pumps for SFE applications is that they hold only a specified amount of fluid/charge. For extractions of long duration, a second pump and a controller may be needed to maintain continuous operation.

Many pump types are limited in their ability to deliver pressurized fluid flows above 2 to 4 ml/min at the upper pressure limit. This corresponds to flow rates of approximately 1 to 2 l/min of expanded gas after fluid decompression (1 ml SF equals approximately 500 ml of expanded gas). In the author's experience, some samples of toxicological interest, such as polar pharmaceuticals in tissues, require significantly higher flow rates to achieve high recoveries. Fluid flow rates in the range of 4 to 6 ml/min (2 to 3 l/min expanded gas) are routinely used for this purpose. An SFE instrument capable of performing multiple extractions simultaneously would require a pump with a much higher performance capability than that for a single vessel instrument. For instance, a two-vessel instrument would need a pump capable of delivering a minimum of 12 ml/min of pressurized fluid.

To achieve uniform performance from pumps pressurizing liquid CO_2, it may be necessary to cool the pump head and fluid transfer lines (–10 to –15°C). This is necessary to prevent cavitation (e.g., gas entrainment) in the pump head and transfer lines. Some SFE instruments are designed to pump CO_2 without the aid of cooling, using instead CO_2 cylinders equipped with a helium headspace. Helium is added to the cylinder above the liquid CO_2, which increases the cylinder pressure from about 6.2 MPa to 13.8 MPa. The additional pressure provided by the helium prevents the liquid CO_2 from cavitating in the pump head; however, recent investigations[53,54] have indicated that the use of helium in CO_2 cylinders may retard the solubility of certain analytes. Although this effect may not be universal, these findings suggest that the use of this type of system be carefully considered, especially in critical applications such as those involving toxicological specimens.

A type of pump not discussed thus far that meets the criteria for many analytical SFE applications is the air-activated liquid pump of the type made by the Haskel* Corporation and other manufacturers. This inexpensive pump may be operated at high flow rates (>20 ml/min) and at pressures above 68 MPa. The pump head of these devices may be cooled, thus alleviating the need for helium headspace CO_2. The incorporation of such pumps into commercial and laboratory-assembled SFE instruments affords the analyst greater flexibility than reciprocating or syringe pumps in selecting parameters required for experimental methods development.

An auxiliary modifier pump is shown as part of the SFE apparatus in Figure 5–3a. For many applications, it is necessary to increase the polarity of the SF by the addition of a modifier or cosolvent, as discussed in the section on "Increasing the Solvating Power of SFs." A discussion of this and alternate modifier addition techniques is included in the section on "Introduction of Modifiers in SFE."

2. Constant Temperature Ovens

The choice of oven size and design assumes significant importance when the samples are biological in origin. For environmental samples, such as soils, river sediment, etc., the sample typically is placed directly in the extraction vessel without further pretreatment. Generally, samples of this type require extraction vessels having a capacity no greater than 10 ml, which may be the upper volume limit for some commercial SFE instruments. Likewise, hair samples may be powdered or simply cut into short segments and placed unamended in small-capacity SFE vessels. In contrast, tissue samples must be dispersed over a support or desiccant material such as Hydromatrix or sodium sulfate to achieve optimum extraction of the target analyte. Typically, these tissue-support mixtures

* Certain commercial equipment, instruments, or materials are identified in this paper to adequately specify the experimental procedure. Such identification does not imply endorsement by NIST or the USDA, nor does it imply that the equipment, instruments, or materials are necessarily the best available for the purpose.

may have volumes of 20 to 30 ml, necessitating significantly larger extraction vessels (see Figure 5–4 and the discussion in "Pre-SFE Sample Preparation"). Therefore, if the SFE instrument is to be used for urine, blood, or tissue samples, an oven of sufficient size to accommodate larger extraction vessels must be considered.

3. Extraction Vessels

The first requirement of any SFE vessel is that it be able to withstand the stresses of high pressures. Today, a wide selection of vessels for working pressures (WP) up to 68 MPa (10,000 psi) are available from suppliers such as Keystone Scientific and Valco Instruments Co. These vessels, typically fabricated from 316 stainless steel tubing, have been designed with burst pressures of at least four times the allowable WP, giving the vessels a considerable safety margin. Some commercially available vessels have WPs lower than 68 MPa. However, even if the intended application for the vessel is less than that pressure, it is still advisable to use the higher rated vessels.

Vessels of the same internal volume (1 to 50 ml) are available in sizes ranging from long and narrow to short and wide. Some controversy exists in the literature regarding the optimum vessel geometry for analyte extraction.[55,56] Reported examples describe the influence of vessel geometry on the SFE recoveries of analytes such as PAHs from environmental matrices; however, the findings may not be entirely relevant to biological matrices. Vessel geometry requirements may also be influenced by the method used for analyte trapping. For example, methods have been developed whereby the extracted solutes are trapped on sorbents placed in the same extraction vessel as the sample matrix (see "Applications of SFE in Analytical Toxicology"). For these applications, long, narrow vessels with 14-mm internal diameters provide the best results.

4. Variable and Linear Restrictors

Restrictor design is a critical element in the efficient operation of any SFE system. These devices control the system pressure and fluid flow. In their most elementary form, restrictors may

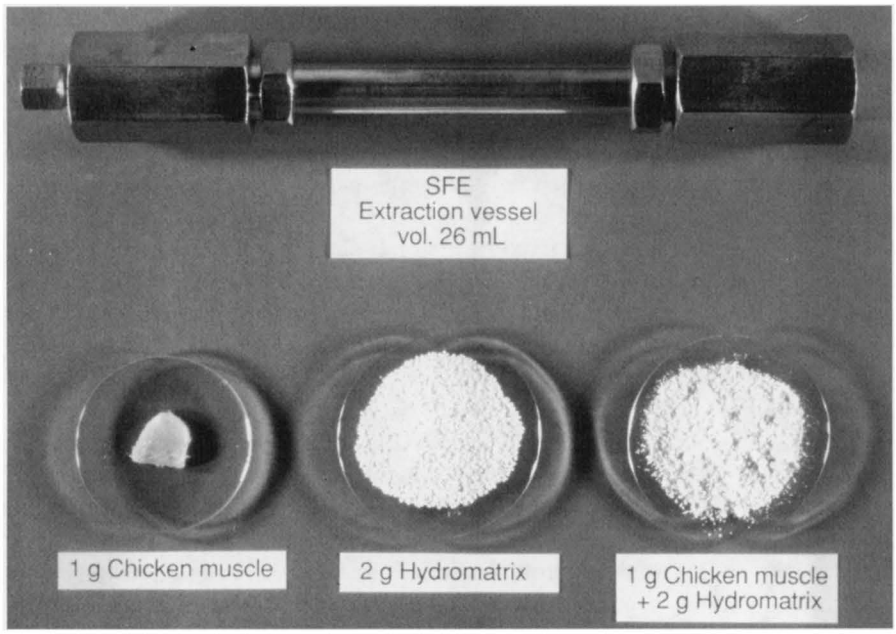

FIGURE 5–4. Pre-SFE processing of a tissue sample. (Left) Homogenized chicken muscle sample and (right) blended tissue/sorbent mixture.

consist simply of a length of capillary tubing composed of either fused silica or stainless steel. Figure 5–3a depicts an SFE system controlled by a linear restrictor. The back pressure and flow rates are determined by the internal diameter of the tubing, and expanded gas flow rates typically range from 100 ml/min to 2 l/min (i.e., fluid flows of 0.2 to 4 ml/min). Linear restrictors are simple to construct; however, in practice, they pose many difficulties, including plugging, which is the major problem observed with these restrictors. Plugging may be caused by restricted flow because of the presence of viscous solutes (e.g., fats), analyte precipitation in the restrictor or connective tubing, or blockage at the tip of the restrictor caused by ice formation during fluid decompression. Because of the cooling effects that occur during SF decompression, the restrictor should ideally be heated. Uniformly heating a linear restrictor is difficult to achieve, especially when it is constructed from fused silica, which acts as more of an insulator than a heat conductor. Aluminum-clad fused silica tubing, available from Scientific Glass Engineering, offers improved heat transfer properties and has been successfully used with no additional external heating for the SFE of cocaine from hair,[51] even when water was used as a modifier. Immersion of the restrictor tip in a solvent bath (Figure 5–3a) may reduce some plugging effects; however, many difficulties associated with these devices are inherent in the basic restrictor design.

In recent years, variable restrictors have gained popularity over the fixed linear variety. A variable restrictor has an orifice whose opening may be varied in size through manual or automated operations. This feature helps prevent the plugging problems associated with fixed restrictors. In addition, a wide range of pressure and flow options are obtainable with one device, whereas fixed restrictors require tubing of different internal diameters for every pressure and flow rate combination.

Manual variable restrictors are more commonly known as micrometering valves. Some models of these valves can be used at pressures up to 68 MPa (10,000 psi). Like fixed restrictors, they must also be heated to ensure uniform fluid flow. Manually adjusted valves of the types sold by Autoclave Engineers and other suppliers are a good choice when obtaining components for a laboratory-assembled SFE or when considering the purchase of a manually operated commercial unit.

Many of the automated SFE instruments currently available use electronically adjusted variable restrictors in their designs. Theoretically, these devices should automate certain flow sequences and require less operator attention than manual valves. However, such devices add significantly to the cost of SFE instruments and may require frequent maintenance.

5. Analyte Collection Traps

Trapping the extracted analytes is the final step in an SFE sequence, and this trapping step must, obviously, be quantitative. Both the physical properties of the analyte and post-SFE sample processing needs must be considered when designing an analyte collector. The collector shown in Figure 5–3a represents the earliest type of analyte trap devised for SFE, referred to as off-line solvent trapping. In the representation, a fixed restrictor is simply immersed in a vial containing an organic solvent. The decompressed gaseous SF bubbles through the collection solvent and is vented to the atmosphere, whereas the extracted analytes are deposited in the solvent. The organic solvent layer is subsequently removed or reduced by evaporation and, if necessary, further post-SFE cleanup operations are performed.

The solvent trapping method depicted in Figure 5–3a may be difficult to perform successfully and may result in analyte loss, especially if the analyte has a high vapor pressure or high volatility. Solvent trapping can only be used with relatively low extraction flow rates, because a 1.0 ml/min flow rate of a SF becomes approximately 500 ml/min in the gaseous state; higher flow rates may thus result in both analyte and solvent being swept from the collection vial. External cooling of the vial may reduce but not eliminate this problem. Some investigators have successfully used dry collection vials to retain certain types of less volatile analytes. For instance, when fats are extracted from tissue samples, they may be collected in this manner without significant analyte loss.[57] Therefore, dry analyte trapping may be of interest to toxicologists only for nonvolatile analytes from certain matrices that require little post-SFE processing.

Alternative sorbent-trapping techniques offer potentially attractive advantages over solvent-trapping methods. Commercial instruments are available that use this collection technique. A generic representation of such an SFE instrument is shown in Figure 5-3b. Up to the point where the SF enters the restrictor in this schematic, the device is similar to that depicted in Figure 5–3a, except that in Figure 5–3b the SF enters an electronically controlled variable restrictor that is connected to a column filled with a sorbent material. The sorbent in the column is chosen by the analyst for its affinity for the extracted analytes and may be any of the materials typically used in high-performance liquid chromatography (HPLC) columns. This restrictor/collector design does not provide for sorbent removal from the column for off-line processing. Instead, the restrictor/collector assembly is equipped with a wash station that is used to elute the extracted analytes from the sorbent bed into a collection vial. Variations on this design feature multiple solvent bottles that allow the sorbent bed to be eluted with solvents of increasing polarity, thereby effecting a partial fractionation of the trapped analytes. Solid sorbent trapping using a restrictor/collector assembly offers several advantages over solvent trapping, including reduced potential for loss of volatile analytes, minimal release of solvent vapors into the atmosphere, and greater selectivity in trapping desired analytes.

Despite these advantages, sorbent trapping by the technique shown in Figure 5–3b may pose problems for some applications involving biological matrices:

1. The sorbent column in these instruments may hold only a small amount of sorbent material (<1 g), whereas larger amounts of sorbent may be necessary to trap trace residue(s) in tissue samples containing large amounts of fat.
2. Because the sorbent bed cannot be removed from the instrument for off-line processing, the extracted residue eluted to a liquid sample vial may require transfer to an SPE column for further clean-up.

An SFE instrument designed in Agricultural Research Service/United States Department of Agriculture (ARS/USDA) laboratories[58] was fabricated specifically to address limitations encountered with solvent and sorbent-trapping techniques then available in commercial instrumentation. The focus of this design (Figure 5–3c) was the quantitative extraction of veterinary pharmaceuticals from biological matrices at parts per billion and parts per million levels. The SFE unit extracts two samples simultaneously; however, the representation shown in Figure 5-3c depicts only one extraction channel. This SFE has a manual rather than electronic variable restrictor that can be connected to a standard, off-the-shelf SPE column through an interface adapter. This integral restrictor–collector arrangement allows the decompressed gas, laden with extracted analytes, to be focused directly above the sorbent bed of the SPE column, thus reducing the potential for analyte losses as may be caused by additional transfer steps. The SPE column may be filled with varying amounts of any sorbent material selected for maximum analyte retention. SPE columns may be quickly removed from the interface adapter after SFE for further off-line analysis by subsequent manual or automated processing. The section on "Applications of SFE in Analytical Toxicology" describes some extractions from urine and tissues conducted using this instrument. This instrument design was transferred to a manufacturer, and a relatively low-cost commercial version is now available on the market.

B. INTRODUCTION OF MODIFIERS IN SFE

The introduction of modifiers in SFE, either for the purpose of increasing analyte solubility in the SF or for facilitating analyte release from matrix binding sites, is generally accomplished in three different ways.[5,7,8,59] One approach involves the use of premixed cylinders containing known concentrations of organic modifier(s). These cylinders are commercially available and can be connected directly to the SFE supply pump. The modified fluid is thus delivered directly and continuously to the extraction vessel. This approach is not particularly convenient for method development, nor is it economical because a large number of tanks with a range of modifier types and concentra-

tions are necessary if different applications are performed. Recent studies have also demonstrated that the modifier composition changes as the contents are withdrawn from a cylinder because of shifts in phase equilibria,[5,60] which can cause irreproducible results.

A second approach to modifier introduction uses two pumps and a mixing chamber. One pump is used for delivery of the primary SF (usually CO_2), and a second auxiliary pump is used for metered delivery of the modifier into the compressed CO_2 stream (Figure 5–3). The primary SF and modifier are typically mixed in a mixing chamber or tee and delivered on a continuous basis to the extraction vessel. Mixing is generally performed in the liquid phase on a volume–volume basis.[5,59] This approach is costly because of the requirement of a second sophisticated pumping system.

The third and simplest method of modifier introduction involves the addition of a small volume of modifier (e.g., organic solvents, water, derivatization or complexation reagents, buffers, etc.) directly to the sample matrix in the extraction vessel before SFE. The vessel is then sealed, the system is pressurized, and a static extraction step (see "Modes of Extraction in SFE: Static and Dynamic") is generally performed for an appropriate time period. The static step is followed by dynamic extraction with pure CO_2 to recover the analytes. This approach is inexpensive and offers the most flexibility in terms of method development, because it allows the user to survey a number of different modifiers and concentrations relatively rapidly. The modifier concentration is maximized in the vessel and remains constant during the static extraction period.[5] The disadvantage to this approach is that the modifier *is not* continuously introduced to the sample matrix and is rapidly purged from the extraction vessel during dynamic SFE.[5,7] However, recent studies[59,61] suggest that, when the modifier acts by competing with the analyte for matrix binding sites, this method of modifier addition may be more effective than either of the other two approaches. On the other hand, if the extraction is limited by the solubility of the analyte in the SF, the continuous addition of modifier to the sample by the use of premixed cylinders or a two-pump system will likely yield higher extraction efficiencies.[5,7]

C. MODES OF EXTRACTION IN SFE: STATIC AND DYNAMIC

The types of samples that are potential candidates for SFE seem endless. Examples range from those where the target analyte is loosely affiliated with the matrix, such as certain pesticides in adipose tissues, to those where an analyte seems to be tightly bound to the substrate, such as some drugs in other biological matrices. Each of these sample types requires different extraction strategies and operating parameters that must be determined by the analyst through experimental trials.

To handle the diverse requirements of various sample types, investigators possess the option of using three modes of extraction in SFE: static, dynamic, or a static/dynamic sequence. In the static (no-flow) mode, the off-line shut-off valve to the restrictor is closed (Figure 5–3), and the system is charged to the operating pressure while the oven is set to the desired temperature. The static hold period allows the system to achieve equilibrium before analyte recovery begins; during this "soaking" period, the SF diffuses through the sample matrix, liberating the analyte from the matrix by disrupting analyte–matrix binding interactions. If pure SF-CO_2 is not polar enough to free matrix-bound analytes, then the static mode may be used in conjunction with the pre-SFE addition of modifiers or other reagents to the extraction vessel (see "Introduction of Modifiers in SFE"). The advantages of this method of modifier addition were demonstrated by Ashraf-Khorassani and Taylor[59] in a study comparing two modes of modifier addition for the recovery of PCBs in river sediment: 1) direct addition of the modifier to the extraction vessel before pressurization and 2) continuous solvent addition through a modifier pump. Recoveries of the PCBs were 20% higher by direct addition of methanol to the sample coupled with a static "hold" than when the solvent was delivered through a modifier pump. These results indicated that the initial higher concentration of methanol in the vessel during the static mode more effectively desorbed the PCBs from the sediment samples than the lower concentrations delivered during continuous modifier addition. As previously described, another reported benefit of static addition is the ability to screen rapidly a number of modifiers and modifier concentrations without purging the entire system between runs or purchasing large numbers of CO_2 cylinders containing varying percentages and types of organic modifiers.

For most analytical-scale SF applications, the static extraction mode is seldom used alone. However, in unique cases where it is used without an additional dynamic step, the shut-off valve of Figure 5–3 would be opened and the system would quickly decompress to atmospheric pressure with no additional SF entering the system from the pump. During decompression, analytes soluble in the SF at higher pressures could potentially precipitate and redeposit in the extraction vessel and throughout the fluid lines before reaching the collection trap, resulting in overall poor product recoveries. For that reason, a static extraction period is normally followed by an extended dynamic flow period, during which the pump supplies a continuous stream of fresh SF to the system fluidics. The fresh SF diffuses through the sample matrix in the vessel and solubilizes additional analyte in the process. Samples may be extracted dynamically without resorting to a static hold period; however, many investigators have reported more favorable results using a coupled static/dynamic extraction rather than a simple dynamic step. During method trials, it is advisable to try various combinations of static and dynamic modes with and without the presence of modifiers and/or complexing and derivatizing reagents to achieve satisfactory analyte recoveries.

D. PRE-SFE SAMPLE PREPARATION

The degree and complexity of analyte/sample matrix pretreatment needed before SFE is sample dependent. Samples such as soil or river sediments containing environmental pollutants generally require only a grinding operation to achieve uniform particle size before packing the material in the extraction vessel. Similar techniques are also used with some polymers that may be ground with dry ice or liquid nitrogen to achieve a uniform sample bed.

On the other hand, with the exception of hair, biological samples (such as blood, tissue, and urine) require careful pre-SFE preparation and handling. Unlike soil samples, biological materials such as these cannot be simply packed into the extraction vessel without pretreatment. Although SF-CO_2 possesses high diffusivity and low viscosity, it does not uniformly penetrate dense sample matrices such as bulk tissue, and direct extraction of such samples with no pretreatment will result in poor analyte recoveries. Instead, tissue samples should first be quickly homogenized in a blender with precautions taken to prevent warming. (*Note:* all tissue processing operations should be conducted with chilled samples to prevent pre-SFE analyte losses.) Blood and urine, of course, do not require homogenization and can be mixed directly with an adsorbent material.

The blended tissue sample must then be desiccated and dispersed before it is ready to be packed into the extraction vessel. This step is accomplished by mixing the sample with an adsorbent material that serves two purposes. First, the adsorbent acts as a drying agent, forming a free-flowing mixture that can be uniformly packed in the vessel, and, second, the adsorbent disperses the biological sample over a wide surface area. This gives the SF greater access to the target analyte than would occur in a nonadsorbent-treated sample.

Several types of adsorbents have been used in SFE applications, including sodium sulfate, magnesium sulfate, normal- and reversed-phase SPE sorbents, alumina, and Celite 566 (Hydromatrix from Varian Sample Preparation Products). The choice of adsorbent used for individual sample types is dictated by the nature of the sample, its water and fat content, and other variables. (A few examples of the use of adsorbents with biological matrices will be given in this section. Other illustrations of their uses with blood, urine, and tissues are described in the section on "Applications of SFE in Analytical Toxicology.")

Biological matrices, such as blood, urine, and tissues, generally must be desiccated and dispersed before SFE. Typically, these samples are mixed with an adsorbent such as sodium sulfate, magnesium sulfate, or Hydromatrix. There are advantages and limitations associated with the use of these adsorbents. For instance, sodium sulfate is a good desiccant; however, it may dissolve in the presence of large amounts of water. Magnesium sulfate has also been used as an SFE desiccant; however, it may bind too tightly to the sample matrix and thus restrict fluid flow. Hydromatrix, first reported as an SFE dispersing agent by Hopper and King[62] in 1991, does not have these limitations.

Hydromatrix is a pelletized form of diatomaceous earth that has a large surface area/volume (>200 m^2/g). It reacts physically with water, rather than chemically like desiccants such as sodium sulfate. Hydromatrix is an inexpensive adsorbent, compared with some support materials, which is an important consideration when a large number of samples are routinely processed.

An example[63] of the use of Hydromatrix as a dispersing agent is shown in Figure 5–4. A 1-g chicken muscle sample is displayed in the left petri dish. The middle dish contains 2 g of unmodified Hydromatrix, which was subsequently blended with the chicken muscle sample. The resultant mixture is displayed in the right dish. Note that the water in the tissue sample has been adsorbed by the Hydromatrix, leaving a free-flowing powder. This adsorbed water can be subsequently solubilized by SF-CO_2 during the extraction process. Water can act as a co-solvent for many analytes, and its presence in the SF may be necessary for the success of the extraction (see "Applications of SFE in Analytical Toxicology"). The powdered mixture can be easily poured into an extraction vessel of the type shown at the top of the photograph. Because of the large volume of the tissue/Hydromatrix mixture, a 26-ml extraction vessel was necessary to accommodate this material. Before sealing the extraction vessel, the tissue/Hydromatrix mixture must be tightly compressed with a tamping rod to ensure uniform SF penetration of the sample matrix during SFE.

An analyte in a biological matrix may interact with an adsorbent in a manner that renders it unextractable by the SF. Therefore, before considering an adsorbent for any application, the analyte of interest should first be spiked on the adsorbent surface and subjected to SFE using the same parameters that would be employed to extract the sample matrix. Incomplete recovery from the dry sorbent can occur if water and/or fat normally present in a wet sample matrix are not present. Because water and fat in a sample matrix may act as co-solvents for the target analyte, these constituents should be added in the proper proportions to the analyte spiked on the adsorbent bed. If the analyte cannot be quantitatively recovered after reextraction under these conditions, then other adsorbents should be tested to achieve a satisfactory match between sample and support material.

It was previously stated that mixing a biological sample with an adsorbent disperses the resultant desiccated sample over large surface areas. This effect may have possible negative implications for biological matrices. The desiccation/dispersion process may disrupt cells in tissues and blood, exposing target analytes to enzymatic and/or oxidative degradation. For example, the loss of antibiotics[63] and steroids in tissues[64] and urine[65] was found when such samples were blended with Hydromatrix and left to stand at room temperature in extraction vessels for prolonged periods before SFE. For this reason, certain precautions *must* be taken with biological samples before SFE to ensure that analytes remain intact:

1. Perform all sample handling operations quickly, including mixing the sample with adsorbent and modifiers or reagents. Quickly transfer the sample/adsorbent mixture to the extraction vessel.
2. Perform handling operations (for very labile samples) with samples that are kept refrigerated until they are to be extracted.
3. Purge the extraction vessel with CO_2 from a cylinder source after packing and capping and before installing in the SFE oven to help retard analyte degradation.
4. Keep empty SFE vessels in the refrigerator until they are needed for packing.
5. Return packed vessels to the refrigerator, unless a sample is to be extracted immediately. *Do not* let packed vessels remain at room temperature on the laboratory bench.
6. Do not store packed vessels in a refrigerator for long intervals, because analyte losses from sample/adsorbent mixtures may occur even at low temperatures.

E. ANALYSIS OF SF EXTRACTS

Both "off-line" and "on-line" analytical approaches have been used for the analysis of extracts generated by SFE. Off-line analysis, where the target analytes are collected for subsequent analysis, is easier to perform than on-line analysis from both a method development and hardware standpoint,

and is the more common approach reported in the SFE literature. Any appropriate measurement technology may be used, including GC, GC-MS, HPLC, supercritical fluid chromatography (SFC), and spectroscopic methods. Off-line SFE/immunoassay methods have recently been reported for the rapid screening of analytes in a variety of matrices, including cocaine in hair,[66] phenobarbital in liver tissue,[67] and pesticide residues in meat products.[68] Capillary electrophoresis, with laser-induced fluorescence detection, has been used for the analysis of PAHs extracted from contaminated soils using SFE.[69] In each case, the type and volume of the SFE collection solvent (for SFE methods involving liquid solvent trapping of extracted analytes) or elution solvent (for SFE methods involving solid-phase trapping of extracted analytes) can be tailored to meet the sensitivity and selectivity demands of the specific analytical technique used. Regardless of the analytical method used, potential interferences derived from modifiers, collection or elution solvents, and co-extractables must be carefully monitored.

On-line analysis of SF extracts involves the direct coupling of the extraction step with the measurement step (i.e., analytes are directly transferred from the SFE system to a chromatographic or other analytical system). The direct interfacing of SFE to chromatographic systems is made possible because CO_2 is a gas at ambient conditions and is therefore readily removed. The obvious advantages of on-line analysis are the elimination of sample handling steps between extraction and analysis, and the sensitivity enhancement that arises because the entire extract is transferred to the analytical system. On-line SFE approaches are potentially very useful for trace analysis when only limited amounts of sample are available. SFE has been directly coupled with capillary GC, GC-MS, capillary and packed SFC, and, less commonly, HPLC.[4,8,11,28,70] On-line SFE can be more difficult to implement because of hardware considerations (i.e., interface design). In addition, the use of modifiers in on-line SFE/GC and SFE/GC-MS methods is not straightforward because of the production of large solvent peaks in the chromatographic system that can degrade chromatography and cause overpressure conditions in the MS. Finally, the need for additional post-SFE clean-up steps may preclude direct interfacing approaches for some biological sample applications. Nonetheless, for simple substrates, where only SF-CO_2 is used, the potential exists for automated on-line analysis.

VI. APPLICATIONS OF SFE IN ANALYTICAL TOXICOLOGY

A. SFE OF TISSUE SAMPLES

1. SFE for the Recovery of Pesticides from Tissues

The recovery of chemical residues from tissue samples by SFE presents unique challenges to the analyst, compared with the SFE of analytes from other matrices. The challenges arise because of the varied nature of the constituents found in tissues. Depending on the tissue site (i.e., liver, heart, muscle, adipose, etc.), the sample will contain varying amounts of fat (lipid) and water. SF-CO_2 readily solubilizes the constituents of a lipid mixture,[71] including fatty acids, diglycerides, triglycerides, and minor lipid components (such as pigments and sterols), whereas water is soluble to a lesser extent in this fluid. The chemical residue of interest will, therefore, constitute only a very small portion of the total SFE extract. The target analyte(s) then must be separated from the co-extracted lipids before chromatographic analysis. (For the purposes of this discussion, the collection of analytes after fluid decompression will be referred to as off-line collection or trapping.) Depending on the properties of the target analyte(s), this separation process may require multiple steps to isolate the analyte(s) from the undesired lipid components. To circumvent this problem, researchers have devised various techniques to simplify the post-SFE clean-up process through alternative analyte collection schemes. If a multiple-step clean-up process is required after SFE, the benefits of this technology may be negated, and non-SFE isolation techniques should be considered. In the applications that follow, we will discuss some of the approaches investigators have taken to facilitate the separation of target analytes from unwanted co-extracted artifacts.

The reported applications of SFE for recoveries of chemical residues from tissues have been performed largely with animal, not human, tissues. Methods may first be developed with animal tissues before they are subjected to further evaluation with those of human origin. However, the majority of the reported work on tissues has been driven by other considerations that are more of a regulatory concern. Regulatory agencies in the U.S.[72] and abroad[73] monitor meat and meat products for unlawful levels of veterinary drugs and other chemical residues, typically by LLE or other solvent-based techniques. These agencies must now reduce their consumption of hazardous and toxic solvents,[74,75] prompting investigators to evaluate SFE and other advanced technologies in efforts to minimize solvent usage.

Pesticides are among those compounds whose levels in tissues are under regulatory control. The first report of pesticide residues extracted from tissues by SFE was by Nam et al.[76] in 1988. This report was followed by King[71] in 1989, who isolated similar compounds from lard; Snyder et al.[77] and King et al.[57] in 1993, who quantitatively isolated organchlorine pesticides (OCPs) from chicken tissues; and Hopper and King[62] in 1991, who isolated pesticides from a variety of food products. In each of these studies, a concentrated SFE extract was obtained that contained appreciable amounts of lipid in addition to the target pesticides, necessitating post-SFE clean-up procedures, such as column chromatography, before quantitative pesticide determination by GC. In most of the aforementioned examples, the sample was blended with an adsorbent, placed in the extraction vessel, and extracted with pure SF-CO_2. Lipid components were found to be extractable over a wide range of pressures (20 to 68 MPa), suggesting that the direct approach to pesticide recovery used by these investigators will frequently result in an extract requiring further post-SFE clean-up.

In an effort to avoid problems encountered with co-extracted lipids, France and King[78] devised techniques that generated pesticide extracts (dieldrin, endrin, and heptachlor epoxide) virtually free of interfering artifacts that could be directly analyzed by GC with electron capture detection (GC-ECD). These researchers designed an SFE instrument with the capability of injecting a liquid chicken fat sample directly into an extraction vessel containing a packed bed of either alumina or silica gel. The extracted fat was trapped on the in-line adsorbent bed during the SF-CO_2 extraction, whereas the target pesticides passed through the in-line trap. Quantitative recovery of the pesticides was reported. The authors noted that the in-line silica gel column could be reconditioned for reuse by a post-SFE MeOH purge followed by a 20-min flow of unmodified CO_2.

In another series of studies[68] from the same laboratory, two types of SFE instruments, a pumpless system and a commercial SFE, were used to extract alachlor and carbofuran from bovine liver. The SF effluents from these systems, laden with the extracted pesticides, were bubbled into vials containing a H_2O/MeOH solution that was subsequently filtered through a microfiber membrane to remove co-extracted fat and then directly applied to an enzyme immunoassay (EIA) test kit. Recoveries for alachlor and carbofuran were $100 \pm 7\%$ and $100 \pm 4\%$, respectively.

Researchers have used sorbent materials other than alumina and silica gel to retain fats in extraction vessels as a means of preventing their carryover into analyte collection traps. Murugaverl et al.[79] developed a hyphenated SFE-SFC-MS for the total analysis of bendiocarb in bovine muscle. They found that most of the commonly used adsorbents could not completely retain fats on the in-line sorbent beds, resulting in contamination of the SFC-MS segment of the system. This problem was eliminated by using a combination of normal- and reversed-phase sorbents (diol/C_{18}, 7:93) for fat retention. This combination sorbent bed retained the fats completely, permitting the on-line detection of 1 ppm bendiocarb. Similarly, Alley and Lu[80] conducted an extensive study to evaluate a series of adsorbents for their efficiency in retaining fats. Extraction vessels were packed first with 5 g of the adsorbent, followed by a mixture of the sample and sodium sulfate. The analysis of carp and chicken eggs using silica gel, amino-silica and Florisil adsorbents gave less than 0.1 mg co-extracted fat and recoveries of fortified PCB (Arochlor 1254) that ranged from 85 to 95%; three other adsorbents (alumina, C-18-silica, and CN-silica) gave 9 to 57% co-extracted fat.

To date, only one SFE study of pesticides and PCB levels in human tissues has been reported. Djordjevic et al.[81] assessed the levels of chlorinated pesticides and PCBs in human adipose breast

tissue. Their SFE method was developed for use in epidemiological studies to assess the potential role of these pollutants in breast cancer. In the reported method, the fat in adipose tissues was trapped on a neutral alumina sorbent bed layered above the sample in the extraction vessel. The extraction process occurred in two steps and resulted in the quantitative recovery of eight pesticides and PCBs. These authors reported that a post-SFE clean-up of the extract by adsorption column chromatography was necessary to avoid interferences in the GC-ECD analysis. Their results indicated that levels of these pesticides and PCBs in breast cancer cases were higher than those in the control subjects, suggesting the need for further studies.

Ashraf-Khorassani et al.[82] are pursuing a novel approach for the isolation of pesticides and PCB extracts free of artifactual fat. Instead of using SF-CO_2, they have conducted experiments with MeOH-modified CHF_3. SF-CHF_3 exhibits much less affinity for fat than does CO_2 under similar conditions. (King and Taylor[83] reported that 54.4% of the fat from avian adipose tissue [0.2 g on glass beads] is extracted by CO_2, whereas only 0.45% [w/w] of the fat from the same tissue is extracted using CHF_3). Ashraf-Khorassani and co-workers used 10% MeOH in CHF_3 to extract rendered fat containing OCPs. Unlike the SF-CO_2 methods previously described, no adsorbent was packed in the extraction vessel for fat retention. Using the modified CHF_3, the extracted OCPs, free of fat contaminants, could be analyzed directly by GC-ECD without post-SFE extract clean-up. The authors have only extracted a few samples using MeOH/CHF_3; however, their preliminary findings indicate that CHF_3 may simplify post-SFE analysis of pesticide and PCB extracts.

2. SFE for the Recovery of Drugs and Other Chemical Residues from Tissues

Only a few research groups have reported data on the SFE recovery of pharmaceutical residues from tissues. The majority of these studies have been conducted using animal tissues, with only one reported use of human tissues. Several of these investigations have used conventional off-line collection strategies for extracted analytes. As in the case of pesticide recoveries from tissues using off-line collection, post-SFE purification steps are typically used to prepare the pharmaceutical analytes for chromatographic analysis. However, unlike most SFE applications for pesticides in tissues, additional post-SFE purification of pharmaceutical extracts may not be necessary in all cases, because pharmaceutical compounds are generally polar and lend themselves to alternative collection techniques that may obviate the need for post-SFE clean-up. Examples of the use of alternative collection techniques will be discussed later in this section.

The earliest report describing the isolation of pharmaceutical compounds from animal tissue samples was in 1989 by Ramsey et al.[84] These workers attempted to isolate four veterinary drugs (dienestrol, diethylstilbesterol, hexestrol, and trimethoprim) from freeze-dried pig kidney with pure CO_2 using an on-line SFE/SFC/MS-MS system. The drugs were trapped after SFE on an amino-SPE sorbent column, whereas co-extracted fat was swept to waste. The drugs were eluted from the SPE column directly into the SFC-MS by switching the SF from CO_2 to MeOH-modified CO_2. The drugs were then detected with high specificity by tandem MS. Recoveries were not stated; however, the authors concluded that the detection limits of the method did not meet the stringent controls for drug residues in meat set by regulatory agencies.

Other investigators have also experienced difficulties in extracting pharmaceuticals from freeze-dried or desiccated tissue samples. For example, Jimenez-Carmona et al.[45] attempted to extract clenbuterol with unmodified SF-CO_2 from lyophilized liver (20 μg/g) premixed with an ion-pairing reagent, the ammonium salt of camphorsulfonic acid. Clenbuterol is a β-adrenergic agonist that is used illegally in Europe and the U.S. as a growth promoter in meat-producing animals, and is monitored by both U.S. and European regulatory agencies. Using an SFE instrument containing a variable restrictor similar in design to that shown in Figure 5–3b, Jimenez-Carmona et al. obtained poor clenbuterol recoveries (12%) from the lyophilized liver samples. The authors provided no explanation for the low recoveries. Subsequent work by Parks et al.[85] suggests that the difficulty may have been caused in part by the lack of water in the liver samples used by the previous investigators. Parks et al. used SF-CO_2 for the isolation of the veterinary drug zoalene (3,5-dinitro-*o*-toluamide)

from chicken liver. They found that dehydration of the sample matrix during SFE results in stronger adsorption of the analytes, thereby limiting their extractability. The authors demonstrated this effect by first extracting a liver sample containing no zoalene (SF-CO_2, 60°C, 103 MPa). After SFE, the desiccated liver was recovered from the vessel, fortified with 2.0 μg/g of zoalene, mixed with Na_2SO_4, and reextracted under the same SFE conditions. Analysis of the resultant SFE extract indicated 42% of the zoalene was extracted from the desiccated matrix. The desiccated sample matrix was recovered from the extraction vessel, mixed with 0.85 ml water, and reextracted as before. This third reextraction resulted in an additional 46% recovery of zoalene for a combined yield of 88%. The total yield is about the same as that reported from nondesiccated tissue. This study confirms the critical role water plays in the SFE recovery of polar analytes from tissues.

Guyer[86] further confirmed the importance of the presence of water in the SFE of drugs from tissues, demonstrating 76% clenbuterol recoveries from fortified wet liver (50 ppb) using SF-CO_2. In contrast, he was able to recover only 12% of the drug from freeze-dried liver samples.

Furton et al.[67] developed a rapid SFE method for the determination of phenobarbital in postmortem liver samples. The target drug was extracted by CO_2 at 41.4 MPa and 50°C and decompressed into a MeOH collection solvent (Figure 5–3a). After solvent removal, the residue was analyzed by EIA. Total analysis time for the SFE/EIA method was 90 min, compared with 8 hr for the (20-step) LLE/GC method. Reported recoveries were similar to those obtained by the LLE/GC method currently in use.

The importance of SFE instrument design on the recovery of drugs from tissues was reported by Maxwell et al.[87] in 1992. They compared the recoveries of three veterinary nitrobenzamide antimicrobial drugs from liver tissues using two types of off-line sorbent collectors on the ARS/USDA-designed[58] instrument. One type of collector was separated from the variable restrictor by a length of stainless-steel tubing, whereas the second collector, a standard, commercial SPE column, was interfaced directly to the manual variable restrictor through an integral adapter similar in design to the device shown in Figure 5–3c. The three antimicrobials (aklomide, nitromide, and zoalene) were obtained in yields of 98, 82, and 88%, respectively, using the integral adapter SFE/SPE interface (Figure 5–3c), whereas recoveries of 50, 41, and 52%, respectively, were obtained for the same drugs using the separated collector. Both systems used approximately the same amount of CO_2 to sweep the analytes from the extraction vessels to the collectors.

These results illustrate the point that, after decompression, extracted materials are no longer in the same phase as the decompressed gaseous CO_2, and trace levels of solutes may be lost by deposition in the tubing of an SFE instrument. Focusing the decompressed gas directly above the sorbent bed of an SPE column minimizes this occurrence. Another benefit of collecting SFE extracts on standard SPE columns is that it simplifies post-SFE processing. For example, the complete sequence for post-SFE work-up[87] of the SFE extract containing the three nitrobenzamide antimicrobials is shown in Figure 5–5. The SPE column containing the nitrobenzamides was removed from the SFE and eluted with only a few milliliters of the HPLC mobile phase (MeOH/buffer) used for post-SFE analysis of the nitrobenzamides. The co-extracted fat was not soluble in the buffer and remained on the sorbent bed.

A similar technique for off-line collection was used by Maxwell et al.[88] for the isolation of trace levels of nitrosamines found in cured meat products such as frankfurters, fried bacon, and boneless hams. These compounds are potential carcinogens, and their levels in food products are monitored by federal regulatory agencies using laborious, solvent-intensive methods.[89] An SFE method was developed using the ARS/USDA instrument that extracted frankfurters containing 20 ppb nitrosamines in 17 min with pure SF-CO_2. The analyte/fat extract was trapped on an off-line silica gel SPE column (Figure 5–3c). A post-SFE elution of the SPE column with dichloromethane (DCM)/pentane (1:3) removed the fat from the column. A subsequent elution step with 8 ml of 30% ethyl ether or Et_2O/DCM removed the nitrosamines. Recoveries were 88 to 100% for the ten volatile nitrosamines as determined by GC with chemiluminescence detection (thermal energy analyzer).

Although off-line sorbent collection is a useful technique for trapping many types of chemical residues, endogenous co-extracted material may elute from SPE columns during post-SFE analyte

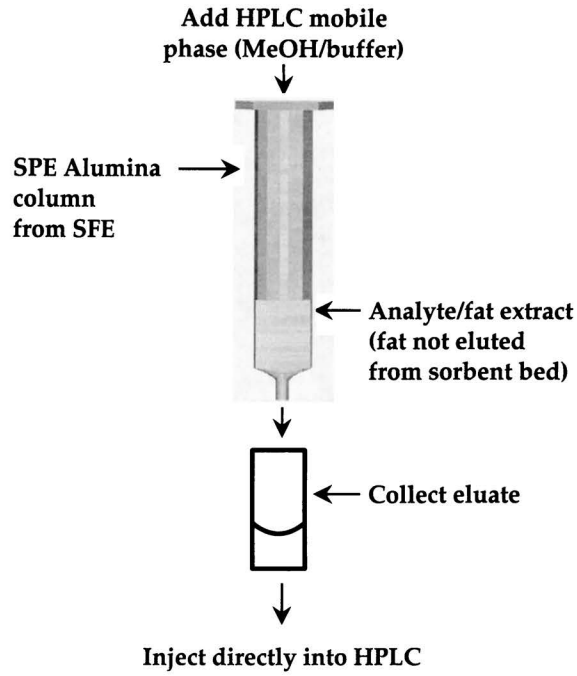

FIGURE 5–5. Post-SFE processing of extracted nitrobenzamide analytes on an SPE column removed from the SFE instrument shown in Figure 5–3c.

purification and interfere with chromatographic detection of the target analytes. It may be possible to minimize this problem by changes in the composition of the eluting solvents; however, if interferences persist, alternative techniques may be required.

Parks and Maxwell[63] recognized this problem during a study of the SFE of three sulfonamide drugs from chicken tissues. In this investigation, recoveries from three tissues (liver, breast, and thigh) were compared. Tissues were fortified with the sulfonamides (sulfamethazine, sulfadimethoxine, and sulfaquinoxaline), mixed with Hydromatrix (Celite 566), and extracted with pure SF-CO_2 at 40°C and 68 MPa. The drugs and co-extracted endogenous material were first collected on off-line alumina SPE columns. Recoveries of the three drugs from the various tissue sites are listed in Table 5–3. The recoveries of all three drugs were low and inconsistent with this off-line collection technique. In addition, sulfonamide peak areas were difficult to integrate because of high background ultraviolet (UV) interferences that may be observed in the HPLC chromatogram shown in Figure 5–6a. Cross et al.[90] also isolated sulfonamides from chicken livers using an off-line solvent-trapping system of the type shown in Figure 5–3a. Recoveries were higher than those reported by Parks and Maxwell;[63] however, to achieve these recoveries, the researchers increased the polarity of the SF by adding 25% MeOH to the CO_2. Incorporation of the MeOH modifier resulted in the need for additional post-SFE clean-up operations to prepare the extract for HPLC analysis. High concentrations of polar modifiers in the SF also limit the choice of off-line collection to solvent trapping, because extracted solutes collected on sorbent beds would be eluted during the extraction process due to the presence of the modifier (Figure 5–6b).

To minimize this problem of co-extracted interferences, Parks and Maxwell[63] devised an alternative trapping technique illustrated in Figure 5–7, which depicts a detailed section of the complete SFE apparatus shown in Figure 5–3c. The extraction vessel in Figure 5–7 is connected through fluid transfer lines to a shut-off valve that is in turn connected to the variable restrictor interfaced to an SPE column. Two sorbent traps are used in sequence to collect extracted material: one consists of a packed sorbent bed contained in the extraction vessel (in-line), and the second is a conventional SPE column attached to the micrometering valve (off-line). In Figure 5–7, SF-CO_2 enters the bottom of the extraction vessel, diffuses through the sample matrix, and solubilizes target analyte(s) and endogenous co-extractables, then contacts the in-line sorbent bed, where the polar target analytes

TABLE 5–3
Recoveries of Three Sulfonamides from Chicken Tissues by SFE

		% ± SD (n = 5)		
Tissue[a]	**SPE[b]**	**Sulfadimethoxine**	**Sulfamethazine**	**Sulfaquinoxaline**
Breast	OFF	79.3 ± 5.6	66.7 ± 4.1	64.4 ± 5.8
	IN	92.0 ± 2.3	86.4 ± 2.5	75.2 ± 4.1
Liver	OFF	69.3 ± 7.0	60.4 ± 3.3	54.1 ± 2.5
	IN	96.9 ± 1.2	89.9 ± 2.3	76.4 ± 3.0
Thigh	OFF	83.9 ± 9.5	74.9 ± 5.1	72.5 ± 7.7
	IN	94.8 ± 2.6	90.8 ± 3.8	79.9 ± 3.2

[a] Fortification level: 1.0 ppm/sulfonamide.
[b] OFF: 2.0 g off-line alumina trap; IN: 2.0 g in-line alumina trap.

Source: From Parks, O. W. and Maxwell, R. J., *J. Chromatogr. Sci.*, 32, 290, 1994. With permission.

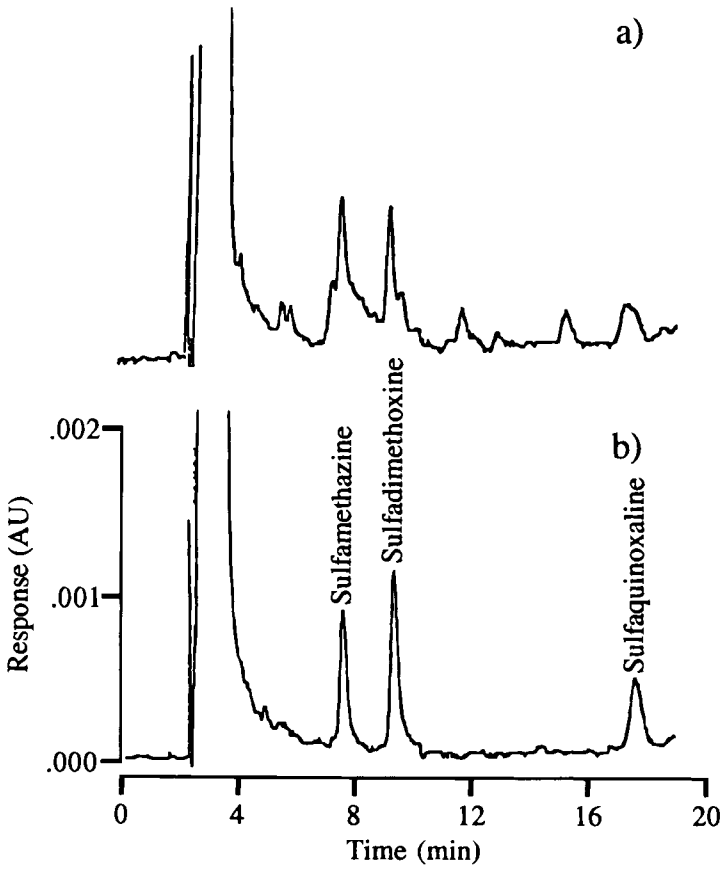

FIGURE 5–6. HPLC-UV chromatograms of sulfonamides from chicken liver extracted by SF-CO_2. a) Sulfonamides collected in an off-line alumina SPE column and b) sulfonamides collected in-line on an alumina sorbent bed.

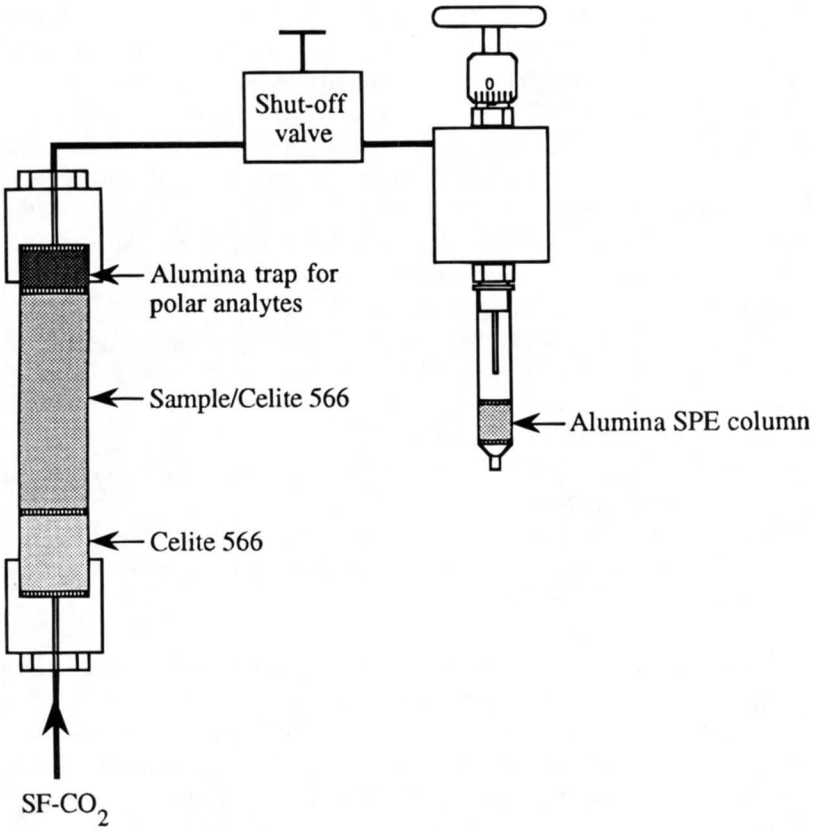

FIGURE 5–7. Schematic illustration of the extraction vessel-micrometering valve/collection assembly section of the SFE shown in Figure 5–3c. The extraction vessel is prepared for polar solute retention on an in-line sorbent bed.

are adsorbed from the SF and retained. The SF, now containing only fat and other endogenous tissue material, is vented through the off-line sorbent bed. Consequently, the analytes of interest are retained on the in-line sorbent bed, whereas interfering endogenous components are collected in the off-line SPE column. The degree to which polar analytes in this process are bound to the in-line adsorbent is dependent on the characteristics of analytes, their affinity for the sorbent, and the density of the SF. This process is one of continuous analyte adsorption and desorption on the in-line bed, necessitating an understanding of analyte–sorbent interactions to ensure successful in-line trapping.

The ability of the in-line technique to retain polar analytes while other endogenous materials remain solubilized is demonstrated by the results in Table 5–3 for the in-line and off-line recovery[63] of sulfonamides from chicken tissues. Recoveries of the three sulfonamides using in-line trapping are uniformly high and consistent from tissue to tissue, whereas the results from the off-line SPE columns are poor and vary from tissue to tissue. Similarly, a comparison of the HPLC chromatograms in Figure 5–6a (off-line recovery) and Figure 5–6b (in-line recovery) illustrates the improved HPLC separation possible with in-line trapping.

Off-line analyte recovery has been used by Magard et al.[91] in the extraction of steroids from animal tissues. Androsterone, a steroid responsible for the boar taint odor in pork, was extracted from fortified boar fat using SF-CO_2. The recovered androsterone was detected by GC-MS. However, whereas the SFE procedure extracted 77% of the steroid, it also co-extracted 10% of the fat, thereby increasing the potential for regular fouling of the GC column and the MS ion source. Although steroids are constituents of fats and are easily extracted by SFE at moderate temperatures and pressures, they too, like sulfonamides, may be retained on in-line sorbent beds. Parks and co-workers[92]

developed an SFE method for the recovery of melengesterol acetate (MGA) in bovine fat at or below the tolerance level of 25 ppb set by federal regulatory agencies.[89] This steroidal hormone is used in feeds to suppress estrus in heifers, and the current regulatory method used for its analysis is a 26-step procedure[93] that requires 1.7 l of organic solvent per sample. The SFE method developed by this team involved blending 1 g of incurred bovine fat with 4 g of Hydromatrix, followed by the addition of 0.75 ml of water. This mixture was poured into an extraction vessel prepacked with an alumina sorbent in a manner similar to that illustrated in Figure 5–7. Extraction of the mixture for 20 min with SF-CO_2 at 68 MPa and 50°C resulted in complete retention of MGA on the in-line trap, whereas co-extracted fat was collected off-line. Recoveries (GC-MS) averaged 98.4 ± 4.5% for incurred samples ranging in MGA content from 25 to 98 ppb.

Although the in-line collection technique is a significant improvement over off-line collection for many types of analytes, some potential problems may occur as a result of:

1. nonuniform packing of sorbent in the extraction vessel;
2. occasional analyte breakthrough because of reduced thickness of sorbent bed, compared with narrower diameter standard SPE columns;
3. possible solute contamination caused by sorbent contact with vessel walls; and
4. potential analyte loss during the post-SFE transfer of the loose sorbent bed from the vessel to an SPE column.

For these reasons, Maxwell and co-workers[87] developed a method for using standard, commercial SPE columns in SFE vessels in place of loose sorbent beds. A representation of an SFE vessel containing an SPE column is shown in Figure 5–8. A Teflon™* sleeve was fabricated to hold the SPE column in the vessel. The sleeve also prevented SF from flowing around instead of through the SPE column. At the end of an SFE experiment, the extraction vessel was uncapped, and the SPE column was removed for further processing. This device was used to trap anabolic steroids effectively from urine[65] and tissue[87] samples. In the tissue studies, chicken liver was fortified with the steroids nortestosterone, testosterone, and methyltestosterone (0.5 μg/g). The tissue sample was mixed with Hydromatrix and packed in an SFE vessel already containing a standard 3 ml SPE column filled with neutral alumina (Figure 5–8). The extraction was performed using CO_2 at 40°C and 27.2 MPa. The extracted steroids were retained on the in-line SPE column, whereas the fat and other solutes were collected off-line after decompression. Clean-up consisted of eluting the SPE column with 3 ml of the HPLC mobile phase (MeOH/H_2O, 7:3). These workers compared the HPLC-UV chromatograms and recoveries obtained from the in-line SPE column eluate with those obtained by two other collection techniques: in-line collection on a loose neutral alumina sorbent bed and off-line collection. The sorbent used to trap the steroids in all three examples was neutral alumina. The HPLC-UV chromatograms for the steroids from these three collection techniques are displayed in Figure 5–9.

Chromatogram a in Figure 5–9 is a trace of the steroids eluted from the off-line SPE column. Note the poor resolution of the steroid peaks in this chromatogram because of co-elution of UV absorbing background interferences. Chromatograms b and c show the steroids eluted from the in-line SPE column and the in-line sorbent bed, respectively. In both chromatograms, the peaks for the three steroids are symmetrical in shape and were easily integrated. Both in-line trapping techniques gave similar results, and either could be successfully used. In-line analyte trapping on standard SPE columns has to date only been tested using pressures up to 30 MPa. Until further testing at higher pressure levels is reported, in-line trapping above 30 MPa should be performed using the loose sorbent packing technique. Regardless of which of the two in-line trapping techniques is used for analyte trapping, they represent a significant improvement for trace residue collection over conventional off-line trapping techniques.

*Registered trademark of E. I. du Pont de Nemours and Company, Inc., Wilmington, DE.

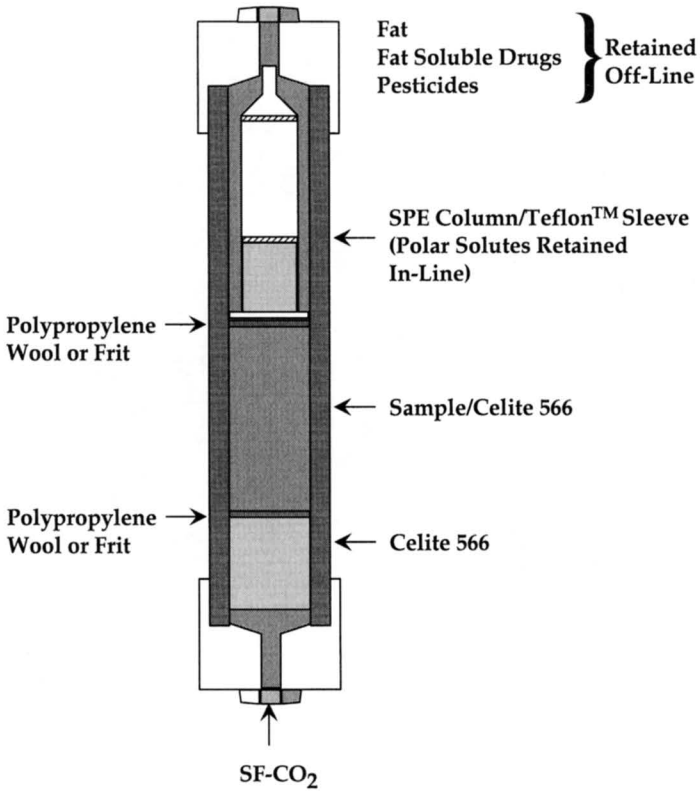

FIGURE 5–8. Cutaway illustration of an extraction vessel prepared for in-line trapping on a standard, commercial SPE column.

B. SFE OF HAIR SAMPLES

Sachs and co-workers[94,95] first reported the application of SFs to the isolation of drugs from hair in 1992. They demonstrated recoveries of opiates and cocaine from hair using CO_2 modified with ethyl acetate, but found that extraction rates and reproducibility of the SFE technique under these conditions were not comparable with wet chemical methods. The approach taken by Sachs was to increase the solvent strength of the SFE fluid by incorporating a modifier that was a good solvent for the target analytes; however, this approach failed to consider the influence of strong analyte–matrix binding interactions on analyte extractability.

Subsequently, Edder et al.[96] reported excellent recoveries of opiates (codeine, morphine, 6-monoacetylmorphine [6-MAM], and ethylmorphine) from drug user and standard reference (fortified) hair using CO_2 modified with a mixture of methanol, TEA, and water (85:6:6:3%, v/v). SFE was performed for 30 min (dynamic) at 40°C and 25 MPa on 50-mg portions of prewashed, pulverized hair. A laboratory-built, two-pump system with a mixing chamber was used to combine and deliver the modified fluid to the extraction chamber. Under the conditions used, the multicomponent extraction fluid is, strictly speaking, in the subcritical rather than supercritical state, and the authors refer to their method as subcritical fluid extraction. Analytes were collected by immersing the restrictor in a few milliliters of methanol (i.e., off-line solvent trapping), and extracts were analyzed using GC-MS after a propionylation derivatization step. Water was again found to be essential for efficient recoveries of the target analytes from the hair matrix.

Based on results obtained on the standard reference hair (prepared by soaking drugs into the hair), the method was found to be linear in the concentration range of 0.5 to 2 ng/mg for the four opiates tested, with coefficients of variation ranging between 3 and 10%. Replicate analyses of blank

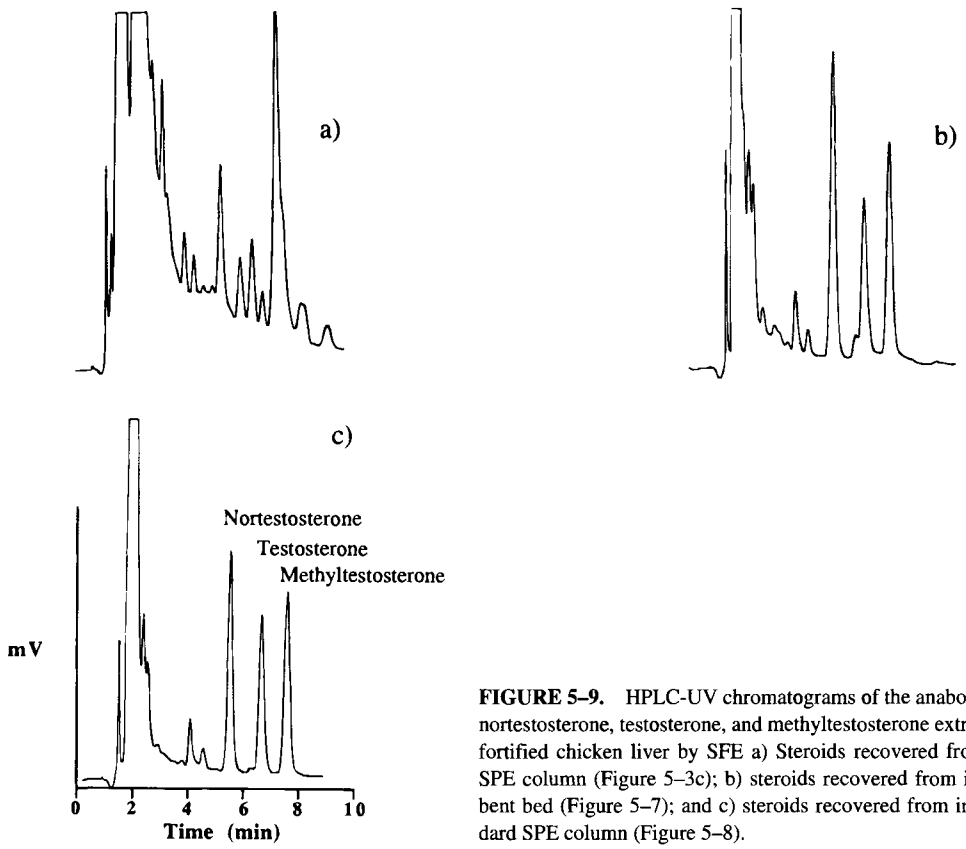

FIGURE 5–9. HPLC-UV chromatograms of the anabolic steroids nortestosterone, testosterone, and methyltestosterone extracted from fortified chicken liver by SFE a) Steroids recovered from off-line SPE column (Figure 5–3c); b) steroids recovered from in-line sorbent bed (Figure 5–7); and c) steroids recovered from in-line standard SPE column (Figure 5–8).

hair established a limit of quantitation at 0.1 ng/mg for codeine, morphine, and ethylmorphine, and 0.2 ng/mg for 6-MAM. The SFE/GC-MS method was applied to hair samples obtained from eight drug users, and recoveries of codeine, morphine, and 6-MAM were demonstrated in the range of 0 to 4 ng/mg. The quantitative efficiency and reproducibility of the SFE method compared favorably with the currently used MeOH extraction and enzymatic digestion/SPE procedures, with some variations. SFE recoveries of morphine were higher than those obtained by MeOH extraction. Compared with enzymatic digestion, SFE recoveries of morphine were slightly higher, whereas those of 6-MAM were slightly lower, suggesting the possibility of a small degree of hydrolysis of 6-MAM to morphine during SFE. SFE performed better than both acidic and basic hydrolysis methods.

Morrison and co-workers[51,52,97] have demonstrated rapid, efficient SFE recoveries of cocaine from hair and have investigated the influence of drug–hair binding interactions on analyte extractability, as well as the role of modifiers in improving extraction efficiency. Consistent with the results obtained for opiates by Edder et al.,[96] this work demonstrated that the addition of water and TEA resulted in dramatic improvements in the recovery of cocaine from drug user and standard reference (fortified) hair. Cocaine was extracted from the hair upon addition of 100 µl of the modifier mixture (15 µl TEA + 85 µl H_2O) directly to the hair matrix before extraction with CO_2, and SFE was performed at 110°C, 40.5 MPa, with a 10-min static and 30-min dynamic extraction period. Extracted cocaine was collected by off-line trapping in MeOH. Figure 5–10 shows the chromatograms obtained by GC with nitrogen-phosphorus detection for sequential SF extracts of a cocaine user's hair. An initial extraction step (chromatogram a) with pure CO_2 illustrated that no significant recovery of cocaine was obtained with the unmodified fluid. Cocaine was recovered in the subsequent extraction (chromatogram b) upon the addition of the TEA/H_2O mixture to the hair

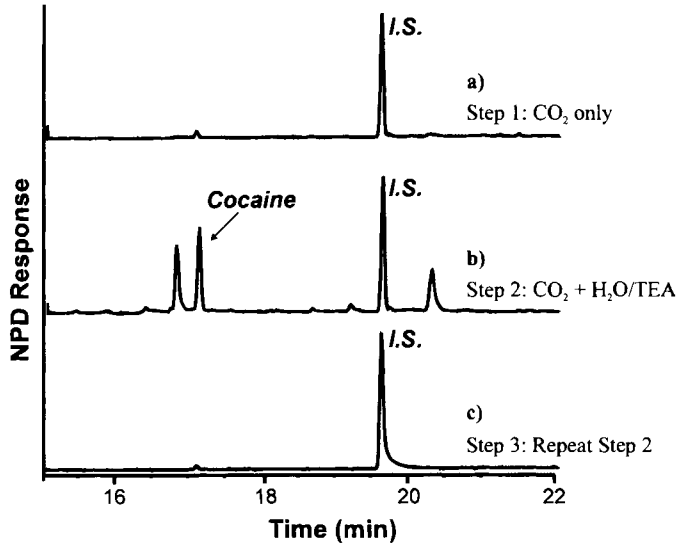

FIGURE 5–10. GC-nitrogen–phosphorus detection chromatograms of sequential SF extracts obtained on a 50-mg sample of a chronic drug user's hair. In all cases, SFE was performed at 40.5 MPa and 110°C with a 10-min static step and 30-min dynamic step. I.S., internal standard (*n*-propylbenzoylecgonine).

sample, and a final extraction (chromatogram c) under the same conditions illustrated that complete extraction of cocaine was obtained during step 2 of this sequence. After SFE, the hair was subjected to a 24-hour acid incubation/SPE procedure; analysis of the resulting extract showed no remaining detectable cocaine.

Further experiments demonstrated that cocaine could be efficiently recovered from an inert matrix such as Teflon™ wool using pure, unmodified SF-CO_2 (Figure 5–11), suggesting that desorption of the drug from matrix binding sites was a rate-limiting step in the extraction of cocaine from hair. It was hypothesized that the role of TEA was one of competitive displacement of cocaine

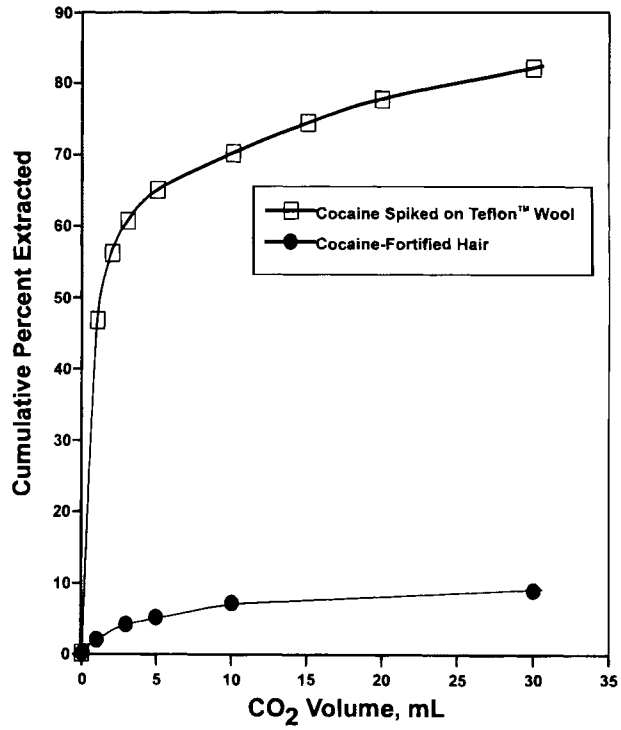

FIGURE 5–11. Extraction profile comparison illustrating the influence of matrix on cocaine extractability using pure SF-CO_2. Hair was fortified with cocaine by soaking it in a dilute dimethylsulfoxide (DMSO) solution of the drug for 1 month, with subsequent removal of the solvent. SFE conditions: 100% CO_2, 40.5 MPa, 110°C.

from hair binding sites and that water aided the extraction process by swelling the hair matrix to improve CO_2 accessibility to those binding sites.

The demonstration of a significant matrix effect led to further investigation to determine whether cocaine present at different discrete sites, or domains, in the hair matrix could be distinguished based on differences in SFE behavior. Figure 5–12 shows the GC-MS single-ion chromatograms obtained for sequential SF extractions performed on a cocaine user's hair that was additionally contaminated with cocaine on its surface (i.e., environmental contamination). As shown in chromatograms a and b, cocaine was recovered with pure CO_2, in sharp contrast to the results obtained in Figure 5–10 for nonexternally contaminated hair. After exhaustive extraction with pure CO_2, additional cocaine was recovered from the hair in Figure 5–12 in a subsequent SFE step with CO_2 + TEA/H_2O (chromatogram d). The CO_2-extractable cocaine fraction is believed to represent cocaine that is physically adsorbed to the hair surface, whereas the CO_2 + TEA/H_2O fraction is believed to represent cocaine chemisorbed at active sites within the hair matrix. These results suggest the potential of SFE for distinguishing external contamination from active drug use. A preextraction step with pure CO_2 is also useful for removing surface oils and waxes that may interfere with subsequent chromatographic analysis, as illustrated in Figure 5–13.

Cirimele et al.[98] subsequently adapted the procedures of Edder et al.[96] and Morrison et al.[51,52] for use on a commercial SFE instrument with off-line collection of extracted drugs on a sorbent trap. They demonstrated recoveries of opiates, cocaine, and cannabinoids from drug user hair under a single set of SFE conditions. Hair samples (50 mg) were placed in 7-ml extraction vessels and subjected to a preclean-up step using pure SF-CO_2 to remove lipophilic endogenous interferences. Subsequently, 1 ml of a modifier solution of MeOH/TEA/H_2O (2:2:1 v/v) was added to the extraction vessel, and the analytes were extracted with CO_2 at a density of 0.68 g/ml and a temperature of 100°C for a 10-min static and 20-min dynamic extraction period. Using GC-MS for quantification, detection limits for hair drug concentrations of 0.3, 0.2, and 0.1 ng/mg were determined for codeine, morphine, and 6-MAM, respectively. Reported relative standard deviations for the SFE/GC-MS method ranged from 13 to 17% for the three drugs.

Morrison and co-workers have also recently investigated off-line SFE-radioimmunoassay (SFE-RIA) as a rapid screening tool for the detection of cocaine residues in hair.[66] Hair was extracted

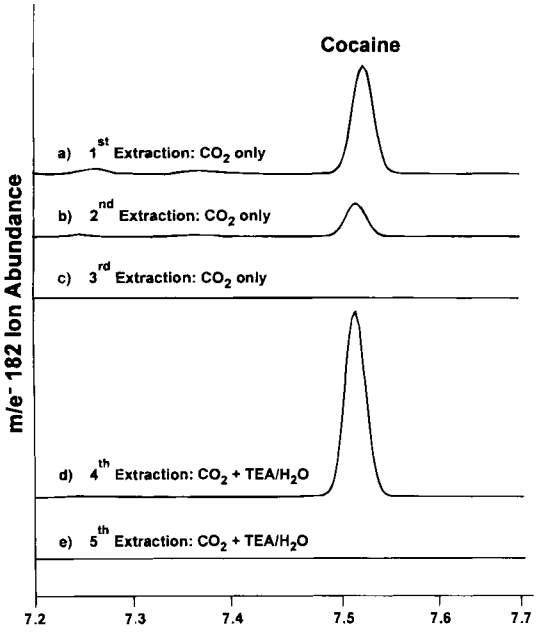

FIGURE 5–12. GC-MS single-ion chromatograms showing sequential SF extracts of a cocaine user's hair that was additionally contaminated on its surface (environmental contamination). SFE conditions: (a–c) CO_2, 40.5 MPa, 110°C, and 10-min static and 30-min dynamic period; (d,e) CO_2 + 100 µl TEA/H_2O (15:85, v/v) added to the sample—40.5 MPa, 110°C, and 10-min static and 30-min dynamic period.

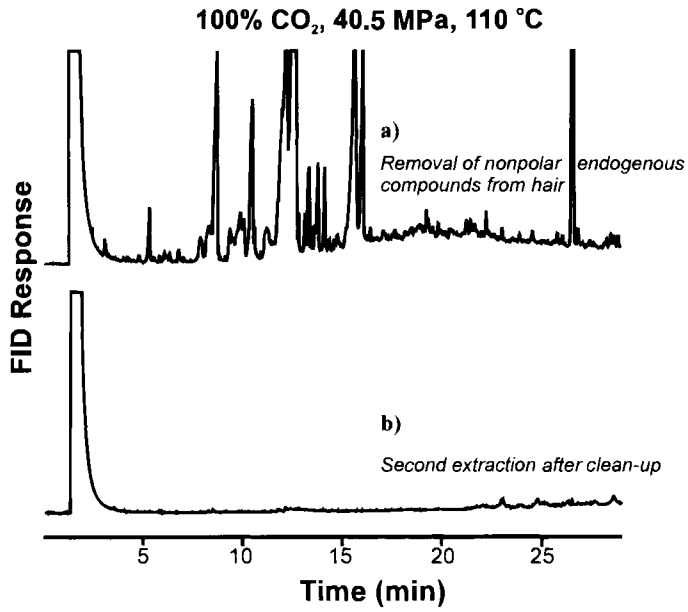

FIGURE 5–13. GC-flame ionization detector (FID) chromatograms showing sequential SF extracts obtained on a 150-mg sample of powdered hair from a non-drug user (blank control). A short preclean-up step with pure CO_2 efficiently removed nonpolar surface oils and waxes from the hair that could potentially interfere with chromatographic analysis.

using the SFE conditions described previously, and extracts were analyzed for the presence of cocaine using a commercially available solid-phase RIA kit. Issues unique to the immunochemical analysis of SFE-generated extracts were studied. MeOH (the SFE collection solvent) had only a minor impact on immunoassay performance; however, the presence of TEA/H_2O (the SFE modifier mixture) profoundly degraded assay performance, producing a 60% suppression in assay response. To preserve RIA sensitivity, SF extracts were evaporated under nitrogen to remove the modifier and reconstituted in MeOH for RIA analysis. SFE-RIA analysis of a series of drug-free hair samples established an RIA cut-off value for distinguishing between a negative and presumptive positive cocaine sample at an SF extract concentration of 1.2 ng/ml, or a hair concentration of 0.07 ng/mg. The robustness of the SFE-RIA method was demonstrated by the analysis of a variety of hair samples from both drug users and nonusers, and the quantitative SFE-RIA findings correlated well with values obtained by an acid incubation/GC-MS method.

C. SFE OF URINE SAMPLES

Direct extraction of analytes from aqueous samples using SFE is a particularly challenging analytical problem for several reasons.[5,70,99,100] First, the extraction vessel must be able to retain the liquid sample and prevent its mobility during the extraction period. Second, although water is only sparingly soluble in SF-CO_2,[101] this limited solubility can be problematic when one considers that, for trace analysis, it would be necessary to extract large aqueous sample volumes to recover sufficient analyte for quantification. Large volumes of SF-CO_2 would consequently be required for extraction, with the potential for carryover of considerable amounts of water to the collection vial or solid-phase trap. The solvated water can additionally cause plugging of linear restrictors because of ice formation during decompression. Finally, the direct isolation of polar analytes from a polar liquid matrix (water) using a relatively nonpolar extractant (CO_2) is a particularly difficult task from an analytical method development standpoint. Using a specially designed CO_2 recycling system and an extaction vessel geometry that minimizes "splashover" of water, Hedrick and Taylor[99,100] have demonstrated the direct extraction of phosphonates, phenols, triprolidine, caffeine, and pseudoephedrine from aqueous solutions. However, the analytes were not at trace levels, and, in addition to the requirement of specialized hardware, this direct approach precludes the use of polar modifiers.

The inherent difficulties associated with direct aqueous SFE have prompted researchers to investigate simpler, more practical alternative strategies for dealing with aqueous matrices, including urine, by SFE. One such approach involves "immobilizing" the aqueous sample by mixing it with an adsorbent material before SFE; the solid adsorbent is subsequently loaded in the extraction vessel and, in this manner, the liquid sample is retained in the vessel for extraction. Using this method, Stolker et al.[65] have demonstrated quantitative SFE recoveries of trace (low ppb) levels of nortestosterone, testosterone, and methyltestosterone from forified bovine urine with in-line trapping of the extracted steroids on neutral alumina. Before SFE, hydrolyzed fortified urine (2 ml) was mixed thoroughly with 2 g of the adsorbent material Hydromatrix until a free-flowing, granular mixture was obtained. In-line trapping of the target analytes (i.e., analytes trapped under dynamic supercritical conditions) was accomplished by incorporating a commercially available neutral alumina SPE column directly in the extraction vessel downstream from the sample (Figure 5–8). SFE was performed at 40°C and 27.2 MPa for a 10-min static period and a dynamic period sufficient to allow passage of 50 l of expanded CO_2 gas as measured at the restrictor outlet. Under these conditions, the extracted steroids were selectively *retained*, whereas co-extracted artifactial material was eluted and trapped off-line. Subsequently, the SPE column (containing the extracted steroids) was removed from the extraction vessel, and the target analytes were eluted using a small volume of MeOH/H_2O. The eluate required no further post-SFE clean-up before HPLC analysis or before derivatization for GC-MS analysis. The HPLC and GC-MS detection limits were 50 ng/ml and 5 ng/ml, respectively. Mean SFE recoveries for the three steroids based on GC-MS quantification ranged from 91 to 94% at the fortification level of 12.5 ng/ml. The method exhibited excellent reproducibility, with relative standard deviations (RSDs) below 6%.

Another strategy for the SFE of aqueous samples involves preconcentrating the target analytes onto solid-phase columns or disks that are subsequently loaded into the extraction vessel and subjected to SFE. This approach is sometimes referred to as SPE with SF elution, or SPE-SFE. Howard and Taylor[102] demonstrated quantitative recoveries of sulfonylurea herbicides from water using the combined SPE-SFE technique. The herbicides were concentrated from water samples (100 ml or 1 l) onto commercially available SPE disks; the disks were subsequently extracted with 2% MeOH-modified CO_2 at 50°C and 35 MPa with a 2-min static and 24-min dynamic SFE period. Sample pH was found to affect analyte recoveries markedly during both the SPE concentration and SFE elution steps. Mean SFE recoveries of the herbicides were 94 to 97% at the 50 μg/l fortification level (i.e., 50 ppb), with RSDs of 6 to 8%.

Edder et al.[103] have demonstrated quantitative SFE recoveries of codeine, morphine, and ethylmorphine from fortified urine samples after adsorption onto preconditioned, commercially available, mixed-mode, solid-phase sorbents selective for basic drugs of abuse. SFE was performed at 40°C and 25 MPa using a quaternary extractant phase composed of CO_2/MeOH/TEA/H_2O (85:6:6:3%, v/v). The presence of water was found to be necessary for efficient analyte recoveries. Recoveries of the three opiates from urine fortified at the 2 ppm level ranged from 96 to 100%, with RSDs below 7%. This method was not suitable for the quantitative recovery of 6-MAM, and the authors postulate that degradation of this analyte occurs during the SFE step because of the polar modifier mixture.

Chatfield et al.[46] recently described the simultaneous SFE and derivatization of acidic drug analytes, including benzimidazoles and propylthiouracil, from aqueous solutions after adsorption of the anionic forms of the analytes onto ion-exchange resins. Methyl iodide was used as the methylation reagent and was added to the top of the resin bed in the extraction vessel before SFE. A 20-min static SFE step was performed at 80°C and 20 MPa for derivatization to occur. The methylated analytes were subsequently eluted with 20 ml of CO_2 under the same conditions. Derivatization/SFE yields for the methylated benzimidazoles and propylthiouracil were 50% and 60%, respectively, suggesting that further optimization is necessary. Nonetheless, this resin-mediated derivatization/SFE approach is potentially very useful for the recovery of drugs and metabolites from urine samples.

D. SFE OF BLOOD SAMPLES

The extraction of blood samples by SFE poses some of the same analytical challenges discussed herein for urine samples. The most common approach has been to preconcentrate the analytes or load the sample onto selective solid-phase sorbents before SFE. Johansen et al.[104] report recoveries of PCBs from blood serum using an on-line SFE-GC method after loading of the serum sample (up to 5 ml) on a C_{18} sorbent. Residual water was retained in the extraction vessel by placing a small amount of basic alumina directly in the vessel. A separate column containing basic alumina was also placed downstream from the sample vessel to retain co-extracted lipid material. Removal of water and lipid material was found to be crucial for maintaining chromatographic performance in the on-line SFE-GC procedure. Extracts obtained by the SFE procedure displayed fewer chromatographic interferences, compared with extracts generated by traditional LLE procedures, resulting in more reliable quantification. Absolute recoveries, however, were slightly lower with the SFE procedure. The method was used to examine total PCB concentrations down to 25 ng/l.

Liu and Wehmeyer[105] have used SPE with SF elution for the trace analysis of the naturally occurring flavonoid compound flavone from dog plasma. The plasma sample (1.0 ml fortified at 50 ng/ml) was preconcentrated on a pre-conditioned commercially available C_{18} column. The SPE column was placed directly in the SFE vessel and eluted with 5% MeOH-modified CO_2 at an extraction temperature and pressure of 50°C and 15 MPa, respectively, and an extraction time of 10 min. Flavone recoveries for the SPE-SFE method ranged from 89 to 96% for concentrations ranging from 10 to 250 ng/ml. The method performance compared favorably with traditional aqueous-organic solvent SPE elution. The advantages of the SPE-SFE elution method included easier removal of the eluent after extraction and the potential for increased selectivity by varying extraction pressure and temperature.

The SFE of temazepam from whole blood has been reported by Scott and Oliver[106] after loading of the blood sample onto a selective sorbent material. The sorbent was subsequently eluted with 5% ethyl acetate-modified CO_2 for 10 min at an extraction temperature and pressure of 65°C and 20.7 MPa, respectively. Analyte recoveries were greater than 80%. SFE results on authentic forensic blood specimens correlated well with results obtained by traditional SPE over the concentration range of 1 to 8 mg/l.

Combined SPE-SFE has also been demonstrated for the recovery of mebeverine alcohol from blood plasma.[107] The plasma was applied directly to preconditioned C_{18} SPE columns; the columns were subsequently washed to remove protein, and the sorbent was removed and placed in the SFE vessel. The analyte was recovered using 5% MeOH-modified CO_2 at 35.5 MPa and 40°C for a 10-min collection interval. Addition of an organic amine (0.1 ml TEA) to the plasma sample before extraction was necessary to effect release of the analyte from sorbent binding sites.

Finally, Karlsson and co-workers[108] have reported the SFE of corticosteroids from fortified blood plasma (93 nmol/l) after deposition of 500-µl portions of the plasma on filter paper. SFE recoveries exceeding 80% could be obtained from the plasma samples, compared with only 28% SFE recoveries when the pure compound was spiked on filter paper. It was postulated that the improved extractability from plasma was caused by the presence of water in the sample, which can act as a polar modifier in CO_2.

VII. CONCLUSIONS

This extensive review demonstrates that significant progress has been made in the development of analytical SFE technology for the isolation of trace analytes from biological matrices. Resourceful and creative approaches have been used by researchers to improve extraction selectivity and sensitivity for biological applications, illustrating the potential of this technology as a sample preparation tool in analytical toxicology. The recent report by Johnston and co-workers[47] describing the solubilization of a protein by aqueous microemulsion droplets in SF-CO_2 suggests exciting possibilities for the SFE of highly polar molecules. Although the application of SFE in analytical toxicology is relatively new, much can be learned from the voluminous SFE literature

describing its application to environmental and other matrices. The significant advances that have been made in our understanding of analyte–SF–matrix interactions, extraction mechanisms, and the role of modifiers and additives in SFE now permit more rational method development choices and some predictability in terms of selection of optimum extraction conditions. Whereas the potential of SFE for biological sample applications has not been fully exploited, the advances described in this chapter, together with the availability of commercial instrumentation and the current emphasis on hazardous waste and solvent reduction, should help to speed the development and acceptance of SFE as a more routinely used laboratory technique.

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Chapter 6

ANALYSIS OF CLINICAL SPECIMENS USING INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY

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CONTENTS

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I. INTRODUCTION

Increasingly, the medical community as well as the general public have become aware of the biological importance of trace elements.[1–5] The book *Handbook on Metals in Clinical and Analytical Chemistry* (edited by Hans G. Seiler, Astrid Sigel, and Helmut Sigel) is an excellent, well-organized reference that covers a wide spectrum of trace elements and their current methods of analysis.[6] Various modern analysis methods for trace elements and their performance characteristics are covered in yet another good reference book.[7] Inductively coupled plasma (ICP)/mass spectrometry (MS) is emerging as a powerful tool for analysis of trace elements in clinical specimens, including urine, serum, plasma, whole blood, tissues and a variety of other biological specimens of potential clinical importance.[7–10] Analytical concepts developed and refined over a period of many years have been incorporated into the ICP/MS instrumentation that has become available for analysis of clinical specimens. The history of ICP as an ion source dates back more than 100 years to 1884.[11] Excellent technical reviews track the course of development and the extensive knowledge base that has been developed by investigators throughout the world.[6,7,11] The first commercial ICP/MS instruments were announced in 1983,[12] with the early applications centering on ICP/MS technology as a tool for analysis of trace elements in the environment. Building upon the success in measuring environmental contaminants in air and water, this powerful technology has increasingly found application in the analysis of clinical specimens.[6,8,9] To overcome matrix interference and increase specificity and speciation, investigators have proposed front-end coupling of ICP/MS with gas chromatography (GC), liquid chromatography (LC), and high-performance liquid chromatography (HPLC).[6–8,13,14] Technical advances have moved the ICP/MS technology beyond a research tool. It is now attractive for analysis of clinical specimens in a service environment offering:

1. Reliable, user-friendly instrumentation—greatly reduced training is required for routine operation in a service laboratory.
2. Simultaneous measurement of multiple trace elements coupled with relatively high throughput has made ICP/MS financially competitive for analysis of clinical specimens.
3. Cost-effective analyses—competition and volume sales continue to reduce the cost of ICP/MS instrumentation. However, capital expenditures remain high, compared with the atomic absorption and anodic stripping alternatives that are in wide use. Nevertheless, laboratories with sufficient test volumes will be able to amortize the cost of capital and significantly reduce their overall cost per test using ICP/MS instrumentation because labor and supply costs can be reduced.
4. Simplified specimen handling—ICP/MS methods minimize matrix and other interference resulting from the complex matrices common to most biological specimens.
5. Isotope analysis—the ability to measure specific isotopes avoids much of the interference, allows use of the powerful isotope dilution methods,[15,16] and opens the way for *in vitro* investigations using nonradioactive isotopes. Isotope ratio comparisons can quite readily be determined and may prove helpful for identification of the sources of trace element exposures.[10]
6. Outstanding analytical performance—sensitivity, specificity, and precision are excellent making accurate and precise trace element measurements routine at trace element concentrations in parts per billion or, in some cases, even parts per trillion.

Clinical service laboratories performing trace element analyses on clinical specimens would do well to consider this powerful technology. This chapter includes an overview of the principles of

ICP/MS, information on currently available ICP/MS instrument alternatives, and a summary of the uses of ICP/MS technology for the analysis of clinical specimens.

II. INSTRUMENTATION

ICP/MS instrumentation includes three major components: the plasma, the interface, and the mass spectrometer. The plasma with the nebulizer serves as the ion source for the mass spectrometer that selectively detects ions based on their mass-to-charge ratio (*m/z*). In this section, the basic operating principles of inductively coupled plasmas and mass spectrometers will be discussed. The interface for ICP/MS instrumentation is critical;[17] therefore, interface geometry and design will also be addressed.

A. INDUCTIVELY COUPLED PLASMA

The term plasma refers to a hot, partially ionized gas. In the analytical field, argon plasmas are prominent, but mixed (Ar-N_2) and even pure helium-based plasmas are also described.[18] The energy required to maintain the plasma is provided by an electromagnetic field in the form of radiofrequency (ICP) or microwave energy in microwave-induced plasmas, or by a direct current (dc) discharge. The commercially available emission and mass spectrometers are coupled to ICP.

1. Instrumental Design

A typical ICP torch consists of three concentric quartz tubes surrounded by an induction coil, also called the load coil (Figure 6–1). The inner tube is used for sample introduction, whereas a tangential gas flow in the outer tube centers the plasma, as well as prevents the quartz torch from melting. The middle tube carries argon that forms the plasma. This flow is optional, because the other two argon flows can maintain the plasma. ICP was originally developed for atomic emission spectroscopy (AES), which used a vertical torch; but, in ICP/MS applications, a horizontal torch arrangement is adopted.

The ICP is created by the interaction of moving charged species in a magnetic field generated by the high-frequency alternating current (ac) in the induction coil. The magnetic field forces the charged species (electrons and ions) into a circular path called the *eddy current*. When the direction of the current in the coil, and consequently, the direction of the magnetic field reverses, the eddy current also reverses. The high-speed charged species collide with the neutral supporting gas atoms, thus causing further ionization along with intense ohmic heating. Plasma forms when a spark from a Tesla coil seeds the argon with electrons and ions. Once the gas is conducting, the plasma forms spontaneously. Coupling exists between the electromagnetic field generated by the accelerated charged particles and that of the load coil when the impedance of the plasma matches the radiofrequency output. This is necessary to sustain the plasma, and the impedance match is achieved with a tuning circuitry.

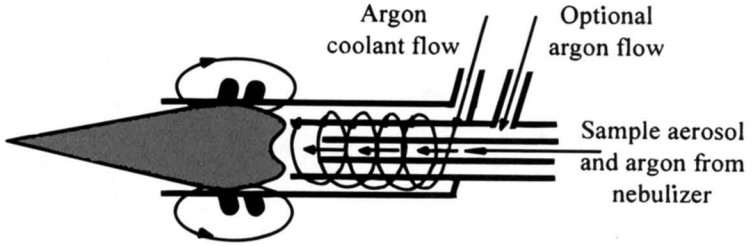

FIGURE 6–1. ICP torch.

2. Inductively Coupled Plasma (ICP) Characteristics

Three different gas flows are involved to maintain a stable plasma. A relatively low flow velocity, ~1 l/min, is used in the inner tube for sample introduction. This can be done with a conventional cross-flow nebulizer, but other methods developed for ICP-AES can also be used. A much higher flow rate, typically 10 l/min, is required in the outer tube. The gas flow in the middle tube (5 to 7 l/min) is optional.

In the U.S., the radiofrequency used to maintain the plasma is regulated; generators of 27.12 or 40.68 MHz are applied. The higher oscillation frequency leads to lower spectral background and more stable plasma, which provides an improved detection limit in AES. Another advantage of the higher frequency is that the coupling between the generator frequency and plasma load is more efficient; therefore, the plasma is more reliable and less sensitive to sample introduction even at lower power level. The increased coupling, however, causes a secondary discharge at the ICP/MS interface (see later), which gives rise to an increased concentration of oxides and doubly charged species. At the same time, shielding of a 40-MHz instrument is more difficult. Therefore, typical commercial ICP/MS instruments operate at 27 MHz, with a nominal power of 1 to 5 kW.[19]

The radiofrequency used also affects the shape of the plasma. In the earlier instruments operated at a lower frequency of about 5 MHz, teardrop-shaped plasma formed with the high temperature region located in the plasma core (Figure 6–2). At this high temperature region, the resistivity is the highest because of the gas expansion; therefore, the sample tends to avoid this region. This results in an inefficient heating of the sample. At higher frequencies, annular or doughnut-shaped plasmas are obtained. The sample particles travel through a narrow axial channel of high temperature in about 2 to 3 ms, which leads to more efficient desolvation and volatilization.

Depending on the methodology used, various plasma temperatures have been reported, but all authors agreed that plasma temperatures are significantly higher than those obtained by high temperature flames.[20] The most reliable descriptors are electron and gas-kinetic temperatures (T_e, T_{gas}), because their measurement does not require the assumption of thermal equilibrium that is not always achieved in plasmas. Typical electron temperatures are 7,000 to 11,000 K for ICPs. Such high temperatures provide complete desolvation and evaporation, as well as efficient ionization.

Another important property for characterizing ICPs is the electron number density (n_e) that will, obviously, determine the concentration of singly and doubly ionized species. In the literature,

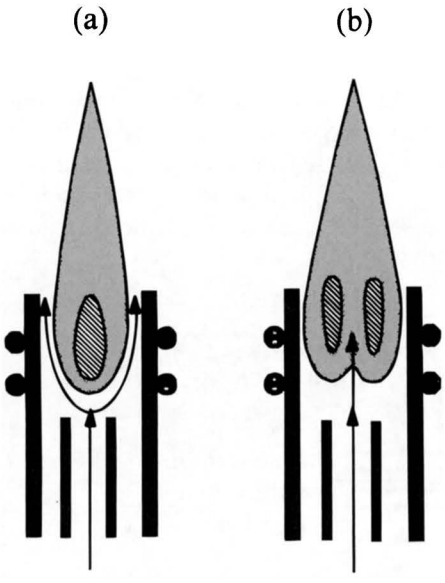

FIGURE 6–2. Plasma shapes at 5 MHz (a) and 27 MHz (b).

accepted n_e values range from 10^{14} to 10^{16} cm^{-3}.[20] These high values explain the lack of ionization interferences (see later), compared with flame atomic absorption spectroscopy (AAS). Because the temperature and electron density values vary across the plasma, it is also important which portion is sampled by the mass spectrometer.

B. INTERFACE BETWEEN THE ICP AND THE MASS SPECTROMETER

Interfacing ICP with mass spectrometers is not a trivial task, because ICP operates at atmospheric pressure and very high temperature. MS operates at a lower temperature and very low pressure.

1. Interface Design

The two-stage interface between the plasma and the mass spectrometer consists of a sampling cone on the tip of a water-cooled front plate, and a sharp-edged aperture in a skimmer cone (Figure 6–3).

The first aperture is typically 0.5 to 1 mm in diameter and located on the torch axis at 5 to 10 mm from the end of the load coil (i.e., the sampler cone is actually in the plasma flame). If a smaller sampling orifice is used, a cooled boundary layer of argon forms over the sampler, and the observed mass spectrum corresponds to the ionic composition of this cold layer rather than to that of the bulk plasma. However, when attempts were made to increase the diameter of this aperture, a current flow between the plasma and the orifices (i.e., a secondary discharge) was observed.[11]

This "pinch" effect produced very high continuum background, a high concentration of doubly charged ions, and very high ion energies that reduced the resolution of the quadrupole filter. Also, by degrading the sampler, it gave rise to very high level of ion signals of the cone material. The secondary discharge was caused by the high radiofrequency potential of the plasma. The plasma potential, which can be several hundreds of volts, is derived from the coupling of the radiofrequency voltage of the load coil to the plasma through the stray capacitance between the coil and the plasma.

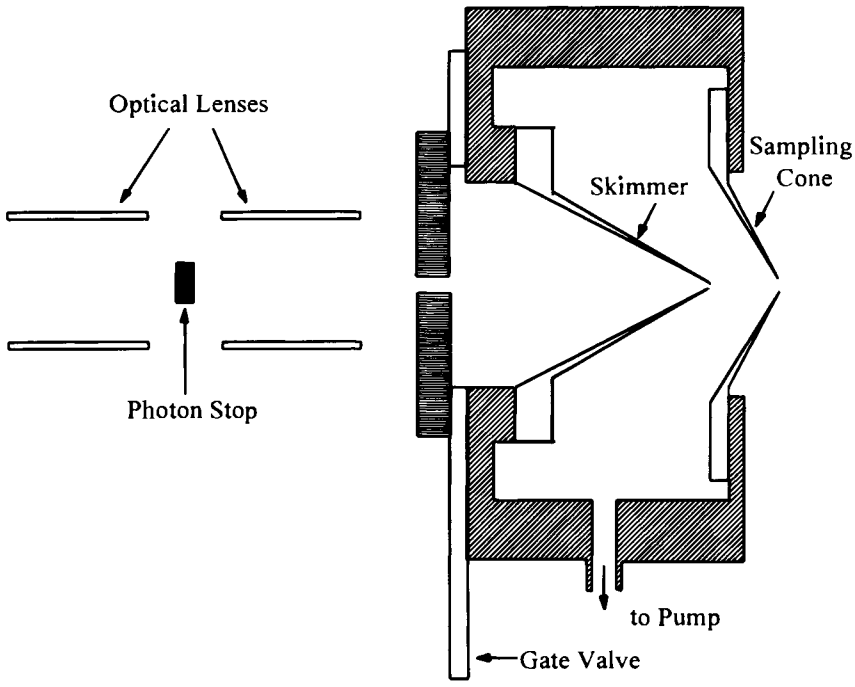

FIGURE 6–3. Interface design for ICP/M.

In current instrument designs, to eliminate the secondary discharge, the center of the load coil is grounded.[21] With this modified circuit, half of the load coil is positive when the other half is negative with respect to ground, and each half drives the plasma potential in opposite directions, thus resulting a low net plasma voltage. The buildup of high plasma potentials, and consequently, the secondary discharge can be prevented by multilayer load coils that shield the plasma from the field of the outer turn. Although this coil design certainly reduces the secondary discharge, the ignition of the plasma is more difficult because of the shielding.

The plasma tail flame extends into the first vacuum stage and forms a jet that is intercepted by the skimmer. The pressure between the sampler and the skimmer cone is 1 to 3 torr, and most of the gas entering this stage is pumped away. At this point, an electrostatic field is not used to separate the neutral and charged particles. The diameter of the skimmer aperture is typically 1 mm, and the pressure behind it is around 5×10^{-4} torr. Behind the skimmer, an ion lens focuses the charged species through a final aperture into the quadrupole mass analyzer.

C. MASS SPECTROMETRY

MS has been a reliable tool for qualitative and quantitative determination of elements based on their *m/z* since the mid-1950s. Based on different physical principles to separate ions with diverse *m/z* values, various instruments have been developed. The so-called quadrupole ion traps are used in the commercially available ICP mass spectrometers.

1. Quadrupole MS Operation

Quadrupole mass spectrometers use path stability as a means to separate ions according to their *m/z*. In the quadrupole ion trap (Figure 6–4), four short electrodes are used that are most commonly four short, parallel metal rods, with a typical diameter of 6 mm. In some designs, the electrodes are hyperbolically shaped to create a more homogeneous quadrupole field.[22] The opposite electrodes are electrically connected, and attached, respectively, to the positive and negative poles of a variable dc source. In addition to the dc voltage (U), variable radiofrequency ac potentials (V), 180 degrees out of phase, are applied to each pair of electrodes. The fields of the two pairs of electrodes combine and create a quadrupole field.

Ions are injected from the source along the axis of the quadrupole ion trap. The charged particles are under the influence of the quadrupole field that forces them to oscillate along the central axis of travel. Depending on their *m/z* ratio, ions display different trajectories. Some of these trajectories are unstable (i.e., they tend toward infinite displacement from the center of the traveling axis). These ions are lost and not detected (e.g., through collision with an electrode). Ions that are successfully transmitted through the analyzer are said to possess stable trajectories. These ions give rise to a signal in the detection system. The path stability of an ion depends on the radiofrequency (Ω), and the magnitude of the dc and radiofrequency potentials (U and V).[22] The ratio of the dc and radiofrequency potentials (U/V) determines the resolution of the instrument. At zero dc potential ($U = 0$), wide bands of *m/z* values are transmitted; but, as the ratio U/V increases, the resolution of a single *m/z* value also increases. A mass spectrum can be generated in two ways: either by scanning U and V while keeping their ratio, as well as the radiofrequency constant, or by scanning the frequency and holding U and V constant.

2. Quadrupole MS Characteristics

Ideally, a mass analyzer should be able to separate particles with small mass differences while still allowing a sufficient number of them to pass through the analyzer to yield a measurable signal. This capability of the mass analyzer is usually referred to as resolution, $m/\Delta m$, where m and $m + \Delta m$ refer to the masses of two particles with just separable peaks of equal size. Theoretically, the mass resolution of a quadrupole filter can be infinite by adjusting the U/V ratio; however, the resolution is limited by the number of radiofrequency cycles the ion spends in the quadrupole field. As the number of cycles decreases, the resolution also decreases.[12] Clearly, the resolution degrades when

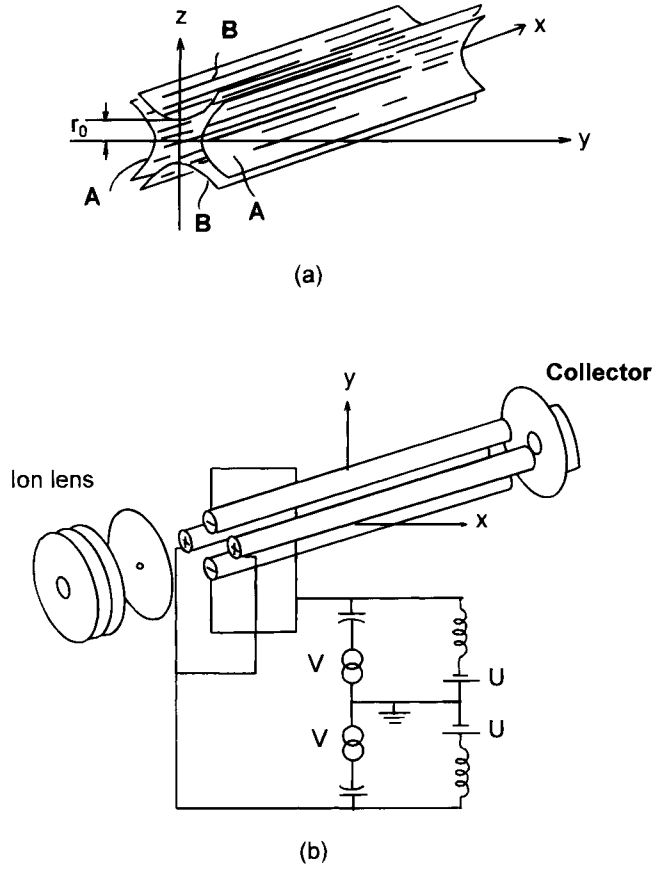

FIGURE 6–4. Quadrupole mass filter. (a) The arrangement of four electrodes (A, A, B, and B) of hyperboloidal shape; the electrodes are at a distance r_0 from the x axis. (b) Schematic arrangement of the mass filter. (From Knewstubb, P. F., *Mass Spectrometry and Ion-Molecule Reactions*, Cambridge University Press, New York. With permission.)

high-energy particles enter the mass filter. Manufacturers commonly set the resolution to 1 amu across the mass range of 0 to 250 Da (i.e., the absolute resolution varies).

Because the scanning is completely electrical, the entire mass spectrum range can be done in a rather short period of time. The scanning of the 0 to 250 Da mass range can be performed in about 30 ms, but usually a scan period of 100 ms or 1 s is chosen. The total number of scans for the integrated data collection is typically set to 1 min, but for more dilute samples it can extend to 5 to 10 min. This fast scan rate allows several repeated mass scans, thus better representation of the mass distribution, even when the transient signal is only a couple of seconds, which is the case for GC/ICP/MS, LC/ICP/MS, and other coupled instrument systems.

III. INSTRUMENT ALTERNATIVES

In 1996, eight different manufacturers are marketing ICP/MS instrumentation in North America.[23] Three companies are actively marketing ICP/MS instruments for analysis of clinical specimens in the service laboratory environment: Perkin–Elmer Corporation (Norwalk, CT); Hewlett–Packard Company (Wilmington, DE); and Varian (Sugar Land, TX). In 1994, Perkin–Elmer introduced their newest version designed and manufactured by Perkin–Elmer SCIEX. Building on their ICP/MS experience since 1983 with earlier models (ELANs 250, 500, and 5000), the ELAN 6000 offers improved computer control, and increased sensitivities plus an optional flow injection system that facilitates microsampling and matrix separation with automatic analyte pre-

concentration. The Hewlett–Packard model (HP 4500) features a compact bench-top ICP/MS instrument package designed by the Hewlett–Packard and Yokogawa Electric partnership venture for routine use in the demanding environment of the service laboratory. Building on experience with ICP atomic emission spectrometers, Varian designed an ICP/MS unit designated the *ULTRA ICP/MS Package Spectrometer System* to compete in this emerging instrument market.

Each of these instrument alternatives differs somewhat in design details, but they each offer automated instrumentation that requires small sample volumes, features user-friendly operation, and is relatively fast and efficient. Direct operating costs per test for disposables and labor are generally less than for atomic absorption and anodic stripping methods. However, only clinical service laboratories performing at least 1,000 trace element analyses per month will likely be able to justify the relatively large capital investment. Competition and volume sales will continue to bring down the capital costs; but, before clinical laboratories invest in ICP/MS instrumentation, they should conduct a careful financial analysis to determine the return on the $190,000 to $240,000 U.S. dollar investment that will be required to establish ICP/MS technology in a service laboratory.

Although not yet widely used in clinical service laboratories, researchers have developed a number of different ways to process specimens before their introduction to the ICP/MS to overcome potential interference. Multielement flow injection analysis and elemental speciation by reversed-phase LC was reported in 1986.[13] Since that time many enhancements have been proposed. A 1995 review summarizes electrothermal vaporization and laser ablation techniques for direct introduction of solid samples.[8] Several techniques have been reported for separation of specific analytes from the potentially interfering matrices common to biological specimens, including precipitation, solvent extraction, anodic stripping voltammetry, chelation, ion exchange, isotope selection,[15,16] and chromatographic methods including HPLC, GC and gas-liquid chromatography.[8,13,14] In our laboratory, the selection of specific isotopes has proven useful to avoid some specific polyatomic masses that interfere. For example a $^{23}Na^{40}Ar$ interference with urine and to a lesser extent serum copper measurements using the most abundant ^{63}Cu isotope can be avoided by selecting the less abundant ^{65}Cu isotope.[24] Similarly, by selecting ^{82}Se from the six selenium isotopes, interference in urine and serum is avoided.[25]

IV. ANALYSIS OF CLINICAL SPECIMENS

Determination of elements in chemically complex materials requires analytical methods that do not suffer from matrix interferences. Obviously, there is no such ideal analytical technique; however, ICP/MS suffers from less matrix effects than the other elemental analytical methods. This makes the technique very advantageous for the analysis of biological, geological, and environmental samples. A complete review of all these applications is not our purpose; therefore, we limit the discussion to the analysis of clinical specimens.

A. APPLICATIONS

Analysis to determine concentrations of biologically important elements such as magnesium, iron, zinc, and copper have been used in clinical medicine for many years. There is increasing interest in monitoring occupational and environmental exposures to many trace elements. As their biological impacts have become recognized, the need to trace element measurements in various tissues and body fluids has increased. AAS is the most common method for the quantitative analysis of trace elements, because it provides accurate and precise results at relatively low cost. In a service laboratory, the wide spectrum of the trace elements having biological importance can be more conveniently analyzed with multielement techniques, such as ICP/MS that is capable of the determination of most of the elements in the periodic table. Clearly, simultaneous multielement analysis requires careful matrix matching and optimization of the instrumental settings in selected mass ranges. However, the excellent sensitivity and low detection limit of ICP/MS for many elements, plus the relatively high throughput, make it the method of choice in the analysis of trace elements in clinical specimens.

Several procedures have been developed and published since the advent of ICP/MS describing the analysis of various clinical specimens with ICP/MS, some of which are summarized in Table 6–1. From the table, it is obvious that a wide variety of elements can be analyzed in blood, plasma, urine, and even in less frequently used specimens, such as bone, tooth, and other tissues. Because of the sensitivity of ICP/MS, generally no derivatization is needed; the samples, either body fluid or solid tissue, are digested or diluted with mineral acid, and this step is followed by the introduction of the solution into the plasma.

B. SAMPLE PREPARATION AND SEPARATION

Unless speciation is required, sample preparation for elemental analysis of biological specimens via ICP/MS requires relatively little specimen preparation. Body fluids are diluted, and the proteins are acid-precipitated, typically with nitric acid, whereas tissue samples are digested in nitric acid with the addition of other acids if required. Most trace elements are released from their biological complexes by the acid precipitation conditions. Digestion with nitric acid gives rise to the same interfering peaks as deionized water,[55] whereas other mineral acids generate more interfering species; but, hydrochloric, sulfuric, and phosphoric acids can also be used. Because all of them introduce various polyatomic interfering species (e.g., ClO^+, $ClOH^+$, $ArCl^+$, ArS^+, and ArP^+), the analyte and its concentration will determine the most suitable acid. Although cold digestion (i.e., digestion at room temperature) can be appropriate for urine or blood specimens, the digestion of tissue samples can be promoted via microwave energy or by simple heating (hot digestion). The concentration of the acid in the digestive mixture should be determined experimentally, but typically is 5 to 15%.

Regulations and public pressure are forcing more and more employers to screen employees exposed in the work environment; therefore, it is not surprising that the number of tests requested (e.g., whole blood lead analysis) is rapidly increasing.[56] Consequently, there is a need for easy specimen collection and handling for this kind of screening tests. One of the current trends is sample collection on filter paper.[57–59] In our laboratory, a reproducible portion of the filter paper containing the specimen is placed in an ethylenediamine tetroacetic acid (EDTA) solution to remove the metal

TABLE 6–1
Applications of ICP/MS in the Analysis of Clinical Specimens

Specimen	Elements	Refs.
Serum	Al, B, Br, Ca, Co, Cr, Cs, Cu, Fe, I, Li, Mg, Mn, Mo, Pb, Rb, Sc, Sn, Sr, Ti, Zn	26–28
Plasma	Br, I, Mg, Pt, rare earths	30–32
Blood	Al, B, Br, Cr, Cs, Cu, Fe, Hg, I, Li, Mg, Pb, Rb, Sc, Sn, Sr, Ti, Zn	28, 33–36
Urine	As, Au, Br, Hg, I, Mg, Mo, Ni, Pb, V, rare earths	29–31, 34, 36–41
Liver	Fe	42
Heart	Fe	42
Hair	As, Cd, Co, Cr, Hg, Ni, Pb, Th	43, 44
Feces	B, Th, Zn	35, 45–47
Breast milk	Zn	35
Hepatocyte	Fe	42
Erythrocyte	Cs, Li, Mg, Se	31, 48
Teeth	Ag, Hg	49
Bone	Ba, Ca, Fe, K, Mg, Na, P, Pb, Sn, Sr, Zn	50, 51
Sweat	Pb	36
Saliva	Ag, Au, Bi, Cd, Mo, Ni, Sn, Zr	28
Hippocampal tissue	Al, Se, Si, Sn, Zn	52
Bovine liver	Al, Br, Ca, Cr, Cs, Cu, Fe, Li, Mg, Mn, Rb, Zn	27
Fish liver	As, Cd, Co, Cu, Cr, Fe, Hg, Mg, Mn, Na, Ni, Pb, Zn	53
Fish muscle	As, Cd, Co, Cu, Cr, Fe, Hg, Mg, Mn, Na, Ni, Pb, Zn	54

ion(s) of interest. Because no wet ashing is necessary, the handling of hazardous concentrated acid is avoided.

Stringent collection techniques must be followed to avoid contamination during the collection and handling of specimens to be analyzed for trace elements. Contamination from the collection site and the collection containers must be avoided. Furthermore, specimen storage in inappropriate containers can be major sources of contamination.

Sample preparation time is affected mainly by the specimen and the digesting conditions (cold or hot digestion), and is determined experimentally. If there is particulate matter remaining after the digestion step, the sample should be centrifuged and the supernatant should be used for further analysis. Methods must be adjusted to avoid co-precipitation of trace elements with residues. Controls should be handled in the same manner as the sample. After the digestion step, the acid solutions formed from the sample and controls are introduced into the plasma via the nebulization system.

C. METHODS FOR ANALYSIS OF CLINICAL SPECIMENS

In the following section, a representative collection of analytical procedures for elemental analysis of different clinical specimens is presented.

1. Calibration

Calibration and quantitation are done with external or internal standards, the standard addition method, or with the isotope dilution method.

With *external standardization*, a series of dilutions with known analyte concentration is prepared (external standards), and the analyte signal is measured. It is important that the matrix of the standards is carefully matched to the biological matrix; otherwise, matrix effects present in the sample, but not in the standards, can alter the analytical signal relative to that from the standards.

In the *internal standard method*, known concentration of a reference species is added to all samples, standards, and blanks. For ICP/MS, an element that is normally not present in the sample (typically beryllium, cobalt, indium, thallium, or yttrium[10,34,53]) is chosen. The internal standard compensates for several types of random and systematic errors. It also reduces the effect of interfering species, assuming that the internal standard is affected the same way as the analyte.

In the *standard addition* technique, the sample is spiked with known amount(s) of the analyte. Standard addition is used when the sample matrix is difficult to reproduce, because the sample serves as the "standard." The method is less influenced by matrix effects than the external calibration, but it is more labor intensive.

MS is well suited for *isotope dilution* methods that are based on the addition of a known amount of enriched isotope, the "spike," to the specimen to be analyzed. Isotope dilution technique is fairly insensitive to matrix interference; therefore, it can be used for analyzing in complex matrices. Because of the importance of this technique, a more detailed discussion is presented later in this chapter.

2. Clinical Specimens

To interpret trace element results effectively, the dramatic concentration differences between specimens must be understood (Table 6–2). Some trace elements are equally distributed in plasma, serum, and erythrocytes; others have very different concentrations in those blood components. Table 6–2 was derived from reported concentration ranges.[60,61]

a. Urine

For many trace elements, the urinary tract is the major pathway of excretion; therefore, urine can be effectively tested to address potential poisoning or environmental exposure of a variety of trace elements. For analysis purposes, 24-hour collection is preferred, and the samples are typically acidified to pH2 with hydrochloric acid. After dilution with 5% nitric acid, the sample is ready for analy-

TABLE 6–2
Relative Distribution of Selected Elements in Circulation (Blood Concentration = 100%)

Element	Blood	Percentage		
		Plasma	Serum	Erythrocyte
Arsenic	100	—	70	—
Bismuth	100	30	—	—
Chromium	100	—	2	200
Copper[a]	—	100	100	110
Lead	100	6	—	—
Manganese	100	8	5	160
Mercury	100	120		330
Molybdenum	100	75		850
Zinc[a]	—	100	110	1400

[a] Plasma concentration is taken as 100%.

sis. Precipitate may form if the protein concentration is high; in this case, the precipitate should be removed by centrifugation. Random urine specimens can also be analyzed, but interpretation is often more difficult, especially for moderate analyte elevations. Urine creatinine values are used to normalize analyte concentrations in random urine collections.

b. Blood

Blood plays an important role in the transport and metabolism of numerous chemical species. It is readily accessible and thus submitted frequently for elemental analysis.

The collection tube is acid washed and closed with special rubber stoppers to minimize contamination. For collection, special vacutainers for trace elements are available. The preferred anticoagulant is EDTA, because heparin does not prevent the formation of microclots that interfere with the sampling of the specimen. Note that prolonged storage in glass containers can significantly alter some trace element concentrations. The specimen is treated with mineral acid to release the trace elements, diluted with deionized water, centrifuged to remove particulate matter, and the supernatant is used for analysis.

c. Serum and Plasma

Serum and plasma concentration are generally similar for most trace elements; but, for some elements, erythrocyte and blood concentrations can be quite different (Table 6–2). For example, erythrocyte zinc levels are at least ten times higher than serum or plasma levels that causes zinc concentration to be 5 to 15% higher in serum than in plasma, because zinc is released from erythrocytes and platelets during clotting.[60] Care must be taken to avoid hemolysis when serum or plasma specimens are to be analyzed, because several elements (e.g., zinc, magnesium, and iron) are present at much greater concentration in red blood cells than in plasma. In these cases, any hemolysis causes erroneous overestimation of the serum or plasma values.

For specimen preparation, first the serum or plasma proteins are precipitated with nitric acid. A notable exception is mercury, which requires hydrochloric acid precipitation.[34] Then, the sample is diluted with deionized water, the precipitate is removed by centrifugation, and the supernatant is used for the analysis.

d. Hair

Scalp hair may be a suitable biological sample for the estimation of long-term intake or exposure to some trace elements such as arsenic and mercury, but representative sampling and external contamination during sample collection, preparation, and handling are serious challenges. A very

informative characteristic of hair sample is the distribution profile of trace elements along the length of the hair that can be used for estimating the time of exposure or poisoning.[43] Overinterpretation has been a problem for hair specimens; furthermore, contamination issues are intensified by their treatment, care, exposure, etc.

Hair is washed with ether or Triton X-110 before digestion. Then, a small section of a single hair strand is wet-ashed and dissolved overnight in a small volume (typically 50 µl) of nitric acid at room temperature. Because it is not possible to measure accurately the weight of a short piece of a single strand of hair, the weight of the sample can be estimated from its length, given that the weight of the whole strand is known. After digestion, the acidic solution is diluted with deionized water and introduced into the plasma. Using this procedure Yoshinaga et al.[43] were able to quantitate copper, zinc, arsenic, cadmium, antimony, mercury, thallium, and lead, with detection limits ranging from 0.002 µg/l (ppb) for cadmium to 2 µg/l for zinc.

e. Bone and Teeth

Several metals (including aluminum, arsenic, bismuth, lead, strontium, and titanium) accumulate and can be assessed in bones. Analysis of metals in bone provides quantitative information that is very difficult to interpret, because adequate reference information is generally not available. In the absence of suitable reference information, normal control specimens of the same type must be run under the same conditions to assist with interpretation.

Typical sample preparation starts with drying. Then, the dried sample is pulverized and a small portion of it is wet-ashed with hot nitric acid and hydrogen peroxide as an oxidant to promote digestion.

f. Other Tissues

Several toxic elements are concentrated in the liver and kidney. The liver preferentially accumulates silver, arsenic, cobalt, mercury, manganese, molybdenum, selenium, and tin; whereas silver, gold, bismuth, cadmium, cobalt, cesium, mercury manganese, molybdenum, selenium, and tin accrue in the kidney. Analysis of certain elements in other tissues, such as in the brain (e.g., aluminum and mercury), placenta (e.g., calcium, copper, and iron), and even in feces (e.g., cadmium), breast milk (e.g., calcium, potassium, and iron), and urinary stones (e.g., cadmium, manganese, and lead), has also been described in the literature (Table 6–1).

Sample digestion conditions depend on the type of specimen. Typically nitric acid is used, and digestion is promoted with heating and the addition of hydrogen peroxide. Reference ranges are generally not accessible; therefore, results must be compared with appropriate reference specimens.

D. ANALYTICAL PERFORMANCE ON CLINICAL SPECIMENS

1. Sensitivity

With ICP/MS methods, detection limits for most of the elements are in the 1 to 100 ng/l range, similar to or lower than AAS and anodic stripping voltammetry (ASV), and superior to ICP-optical emission spectroscopy.[17] Detection limits of some biologically important trace metals in biological specimens are summarized in Table 6–3. Practical detection limits in clinical specimens are often an order of magnitude higher than in aqueous solutions.[56]

2. Precision—Accuracy

Both short-term (30-min) and long-term (4 hours) instrument stabilities are typically below 5%.[7] With careful standardization, precision of 2 to 3% can be routinely achieved. Using internal standards, this can be improved further to 1%. Isotope ratios can be measured much more precisely, generally with 0.1 to 1% coefficient of variation (CV) for stable isotopes.[7] For clinical specimens, 5 to 15% overall, CV values have been reported.

Accuracy depends on the quality of the standards, interferences, contamination, etc.

TABLE 6–3
Sensitivity, Percentage, Precision, and CV of ICP/MS in the Analysis of Selected Trace Elements in Clinical Specimens.

Element	Specimen	Detection limit	RSD[a] (%)	Refs.
Platinum	Plasma	0.05 µg/l		32
Bromine	Plasma, urine	52 µg/l	5	30
Iodine	Plasma, urine	1.6 µg/l		
Mercury	Urine	0.2 µg/l	5–16	34
Arsenic	Urine	0.004 µg/l		41
Nickel		0.03 µg/l		
Vanadium		0.01 µg/l		
Lead	Blood	0.15 µg/l	10	33
Antimony	Hair	0.004 pg	15	43
Arsenic		0.04 pg		
Cadmium		0.002 pg		
Copper		0.3 pg		
Lead		0.02 pg		
Mercury		0.1 pg		
Thallium		0.0004 pg		
Zinc		2 pg		
Bismuth	Serum	0.007 µg/l	5.7–13.6	62
Thallium	Hair	0.001 µg/l		63

[a] RSD, relative standard deviation.

3. Useful Range

The linear calibration range is limited by the mass spectrometer detector, but typically spans over 6 to 10 orders of magnitude,[23] which is more than sufficient for the concentration ranges found in clinical specimens. For daily calibration, standards are included that cover the range of values that might be encountered in biological specimens.

V. ADVANTAGES AND DISADVANTAGES OF ICP/MS ANALYSIS FOR CLINICAL SPECIMENS

A. THROUGHPUT

In busy service laboratories, many specimens must be analyzed every day, and labor is generally the greatest component of total cost. Therefore, productivity, which might be assessed by the number of performed tests per paid labor hour, is critically important. Overall ICP/MS throughput is influenced by the time for sample preparation, instrument stability and reliability, as well as the actual instrument analysis time. It is our experience that one person can perform the required specimen preparation on about 100 blood or urine specimens for ICP/MS analysis in 1 hour. After the daily morning calibration for each element to be tested, instrument stability is generally adequate to run several hours before control results, which are used to monitor drift and indicate that recalibration is required. About 10 to 20 min/day are required for routine maintenance of the instrument, which consists primarily of cleaning the torch and the sampler cone. Analysis time for each specimen is 0.5 to 5 s.[10] Approximately 40 specimens can be analyzed per hour, with simultaneous measurements possible for several elements. Therefore, the ICP/MS becomes a productive tool in a service laboratory.

B. SENSITIVITY—SPECIFICITY

Detection limits of several elements with ICP/MS are in the nanogram per liter (ppb) range, thus ICP/MS is a very sensitive technique. The reported detection limits for many elements are listed in Table 6–3.

With proper mass selection and specimen preparation to eliminate potential interference good specificity can be achieved, especially above 85 m/z where the interference from polyatomic species is negligible.[55]

C. MULTIPLE ELEMENT MEASUREMENT

Multielement analysis is one of the major advantages of ICP/MS. Simultaneous detection of up to 15 elements in serum and whole blood has been reported.[28] The ability to analyze multiple elements simultaneously is affected by the sample matrix and selectivity of the detector. Detection limits may be increased because of interaction between the elements to be analyzed.[64]

D. ISOTOPE MEASUREMENT

Isotope measurement with ICP/MS technology is becoming a useful tool in selected areas of laboratory analysis.

1. Isotope and Isotope Ratio Measurements

By selecting a specific isotope, interferences may be eliminated.

ICP/MS has also been used to measure isotope tracers to study the absorption and metabolism of iron and magnesium.[31,65–67] Measuring stable isotopes instead of radioactive isotopes eliminates several problems in handling, storing, and disposing of hazardous radioactive material; at the same time, this method does not have the risk associated with the use of radioactive isotopes.

Also, isotope ratio measurements can potentially be useful for determining the source of exposure. Reinhard and Ghazi[50] have analyzed lead in skeletal remains and artifacts to evaluate the source of lead exposure. A much wider study has been conducted by al Saleh et al.[68] to identify the lead-contained petrol as the source of lead exposure among children in Saudi Arabia.

2. Isotope Dilution

Isotope dilution mass spectrometry (IDMS) was developed in the 1950s, and since then has found application in certification of Standard Reference Materials.[16]

IDMS is based on the addition of a known amount of enriched isotope, the "spike," to the sample. After equilibration, the altered isotopic ratio, R_m, is given by the following relationship:

$$R_m = \frac{A_x c_x m_x + A_s c_s m_s}{B_x c_x m_x + B_s c_s m_s}, \tag{1}$$

where subscripts x and s refer to the sample and the spike, respectively; A and B are the atomic fractions of isotope A and B; c is the concentration of the element; and m is the weight. The concentration of the element in the sample can be calculated from:

$$c_x = \left(\frac{c_s m_s}{m_x}\right)\left(\frac{A_s - R_m B_s}{R_m B_x - A_x}\right). \tag{2}$$

In principle, IDMS can be applied to elements with at least two stable isotopes. The primary source of enriched isotopes has been the Oak Ridge National Laboratory Electromagnetic Isotope Enrichment Facility.

The two major sources of error in IDMS comes from the sample preparation and from the mass spectrometric analysis.[16] Care should be taken that the sample and the spike equilibrate completely before analysis. The mixture of spike and sample has to be optimized to avoid "overspiking" or "underspiking" (i.e., the spiked sample ratio approaches that of the spike or natural isotope ratio). Isobaric interferences in the mass spectrum can create a problem that can be overcome by a preconcentration or separation step. Accuracy and precision for isotope ratio measurements are typically 0.25%, with detection limits in the parts per billion range. The additional cost of the enriched spike

is about $10 per milligram. It is very appealing that, in ICP/MS, a multielement "master" spike can be prepared to determine more than 20 elements simultaneously.

E. EQUIPMENT COST

The cost of instrumentation is a disadvantage of ICP/MS when compared with AAS. ICP/MS units cost approximately $200,000, whereas AAS instruments can be purchased for 25 to 30% of that amount. Instrument operation, caused by the high argon consumption, adds approximately $.20/patient specimen analyzed depending on the testing volume. ICP/MS instrumentation requires a large initial investment, but the potential labor-saving made possible by the relatively high throughput and simultaneous measurements renders this method cost-effective for use in clinical laboratories.

F. INTERFERENCES—MATRIX EFFECTS

ICP/MS suffers from interferences and various matrix effects. Mass overlaps from elements (isobaric overlap), polyatomic species, and doubly charged ions are the major problems. The quadrupole mass detector is able to separate species that differ about 1 amu.[23] Higher resolution can be achieved with single- or double-focusing mass analyzers, however, the significantly higher equipment cost cannot be justified in a production laboratory.

Isobaric interferences can be overcome using computer software corrections that are usually not very ideal. With the software correction method, the signal caused by the contaminant is estimated at a different *m/z* peak, then its contribution is subtracted from the analyte signal. Another, more accurate, way to overcome isobaric interferences is to select a different isotope of the analyte, which is not affected by interfering species. For example, measurement of $^{58}Ni^{+}$ (57.9353) is seriously affected by $^{58}Fe^{+}$ (57.9333). Although it is a minor iron isotope, iron is usually present in biological specimens at a much higher concentration level than nickel, which makes the use of this nickel isotope impractical. The iron interference can be eliminated by measuring another nickel isotope, ^{62}Ni.[10]

More problematic are the polyatomic species that are created by recombination events in the plasma. Oxides ($ArOH^{+}$, CO^{+}, and NOH^{+}) arise from aqueous solutions, chlorides, nitrides, and sulfides ($ArCl^{+}$, ClO^{+}, NOH^{+}, SH^{+}, and SO^{+}) from the corresponding mineral acids used to process the specimens,[55] and argon species (Ar^{+}, ArH^{+}, and Ar_2^{+}) from the plasma itself. Because many of these species are present in the plasma, numerous possible interferences can occur. For example, the determination of the three most abundant calcium isotopes (^{40}Ca, ^{44}Ca, and ^{42}Ca) is affected by $^{40}Ar^{+}$, $^{12}C^{16}O_2^{+}$, and ArH_2^{+} interferences, respectively.

Although species are separated according to their *m/z*, doubly ionized species are also a source of potential interference. Fortunately, few elements have low enough second ionization potentials to form doubly charged ions at the plasma temperature. Nevertheless, if large amounts of $^{48}Ca^{2+}$ are produced in the plasma, this doubly charged ion can cause a significant increase in the $^{24}Mg^{+}$ signal, for example.[10]

Any element, if present at high concentration, can cause interference with ICP/MS. High salt concentration can suppress the nebulization efficiency and can potentially plug the sampling orifice.[69] Easily ionized elements can enhance or suppress the analyte signal,[54] but suppression is more commonly observed. These elements contribute significantly to the electron density in the plasma and consequently decrease the ionization of the analyte. Over the years, several solutions have been suggested to overcome this signal suppression that is more pronounced with ICP/MS than with ICP/AES. These include decreasing the nebulizer flow rate and adjusting the ion lens voltages, both of which lead to reduced sensitivity. Addition of an internal standard has proven to be an effective means to correct for interferences, but this requires extra sample manipulation and increases the risk of contamination. It has been suggested that certain background peaks can be monitored to correct for these ion sampling effects the same way as an internal standard.[54]

G. COMPARISONS OF TRACE ELEMENT METHODS

Other methods most often identified for trace element determination in clinical specimens include: AAS, ICP-AES, ASV, neutron activation analysis (NAA), and atomic fluorescence spectrophotometry (AFS).

AAS is widely used for determination of trace elements. Typical detection limits are in the nanogram per liter range; precision with electrothermal atomization is 1 to 5%. Both detection limits and precision are similar to those obtained with ICP/MS, with less instrumentation costs than ICP/MS. However, there are two major drawbacks. AAS methods generally have a fairly limited linear range, therefore requiring nonlinear calibration curves and frequent sample dilutions. Also, matrix effects are more pronounced in AAS. Although multielement determination is possible, in a practical sense AAS is not a multielement technique. This seriously limits the sample throughput, which is inferior to the throughput of ICP/MS.

ICP-AES is 10 to 100 times less sensitive than ICP/MS.[17] This is primarily caused by the high continuous background originating from the recombination of ions and electrons. Furthermore, spectral overlapping presents another problem, because each element displays about 100 emission lines. Still the method is capable for multielement determination with a precision of 3%. Its linear range extends to six orders of magnitude, but the fairly high instrumentation and operation cost with an inferior detection limit make it a less attractive alternative for trace element determination.

Accuracy, precision, and sensitivity achievable by ASV is comparable, sometimes even superior to AAS and ICP/MS. Instrumentation is relatively inexpensive, and in theory 4 to 6 elements can be determined simultaneously. However, multielement methods for clinical samples are not available. Unfortunately, the method can only be applied to electoactive elements, and requires relatively high effort for sample preparation and instrumentation maintenance. Reagent costs per test are generally high, and throughput is low compared to other methods.

NAA for trace element determination can provide very accurate results with good precision. The method can be nondestructive and no sample preparation; therefore, no blank is required. NAA is a true multielement technique. Its major drawbacks are 1) detection limits are higher than for ICP/MS or AAS, 2) NAA is not capable of chemical speciation, 3) high cost, and 4) limited access to facilities to conduct NAA.

AFS has the wide linear range of atomic emission techniques, as well as the high selectivity of atomic absorption techniques. However, the lack of commercially available instruments and the limited application (only for couple elements) hinder the use of AFS in clinical laboratories at this time.

The widespread interest in blood lead analysis for the monitoring of children at risk of lead poisoning has stimulated many investigators to develop alternatives analysis methods. The potential of using trace element activation and inhibition of enzymes as the basis for trace element measurements is receiving increasing attention[70] and could in the future lead to attractive alternative methods. In years to come, new discoveries will bring many advances in trace element analyses.

VI. CONCLUSIONS

As the knowledge base relating to the biological import of trace elements in the environment expands, the demand for accurate, sensitive, specific, rapid, and efficient analysis of a wide variety of biological specimens will increase. ICP/MS is emerging as an attractive technology for analysis of trace elements in clinical specimens. Commercial ICP/MS instruments are now available that can perform simultaneous measurements of multiple trace elements and their isotopes. Service laboratories analyzing a variety of trace elements in a large number of specimens will find ICP/MS to be a cost-effective technology. Even though instrumentation costs are high, the efficiency of ICP/MS will result in significant savings in labor. However, because of the potential for interference from the complex matrices of biological specimens and the possibility of isobaric, polyatomic, and doubly charged ion interferences, ICP/MS method must be validated for each type of clinical specimen.

Methods are now available for analysis of many trace elements in blood, urine, bone, tissues, and other biological specimens, and improvements are rapidly emerging as investigators are coupling ICP/MS with several other analytical technologies. The future for ICP/MS has never been brighter.

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Chapter 7

FOURIER TRANSFORM INFRARED STUDIES FOR DRUG HAIR ANALYSIS

Kathryn S. Kalasinsky

CONTENTS

I. INTRODUCTION

Hair has become an increasingly popular matrix for drug analysis. This trend can be attributed to the many unique features of hair. The collection of hair specimens is much less intrusive than observed urine collections or blood collections by needle punctures. Adulteration of the hair specimen is much more difficult because of its physical nature. Replacement of the sample for a second analysis is possible because there is little change in a second hair sample. Drugs seem to have an uncommon stability when trapped within the hair matrix.[1] The hair matrix holds a near permanent record of drug usage, whereas urinalysis or blood analysis provides only current drug use. Long-term drug history may be obtained from hair by segmental analysis.[2] This drug history may be useful when acurate recorded drug usage is desired, either for clinical or forensic purposes. This historical information is not easily available by any other means.

Although these advantages have spawned much interest in hair drug analyses, many disadvantages are also inherent in the analysis of hair for drugs. Hair is not very homogeneous, because hair development varies depending on the stage of growth of an individual hair follicle. Hair composition varies depending on personal physical status; thus, drug affinity varies.[3] This may produce results in ethnic and sexual bias in hair testing. Furthermore, hair is exposed to the environment and is vulnerable to contamination. Decontamination procedures vary in their ability to remove external contamination and in some cases remove the drugs deposited by ingestion that are the desired product of analysis.[4] Cosmetic hair treatments affect hair composition and possibly drug retention. All of these factors may influence analysis of hair for drugs.

Another problem in hair analysis can be attributed to the multiple possible mechanisms of drug incorporation into the hair.[5] Several proposed routes of drug incorporation have been postulated, including diffusion from the bloodstream into the hair root, deposition from sweat at the scalp, deposition from the oily secretions of the sebaceous gland, and a combination of environmental factors. Unique drug binding in the hair matrix is evidenced as more parent drug and less metabolites are typically found with hair analysis.[6,7] This gives the hair matrix an advantage in drug identification

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over the traditional analyses. If the parent drug is still contained in the matrix, the question of whether a metabolite came from the drug or an alternate source is no longer a question. The binding of the drug to the hair matrix is still being investigated. It has been proposed that this involves the protein, melanin, and/or lipids. Many of these variables have contributed to wide range of results in dose–concentration and dose–time studies for hair testing.[8,9] The primary missing factor is the unknown pharmacokinetics and pharmacodynamics of drug incorporation and distribution in hair. To try to understand some of these factors, Fourier transform infrared (FTIR) studies of hair were initiated.

II. TECHNIQUE

FTIR spectroscopy in combination with microscopy (as a focusing tool) has been used as a unique method of analysis for drugs of abuse in hair. The systematic approach can be described as follows: (1) obtain the hair specimen and a drug-free hair to use as a reference; (2) microtome the hair into thin sections; (3) obtain infrared microscopy data on the reference and sample hair sections; (4) spectrally subtract the drug-free hair data from the sample hair data; (5) examine the resultant subtraction spectrum and compare with reference spectra of pure drugs for identification; (6) if a drug(s) is identified, follow-up with a spectral mapping experiment across or down the hair for drug distribution determination; and (7) plot the map of the occurrence of hair protein infrared frequency bands vs. the primary infrared identification bands of the drugs. This yields an image or visualization of the drug location across or down the shaft of the hair. Detailed information of these various steps is included herein.

This technique has the ability to analyze the interior portion of a hair shaft by using hair that has been prepared by microtoming in either cross-sectional or lateral directions. These hair preparations are similar to those of standard tissue sections prepared in paraffin for optical microscopy and have been described previously.[10,11] The diameter of a single hair is approximately 100 μm, with a central medulla diameter of about 20 μm. The bulk of the hair is cortex material with a cuticle outer sheath. The typical area chosen for infrared analysis will range from 20 μm in diameter to encompass the medulla area to about 100 μm in diameter to encompass the bulk of the hair shaft. In lateral cuts, an elongated or rectangular aperture can be use for maximum absorbance throughput without compromising the spatial definition desired. FTIR microscopy allows the investigator to obtain an infrared spectrum of an exact spot of the internal hair shaft chosen by using the viewing microscope. Analysis of only the internal hair shaft area minimizes the influence that external contamination will have to the resultant data. This gives the specificity and selectivity needed. The problem of using infrared techniques is that the matrix material is also viewed by the infrared microscope at the same time as the analyte and must be eliminated through spectral subtraction of drug-free reference hair. The infrared data for this reference material must be collected in the same manner as the samples (i.e., microtomed hair sections on infrared reflecting slides using an infrared microscope system).[10,11] The sensitivity of the technique has not been determined, because it would require quantitated reference material with homogeneous distribution of drug throughout. This material is not available, because these experiments show an uneven distribution on the micron scale based on many factors in both drug-ingested hair and drug-doped hair. (Sample homogeneity within a single hair shaft will be addressed in a later section.) The sensitivity is considerable, because the material is a microtomed section only 5 μm thick, with an analysis area of approximately 20 μm × 100 μm. A very rough estimate of the amount of drug material in a single infrared spectrum collected in this manner is the low picogram to femtogram range.

Infrared data yield functional group frequencies that are specific for various different compound classes. For instance, carbonyl compounds give rise to a very strong band in the 1900 to 1550 cm^{-1} region of the infrared spectrum because of the C = O bond of the molecule. By using these frequencies that are specific for various drugs and spectrally mapping, a three-dimensional image of the drug location across the hair can be obtained. Spectral maps are simply a series of infrared spectra

collected in a grid pattern across a surface. The resultant series of spectra can be combined and drafted into three-dimensional plots using specific frequencies of each spectrum in the series. These mapped images are collected with a 5- to 10-μm spatial resolution. That is, a spectrum is collected for every 5 to 10 micron shift of the microscope stage. The microscope stage is computerized so such controlled shifts are attainable.

For lateral cuts, a profile of drug presence along the shaft of the hair can also be obtained. This technique can differentially analyze the inner core or medulla of the hair from the bulk cortex material. The medulla is not always continuous along a strand of hair; it is unknown whether it deteriorates after the hair is formed or initially fails to be formed in the root on an intermittent basis. Results from infrared spectral mapping have shown a direct correlation between drug and medulla presence in the hair. The occurrences of drug in nonmedullated hair show a distribution that is considerably different. (Examples are described in the next section.) From these mapped images, multiple routes of drug incorporation into the hair can be postulated. One route would be through the root where the hair shaft is formed, with the drug binding to the medulla material. Another route would be from the exterior through the cuticle via perspiration, body oil, or a combination (including environmental exposure). Spectral data have also shown results that indicate different drugs bind differently with different distribution to the various hair materials.

An FTIR drug hair analysis requires that a reference spectrum of a drug-free hair from a healthy volunteer be obtained for spectral subtraction. These hairs must be prepared in microtome sections analogous to the patient hair preparation for analysis. The resultant infrared spectrum is a typical profile of a protein-containing sample. Hair from the drug user is likewise prepared and the infrared data collected. Its protein profile is similar to that of the drug-free hair. Spectral data are then spectrally subtracted, and the resultant difference spectrum is compared with reference spectra of drugs. The reference drug spectra are collected as dried solutions on a microscope slide. Figure 7–1 shows the spectra of the reference material of benzoylecgonine/6-monoacetyl morphine combination, compared with the difference spectrum of the patient and reference hair. The difference spectrum compares with the reference spectrum in the fingerprint region below 2000 cm^{-1}. The region above 2000 cm^{-1} is not usable because of the strong hydrogen bonding that occurs in the protein of hair samples. Frequencies below 1000 cm^{-1} are typically distorted because of the optical considerations

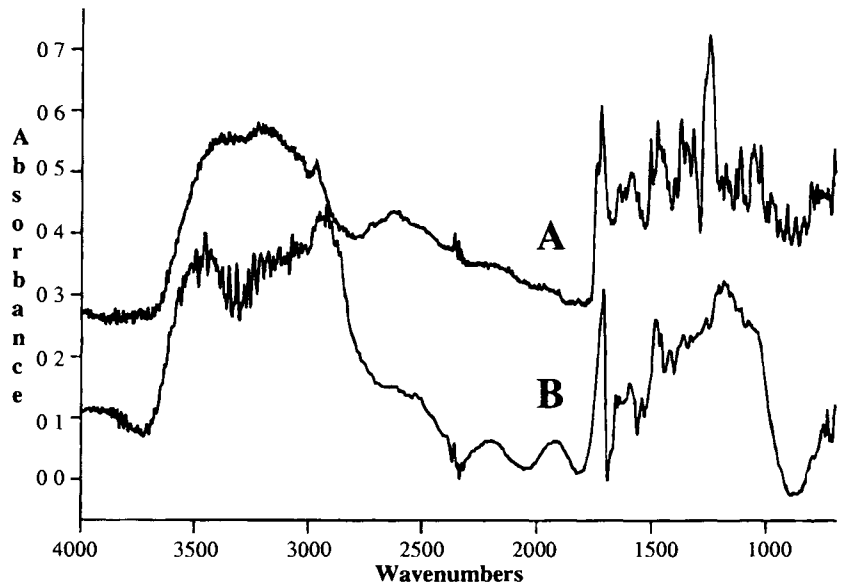

FIGURE 7–1. Infrared spectra of (A) reference spectrum of 6-monoacetyl morphine and benzoylecgonine combination, and (B) difference spectrum of the patient and drug-free hair.

of the experiments. Direct correlation or a high degree of spectral matching between the reference spectra of pure drugs and difference spectra of the hair samples is typically not obtained. This is caused by the binding of the drug to the hair matrix that has not been defined and results in an alteration of the spectral results because of varying molecular configurations. The drugs can usually be identified in hair with a primary frequency match in the 1750 to 1450 cm^{-1} region. Benzoylecgonine can be identified with a primary frequency band centered at 1715 cm^{-1}, and 6-monoacetyl morphine centered at 1735 cm^{-1}. An expanded view of this region shows the two bands in the spectral subtraction of the sample hair. Use of just the primary infrared absorbances for identification in this technique has been tested with consistent positive results by comparing infrared findings to that of quantitated mass spectral data of the sample. These primary frequencies are then used for mapping. A three-dimensional image or visualization of the location of the drug in the hair section can be obtained and will be demonstrated in "Drug Distribution."

For the sample shown in Figure 7–1, FTIR analysis was decisive in determining the drug content of the specimen. Previously, traditional analyses using mass spectrometry (MS) had determined the presence of cocaine and benzoylecgonine at 44.66 ng/mg and 12.20 ng/mg, respectively. The infrared data showed different band frequencies in the 1700 cm^{-1} region, and the resultant difference spectrum did not correlate well with a cocaine/benzoylecgonine reference spectrum. Combining 6-monoacetyl morphine and benzoylecgonine for the infrared reference data improved the correlation significantly in the 1700 cm^{-1} region. An additional subsequent analysis of the sample by MS methods, specifically searching for opiates, found a level of 7.09 ng/mg of 6-monoacetyl morphine and 11.60 ng/mg of morphine. This example shows the specificity of the infrared technique. It also demonstrates that the sampling area is so small that it may not correlate with a traditional analysis, such as mass spectrometry, that use a larger sampling.

III. DRUG DISTRIBUTION

Drug distribution can be visualized by display of the spectral map of various drug frequencies across a hair, analyzed by the FTIR microscopy technique as described herein. Figure 7–2 shows the spectral images of a patient hair mapped for protein vs. cocaine. The lighter portions are profiles representing the presence of the hair protein across the hair shaft, and the shaded areas are drug profiles based on the presence of the identifying drug frequencies. These profiles are created by plotting the absorbance of the primary infrared frequency band for the material (protein vs. drug) in a stepwise fashion as you collect data across the hair. The primary frequency of 1680 cm^{-1} for the amide I band of protein is used for the background profile, and the carbonyl band at 1745 cm^{-1} is used for the cocaine profile. Figure 7–2A is the infrared spectral image of a map across the laterally microtomed hair with medulla, and Figure 7–2B is a map across the same laterally microtomed hair in a section with no medulla. The cocaine is found to be concentrated in the central portion of the hair corresponding to the medulla location. This is a typical profile of the results we have obtained for hydrophobic drugs. The hydrophobic drugs tend to concentrate in sections of medullated hair and do not concentrate in nonmedullated hair. The identifying spectral match for this sample is shown in Figure 7–3. Figure 7–3A is the reference spectrum of cocaine cast on a slide analyzed by FTIR microscopy, and Figure 7–3B is the difference spectrum of the patient and reference hair. The spectra of cocaine in hair are the most difficult to match of the drugs that we have investigated. This is probably because of a high degree of binding that exists between cocaine and the hair matrix. This particular patient sample had only cocaine at this location in the hair shaft. The concentration of the cocaine was also quite high, compared with other FTIR hair analyses for cocaine. This particular location along the hair shaft may be correlated with an exact time of dosing when a large amount of cocaine entered the bloodstream. Figure 7–4 shows the spectral match of the cocaine/benzoylecgonine reference spectrum in trace A relative to the difference spectrum of a patient positive for cocaine and benzoylecgonine in trace B. The spectral correlation is greater when both parent and

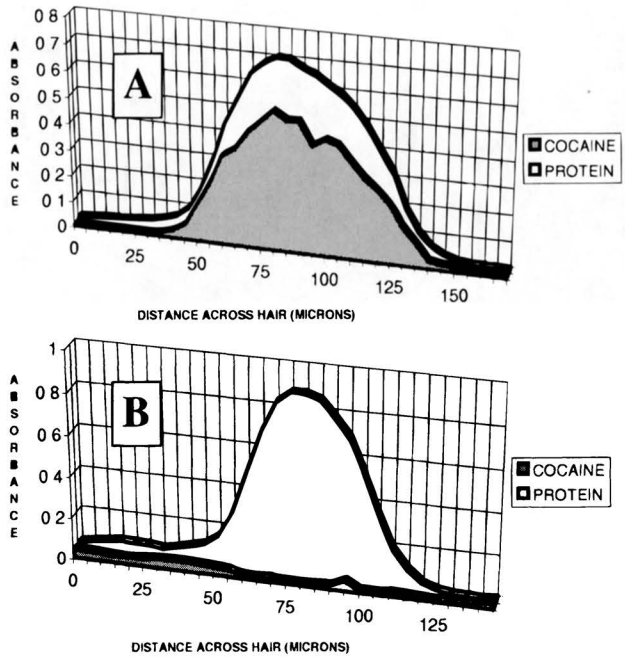

FIGURE 7–2. Patient hair mapped for protein vs. cocaine. Infrared spectral images of (A) single pass across laterally microtomed hair with medulla and (B) single pass across laterally microtomed hair with no medulla. White areas represent the frequency mapped for protein, and the shaded area represents the frequency mapped for drug.

metabolite are present in the reference spectrum used for the analysis. Figure 7–5 shows the spectral mapping image of a hydrophilic drug, amphetamine, as it exists in a medullated section of hair (Figure 7–5A) and a nonmedullated hair (Figure 7–5B). The drug is broadly distributed across the hair, regardless of medullation. This is the typical distribution that we have seen for hydrophilic drugs. The two primary frequencies of amphetamine at 1580 cm^{-1} and 1453 cm^{-1} were used for identification.

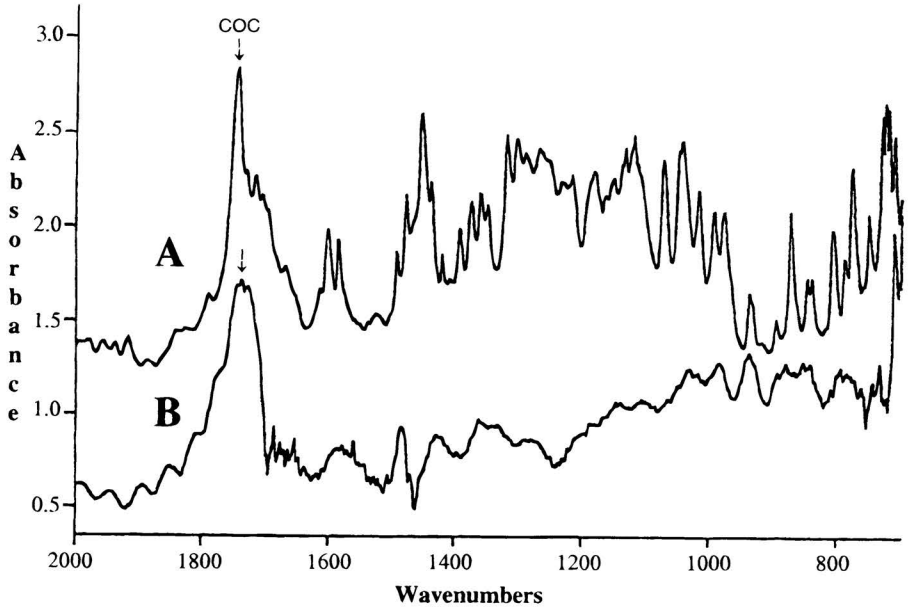

FIGURE 7–3. Infrared spectra of (A) reference spectrum of cocaine, and (B) difference spectrum of the patient and drug-free hair.

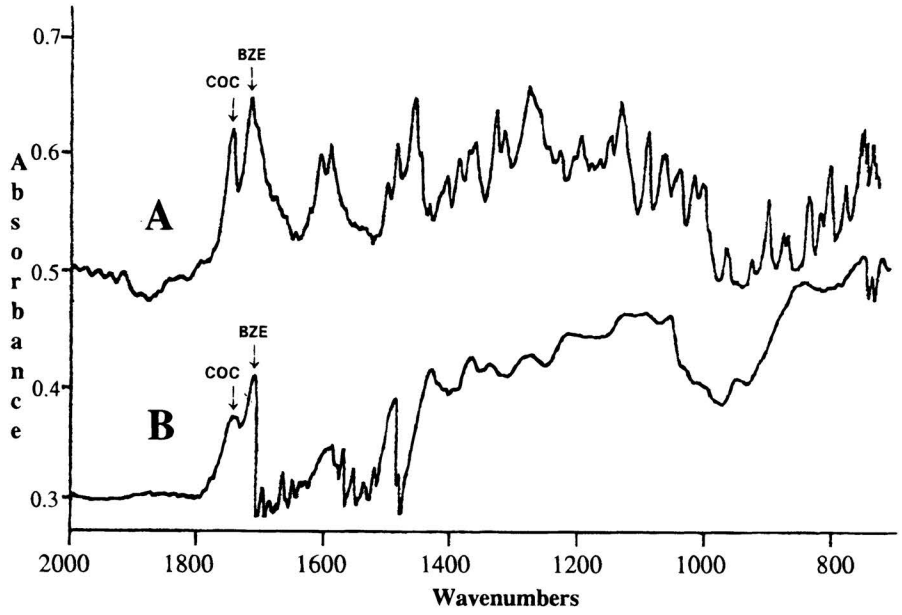

FIGURE 7–4. Infrared spectra of (A) reference spectrum of cocaine and benzoylecgonine combination, and (B) difference spectrum of the patient and drug-free hair.

Mapping lengthwise down the shaft of a laterally cut hair can yield a drug profile vs. time. A lengthwise profile of a patient's hair positive for cocaine/benzoylecgonine/6-monoacetyl morphine is shown in Figure 7–6. The bar graph shows the relative absorbance of the drugs in the hair. The line graph above the bars is for indication of whether a medulla was in view in the section being measured. Elevation of the line graph indicates full medulla in view. The low level line indicates no medulla in view, and a partial line elevation indicates medulla in partial view. The presence of the

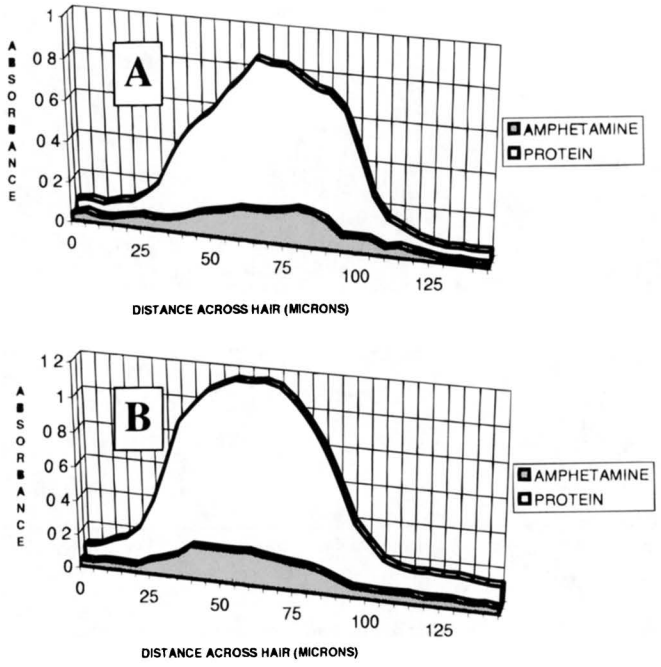

FIGURE 7–5. Patient hair mapped for protein vs. amphetamine. Infrared spectral images of (A) single pass across laterally microtomed hair with medulla and (B) single pass across laterally microtomed hair with no medulla. White areas represent the frequency mapped for protein, and the shaded area represents the frequency mapped for drug.

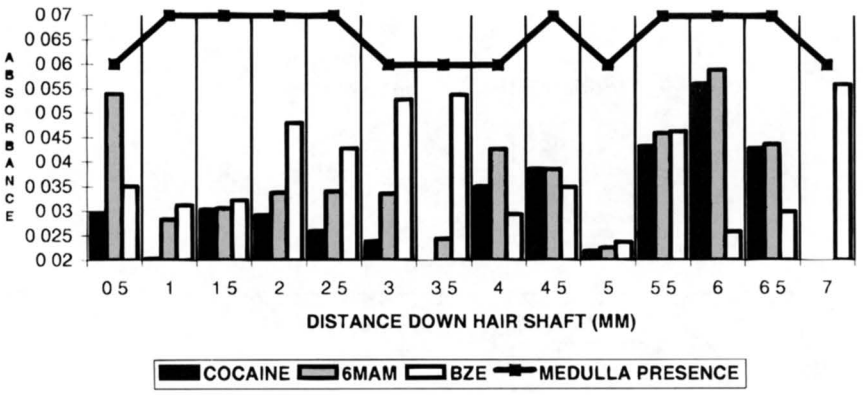

FIGURE 7–6. Bar chart corresponding to infrared absorbance as measured down the shaft of a laterally microtomed hair. Upper trace represents whether the medulla was visible (elevated) in the section being measured or not visible (depressed) in the section being measured. Black areas represent cocaine absorbance; gray areas represent the 6-monoacetyl morphine (6MAM) absorbance found; and white areas represent the benzoylecgonine (BZE) absorbance found.

drugs correlate with the visible view of the medulla. The presence of hydrophobic drugs, cocaine and 6-monoacetyl morphine (black and gray bars), tend to follow the visible observation of medulla presence in the analyzed section of hair. The more hydrophilic drug, benzoylecgonine (white bars), tends to be broadly distributed, regardless of medulla presence.

A postulate can be developed that hydrophobic drugs bind tightly to the medulla of the hair, and hydrophilic drugs are broadly distributed through the cortex of the hair. Our general findings also showed that hydrophilic drugs were in lower concentration than hydrophobic drugs in the hair in general and were harder to locate and identify. This signifies less preferential binding for hydrophilic drugs.

Doped or spiked hair shows the same profile, providing the hair was spiked in a manner that mimics the conditions of naturally occurring doping, such as a sweat model of drug incorporation.[12] Drugs seem to bind to the hair in accordance with their hydrophobicity, regardless of route of entry.

IV. SAMPLING HOMOGENEITY

Sampling homogeneity was tested using a MS method. This involved viewing the hair under a dissection microscope and cutting each hair at the medulla nonmedulla interchange. From these cuttings, two samplings were obtained for each specimen: one with all medullated sections and one with all nonmedullated sections. This separated hair was then analyzed for each patient. Totally medullated hair sections vs. totally nonmedullated sections were compared with an analysis of unseparated hair in a standard MS analysis.

The initial cutting revealed data that were very interesting relative to percentage of medullation in hair. Four patients with a mix of Caucasian and Hispanic ethnicity, all with dark hair, showed an average of 60% medullation in the hair. No Africans were used in this study, because those African hair samples to which we had access were near totally medullated, and there would not be enough of the nonmedullated material for analysis. The medullated portion would mimic the results of the unseparated hair analysis, because the bulk of the hair was medullated. No Asian hair samples were available for the study. Figure 7–7 represents the results in bar graph form. The gray areas are the percentage by weight of medullated segments in a sample, and the white bars indicate the percentage by weight of nonmedullated segments. Figure 7–7A shows the distribution of the percentage of medullation from the four patients used in the study as reported herein. Figure 7–7B shows the percentage of medullation at successive 1-in. segments away from the root of one patient. Variability

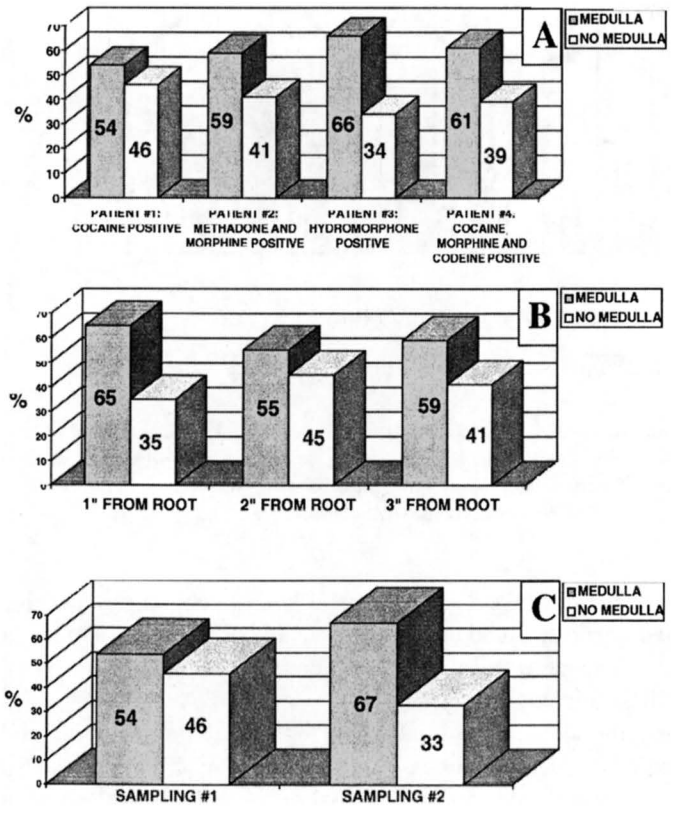

FIGURE 7–7. Bar chart of the percentage of medullated vs. nonmedullated hair in (A) four different patients positive for various drugs of abuse, (B) one patient sampled at 1-in. intervals from the root, and (C) one patient sampled two successive times from same area of hair. Gray area represents the percentage of medullated hair, and white area represents the percentage of nonmedullated hair found.

exists, but again the average is 60% medullation, regardless of the distance from the scalp. This indicates that the medulla does not dry up or deteriorate, but remains at a consistent percentage throughout the hair. Figure 7–7C shows the error range involved with two successive samplings of the same specimen. Percentage ranges from 54% to 67%, but again the average is 60% medullation. This indicates wide variability just in the sampling alone for traditional quantitative hair analyses, such as MS.

Figure 7–8 shows the results of the MS analysis of the separated and nonseparated sections of one of the patients in the sampling homogeneity study. This patient was previously identified as being positive for morphine, codeine, and cocaine by MS. Similar results were found in the MS studies to those of the infrared studies. The hydrophobic drugs concentrated in the medullated areas and the hydrophilic drugs were spread throughout the cortex. Figure 7–8A shows the hydrophilic drugs, morphine (gray) and codeine (white), in an even distribution through the medulla and nonmedullated sections. Those values are corrected for percentages by weight found herein and correlate to the analysis of the nonseparated material (including both medulla and nonmedullated portions in a standard sampling analysis). The variability that is seen can be attributed to sampling error. The hydrophobic drug, cocaine, shown in Figure 7–8B, is concentrated in the medullated sections only and equates to a total nonseparated standard sampling analysis, verifying the results found in the FTIR hair drug analysis.

V. CONCLUSIONS

Infrared microscopy is a unique tool for investigations of drugs of abuse in hair. The technique can analyze the central core of the hair shaft. Drug distribution across the hair or down the shaft can be determined. This technique is not quantitative and will not replace the typical method of extrac-

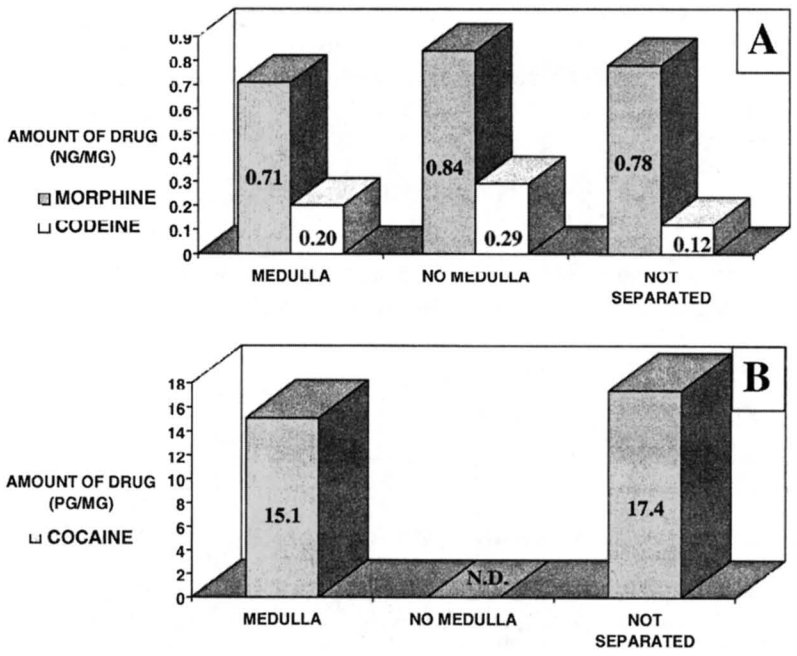

FIGURE 7–8. Bar chart of the amount of drug found in the medullated, nonmedullated, and not separated portions of one patient hair sample for (A) morphine and codeine, and (B) cocaine.

tion followed by analysis for determining concentrations of drugs in a hair. The FTIR hair drug analysis is limited because of the low concentrations of drugs in thin, microtomed hair sections used for the analysis. Chronic drug abuse is necessary for a hair sample to be useful in FTIR studies. The infrared microscopy technique analyzes such a small sample area that the likelihood of detecting casual use is low. It is difficult to obtain high-quality data with this technique because of the hair matrix interference. Reference hair used for spectral subtraction of the matrix must be from a similar hair type as the hair being analyzed.

Infrared microscopy has been used to help determine the kinetics of drug incorporation and distribution in hair. Monitoring infrared functional group frequencies for spectral imaging is a valid detection mode, resulting in a visual map of the drug distribution in the hair. However, finding isolated group frequencies for infrared analysis is difficult, whereas many of the molecular configurations are unknown because of the, as yet, undetermined binding of the drugs to the matrix. This limits its use as a routine analysis of drugs in hair. Its usefulness is in distribution studies.

The results from FTIR drug hair analysis indicated that the presence of the medulla seems to be associated with the presence of hydrophobic drugs, and hydrophilic drugs seem to be distributed throughout the cortex material. Complementary MS analysis on totally medullated or totally nonmedullated hair samples confirms the results found by the infrared studies.

The drug free reference hair that must be used as a subtraction factor for the difference spectrum also adds a variable into the already complex infrared profile. Drug free reference hairs from various individuals of differing race and sex are collected and kept on file and compared to the protein profile of the drug user hair for the subtraction. Matching the race and sex does not always yield the best difference spectrum with fewest artifacts. This indicates additional unknown variables affecting the protein profile of hair samples.Ideally, the best reference hair for any individual would be their own hair in a drug free condition. Generally speaking, this is not available unless a small enough spatially resolved area of the hair can be viewed for data collection that falls in between drug absorption locations. Exploration into state-of-the-art instrumentation that can yield such results is noted below.

Future studies are directed to FTIR microscopy investigations with a synchrotron high-energy source.[13] Initial efforts in this area suggest that the sensitivity can be increased and spatial definition of the drug distribution greatly enhanced. Further studies on the kinetics of drug incorporation and distribution in hair will continue.

ACKNOWLEDGMENT

The opinions or assertations contained herein are the private views of the author and are not to be construed as being official or as reflecting the views of the Department of Defense.

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Chapter 8

SOLID-PHASE EXTRACTION DISKS: SECOND-GENERATION TECHNOLOGY FOR DRUG EXTRACTIONS

Gary L. Lensmeyer

CONTENTS

I. INTRODUCTION

Solid-phase extraction (SPE) sorbents have undoubtedly simplified tedious sample preparation processes common to drug analyses. Most sorbents are commercially available as large particle silica-, polymeric-, resin-, or diatomaceous earth-based materials, loosely packed in columns or cartridges. Benefits of these materials over traditional liquid/liquid extraction systems have been proven several times.[1]

Recent advances in the technology of sorbent chemistry, particle sizing, and enmeshing materials/processes have integrated solid-phase sorbent particles into glass and synthetic fibers. Thin particle-loaded disks (also referred to as "discs" or "membranes") are products of these efforts.[2,3] The disk has demonstrated unique properties and has expanded the capability of customary SPE sorbents introduced in the 1970s. Recognition of the disk as a tool for sample preparation has been documented by many reported applications.

One of the first reported uses of the silica particle-loaded disk was for quantitative applications in environmental analyses.[2] Liter volumes of water were passed through 47-mm diameter disks secured in filtration manifolds. Organic pollutants were retained, concentrated, and eluted for subsequent analysis. Extraction methods for pesticides, herbicides, and other organic pollutants were developed with this new technology.[4–10] Moreover, isolation of alkylbenzenesulfonates and explosives were reported.[11,12] Ease of use, better selectivity, improved concentrating ability, and

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decreased solvent requirements were reasons stated for selecting the disk over traditional liquid/liquid extractions or large-particle, solid-phase sorbents in columns.

An early application of the new disk technology to therapeutic drugs was reported in 1991. Investigators down-sized commercial 47-mm diameter polytetrafluoroethylene (PTFE) octyl (C_8) particle-loaded disks to 11 mm, secured them into holders, and extracted the antiarrhythmic drug amiodarone and its metabolite from serum.[13] The drugs were quantitatively eluted with a small volume of mobile phase that was injected directly onto a high-performance liquid chromatographic (HPLC) column without further concentration or loss of analytical sensitivity. The authors claim the disk offered improved efficiency and concentrating ability because of the geometry of the device. Specifically, small particles of bonded silica enmeshed in fibers gave a high surface area per unit volume that allowed intimate contact of sample with the sorbent. Subsequently, other disk extraction methods for drugs of abuse and drugs monitored clinically were developed and reported.[14–27]

The focus of this chapter will be on descriptions, techniques, and benefits of the SPE disks in analytical toxicology. Investigators' experiences with the various disks in the development of drug assays will also be highlighted.

II. DESCRIPTION OF SOLID PHASE EXTRACTION (SPE) DISKS

A. PRODUCTION OF SORBENTS

Most conventional solid-phase sorbents used for drug extractions are chemically modified forms of silica. A rigid porous material, silica has a high surface area, rapidly equilibrates with solvents, and is relatively stable to changes in temperature and pH within a range of 2 to 7. Short periods of exposure to liquids with a pH outside this range does not seem to have deleterious effects on silica. Newer co-polymer supports—such as styrenedivinylbenzene—have shown potential, but product applications are somewhat limited at this time.

Structurally, unbonded silica used for SPE applications contains free and bound hydroxyls (silanols) and siloxane bonds. To bind a ligand(s) to this material, irregular-shaped (in rare case spherical forms) silica particles are dehydrated to produce a high population of surface hydroxyls that in turn are reacted with mono-, di-, or trifunctional alkoxysilane or chlorosilane reagents of the ligand to be bonded. Covalent ether bonds form with a limited number of the silanols to produce an *un*capped bonded silica. Efficiency of this step is not always consistant, attested by differences in carbon loading for commercial-bonded sorbents. Some manufacturers treat the remaining unreacted silanols with trimethylchlorosilane or similar reagent to generate an *end*-capped sorbent. Unfortunately, a population of unreacted silanols still remain after this two-step bonding process. The heterogeneous surface of the bonded silicas contributes to mixed-mode interactions often observed with analytes on these sorbents. The prevailing interactions are highly dependent on the liquid environment surrounding the sorbent. A diagram of the surface chemistry of silica and a bonding process, wherein a cyanopropyl ligand is covalently attached, are illustrated in Figure 8–1. Bonding processes used commercially are usually proprietary and differ among the various manufacturers. These dissimilarities contribute to the inconsistencies in selectivity for analytes for a given chemical type of sorbent obtain from different sources.

B. SORBENTS ENMESHED IN GLASS AND INERT FIBERS

Thin SPE disks are available commercially in a variety of sizes (typical range = 4- to 90-mm diameter) secured in filtration manifolds, cartridges, columns, or molded plastic luer-lock units. Most current applications in analytical toxicology use disks 4 to 12 mm in diameter, 1 mm or less in thickness, and fitted in polypropylene columns.[3] Maximum sample volume with these devices is usually <10 ml. The ideal sample volume is contingent on the chemistry and the mass of the sorbent contained in the disk, the energy required for interaction of analyte with sorbent ligand(s), and the potential for competition between sample matrix and the analyte for the sorbent ligand. In those cases wherein analyte retention is tentative and sample matrix is disruptive to the analyte–ligand

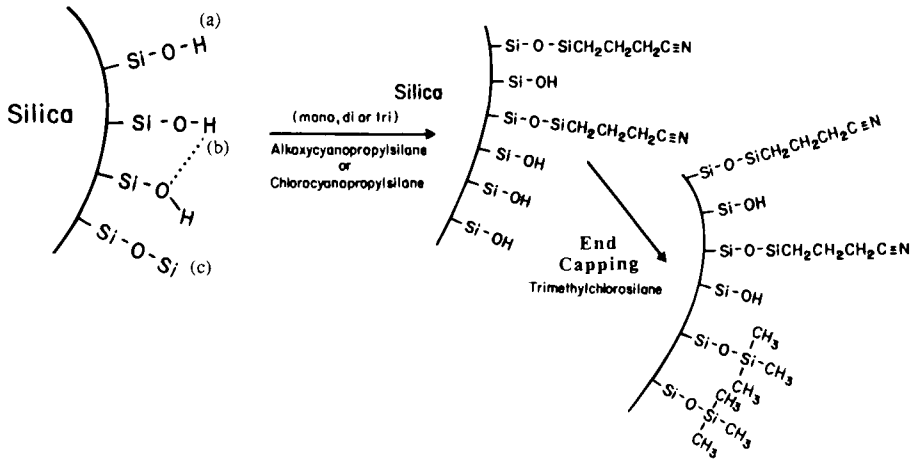

FIGURE 8–1. Surface chemistry of unbonded silica with free (a) and bound (b) hydroxyls and siloxane bonds (c). Representative scheme demonstrating the bonding process of cyanopropyl ligands to silica.

interaction, breakthrough of analyte will be observed at low sample volumes. Selection of a larger size disk can improve extraction recovery and allow use of increased sample volumes. To illustrate this concept, Ensing et al.[20] scaled up the size of the C8 disk to 25 mm to recover efficiently drugs of abuse from 25 ml of urine.

Two popular but distinctly different forms of the SPE disk used for drug extractions are the particle-loaded PTFE and glass fiber disks, available under such trade names as Empore™* and SPEC™**, respectively. Sorbent-loaded polyvinyl chloride and derivatized cellulose membranes are yet other forms of the disk whose utility seem to be limited to biopolymer separations. The vast majority of disk extractions for drugs have been developed with the PTFE and glass fiber products. Accordingly, this discussion will focus on these devices. The SPE concentrator (SPEC) microcolumn product is a rigid glass fiber disk (1.0 mm thick holding 30-μm diameter particle-size silica and bonded silica.[3,28] The Empore extraction disk cartridge has a flexible membrane (0.5 mm thick) with 8-μm diameter average size silica- or polymeric-based sorbents densely entrapped within inert PTFE fibrils: 90% particles and 10% PTFE by weight.[2,29] Both commercial disk products are available in different diameter sizes and a variety of bonded-phase chemistries. Both contain prefilters to minimize plugging of disk with particle-laddened samples.

The SPEC unit has a glass fiber prefilter, and the Empore prefilter contains multilayers of polypropylene sheets of graded pore size. A typical SPE disk secured in cartridge format and a traditional SPE column containing loosely packed, large-particle sorbents are illustrated in Figure 8–2. Photomicrographs of large-diameter (40 μm), irregularly shaped silica particles that are packed in columns and of small particles (8 μm) secured within fibers of a disk are demonstrated in Figure 8–3.

III. GUIDELINES FOR USE OF SPE DISKS

Most commercial SPE disks are used in essentially the same manner; exceptions usually are predicated on differences in construction (composition) of the device. Literature supplied by manufacturers of these products offers a starting point for the novice. Moreover, the disk extraction process is somewhat analogous to that used with traditional large-particle sorbents loosely packed

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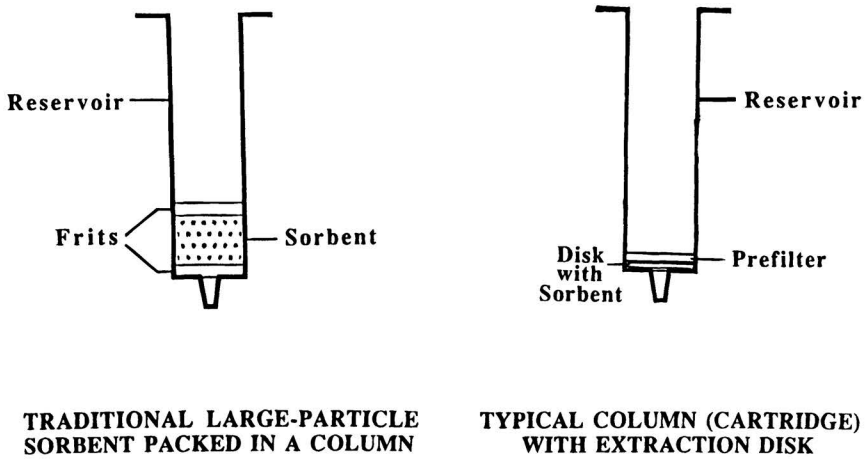

FIGURE 8–2. A traditional SPE column packed with large-particle sorbent and a typical disk product in cartridge format.

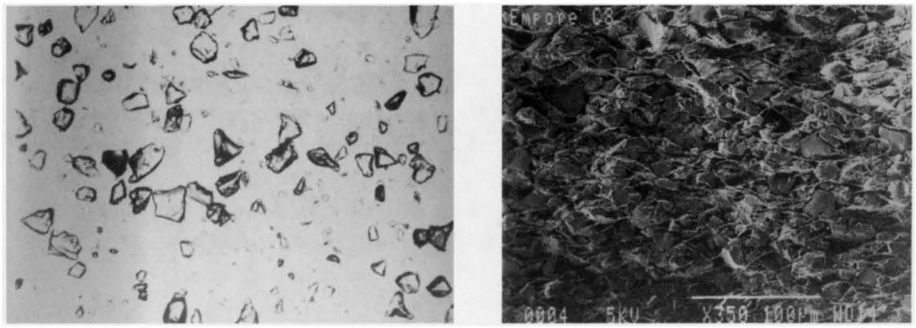

FIGURE 8–3. Photomicrographs of 40-μm (average size) diameter-bonded silica that is typically used loosely packed in columns (left); 8-μm (average size) diameter-bonded silica particles enmeshed in PTFE fibrils of a disk (right). (Original magnification × 350.)

in columns. Theory and technical application of SPE sorbents have been well described previously by Harkey,[27] Van Horne,[30] and Majors.[31] Mayer et al.[36] reviewed membrane-based sample preparation for chromatography as it pertains to several disciplines. Similar reports have surfaced for the SPE disk.[32,33–36] Nonetheless, to take advantage of the new disk technology requires some elaboration of technique to appreciate fully its unique properties. A representative reversed-phase disk extraction protocol, listing the steps for isolation of therapeutically administered and endogenous steroids in serum, is presented in Table 8–1. This scheme is an example that can be followed as each step of a SPE disk extraction is discussed.[16]

A. CHOICE OF SORBENT PHASE

For an efficient extraction, the ideal sorbent type is chosen with several factors in mind: (1) the acidic/basic/neutral (i.e., pK_a) properties of the analyte to be isolated; (2) the environment or matrix of the sample specimen; and (3) hydrophobic/hydrophilic/ionic characteristics of the sorbent ligand and underlying support, usually a silica or polymeric material. Common ligands and solid supports now available in the disk format are listed in Table 8–2.

Nonpolar ligands (i.e., C_{18}, C_8, and C_2) bonded to irregularly shaped silicas comprise the most frequently used solid-phase sorbents; spherical copolymers of styrenedivinylbenzene (i.e., SDB-XC and SDB-RPS) are newer sorbents in this class. Chemical mechanisms for analyte retention on these sorbents involve nonpolar interactions, Van der Waals forces, and secondary interactions. In con-

TABLE 8–1
Protocol for Disk Extraction of Steroids in Serum[a]

Pretreatment sample	
(releases protein-bound steroids)	0.3 ml serum + 0.15 ml of 0.4 *N* HCl containing internal standards (incubate 10 min at room temperature)
Condition disk[b]	0.5 ml methanol followed by 0.5 ml water
Apply sample	Pretreated sample mixture
Wash disk	1. 0.2 ml water 2. 0.5 ml methanol/water (18:82)
Elute	
(two-step process)	0.05 ml acetonitrile followed by 0.15 ml water (combine eluates)
Analysis	Inject 50 µl of eluate onto HPLC column

[a] Extraction of steroids (prednisone, its metabolite prednisolone, and the endogenous steroids cortisol, cortisone, and corticosterone) from serum.[16]

[b] C_8 bonded silica in PTFE fibrils (4 mm diameter, 500 µm thick).

trast, adsorptive interactions (hydrogen bonding and dipole–dipole) of molecules with polar ligands (i.e., silanol, cyanopropyl, and aminopropyl) account for retention of analyte on normal (polar) phase sorbents. Analytes with hydroxyl and amine functional groups can effectively be adsorbed, even though applications using normal phase sorbents in the disk format are somewhat limited at this time and remain to be exploited. Ion-exchange sorbents can be anionic or cationic, weak or strong in their binding ability, and usually require strong displacing reagents for elution caused by higher binding energies than seen with reversed-phase interactions. A variety of mixed-phase sorbents containing both C_{18} (or C_8) and sulfonate moities interspersed within a sorbent particle com-

TABLE 8–2
Common Ligands/Sorbents Available in the SPE Disk[a]

Nonpolar

C_{18}—octyldecyl
C_8—octyl
PH—phenyl
C_2—dimethyl
SDB-XC—poly(styrenedivinylbenzene)[b]
SDB-RPS—reversed-phase sulfonated styrenedivinylbenzene[b]

Polar

CN—cyanopropyl
NH_2—aminopropyl
PSA—primary/secondary amine
Si—silica

Cation exchange

SCX—benzenesulfonic acid
SDB—silver or hydrogen forms[b]

Anion exchange

NH_2—aminopropyl
PSA—primary/secondary amine
SAX or QUAT—quarternary amine

Mixed-Mode Phases

MPC—C_8/benzenesulfonic acid
MP1—nonpolar/strong cation
MP3—slightly polar/strong cation

[a] Specialty ligands and affinity sorbents unique to the biotechnology field are not included.

[b] Co-polymer–based sorbent.

bine ionic, nonpolar, and polar interactions to achieve unique selectivities and a capacity for a given analyte or group of analytes. Herein, the predominating mechanism(s) of interaction are highly influenced by the environment created by the solutions applied to these sorbents.

Capacities of different bonded silicas for an analyte vary widely. Disks of very low bed mass can have reduced capacity for weakly bound analytes. By choosing a more suitable bonded phase, capacity (retention) for these analytes can be improved. The appropriate size of the SPE disk to use for an extraction is predicated on its capacity to retain an analyte in its native sample matrix. When breakthrough of analyte is observed, recovery can be improved by switching to a larger diameter disk, assuming choice of sorbent type and extraction conditions have been well established.[32] Similarly, when the analyte is highly retained and elution volume is large, downsizing to a smaller diameter disk can improve efficiency.

When selecting the best sorbent type, one tries to achieve high selectivity and capacity for the analyte to minimize interferences and yet maintain quantitative recovery. Sometimes, the trial-and-error approach is necessary in the search for a functional sorbent. Analytes often interact with both sorbent ligand and underlying solid support via mixed-mode mechanisms that, unfortunately, are not always predictable. Most important, silica-based sorbents are capable of both polar and ionic interactions.[30]

B. CONDITIONING SORBENT

Wetting a reversed-phase sorbent solvates the covalently bonded ligand and exposes it for interaction with analyte. This conditioning process promotes high recovery of the analyte and allows liquid to flow easily through the disk. To prime the disk, a conditioning solvent (such as methanol or acetonitrile) is passed through, followed by water, a buffer, or other reagent to remove excess conditioning solvent. Volumes of these solutions can be critical. Residual conditioning solvent must be retained within the sorbent after the water/buffer wash passes. Deactivation of the sorbent will occur, and analyte retention will be impaired in cases wherein excessive volumes of an aqueous wash solution are applied. Likewise, care must be taken to prevent drying of the disk before sample application; otherwise, flow of liquids will be restricted.

Normal-phase sorbents (i.e., unbonded silica) are conditioned with nonpolar solvents, usually hexane. Highly polar solvents must be avoided, especially water, which can bind irreversibly and alter the native chemistry/selectivity of the hydrophilic sorbent. Extractions may not be reproducible when polar solvents contact the sorbent.

C. APPLICATION OF SAMPLE

Biological samples will sometimes require pretreatment before extraction with a SPE disk. Pretreatment can facilitate a more robust retention of the analyte on the sorbent and also improve flow of sample through the disk. Commonly used preparation steps include dilution and addition of internal standard[13,14]; hydrolysis of conjugates to free covalently bound analytes[23]; addition of reagents to release analytes highly bound to proteins that compete with sorbent ligand for analyte[16]; protein precipitation; and filtration/centrifugation of particle-laden samples to minimize plugging of disks.[17] In most cases, dilution of a specimen with an appropriate buffer is all that may be required. This step is often essential with viscous samples (serum, plasma, bile, etc.) to improve flow through the disk.

When normal-phase sorbents are used in an extraction, the sample must be contained and applied in a nonpolar matrix for efficient retention of the analyte. Usually, specimens submitted for drug analysis are polar in nature and require a solvent extraction or other pretreatment to facilitate transfer of the analyte to a nonpolar solvent.

A general review of guidelines established for use of the various sorbent types can assist method development with the disk. For extractions using reversed-phase sorbents, the sample environment (pH, ionic strength) is adjusted to promote an uncharged (neutral) form of the analyte. Increasing the ionic strength of the sample matrix can sometimes "salt-out" the more polar analytes and improve

recovery. For ion-exchange sorbents, the sample matrix is adjusted such that one charged species predominates so the analyte is able to interact efficiently with charged ligand of the sorbent. Herein, the pH is adjusted to 2 units above pK_a of anions, 2 units below pK_a of cations, and the ionic strength should be low. With normal-phase (mode) sorbents, the analyte requires a nonpolar environment to facilitate interaction with the bonded polar ligands or native silanols present on the silica support.

The optimal flow rate of liquids through the disks will differ, depending on particle and pore size of sorbent and its packed density in the disk. Traditional large-particle sorbents loosely packed or held in columns or support materials have relatively low resistance to flow. Analyte recovery (retention) with these devices is highly influenced by flow rate because of channeling and the random nature of analyte contact with sorbent particles. Control of flow rate is less of a concern with SPE disks designed to minimize channeling. Devices with small sorbent particles oriented closely to one another to form a dense disk allow intimate contact of sample with sorbent and greater freedom from anomalies associated with variations in flow rate. Although the resistance to flow increases as particle size decreases,[15] capacity per unit weight of sorbent is greater and mass transfer is highly efficient with the disk format.

Forces required to pass liquids through SPE disks vary, depending on geometry of the device. Oftentimes, forces used with the disk are greater than those used with packed columns. Positive pressure applied with a syringe or by centrifugation—typically 85 to 120 g or greater—have been used.[14,16] With centrifugation, the disk cartridge is suspended in a test tube and placed in the carrier of a centrifuge (Figure 8–4).

Alternatively, a vacuum force can pull liquids through the disk; however, the optimal vacuum will vary, depending on disk geometry but typically will be in the range of 2 to 10 in. Hg (glass fiber disk) or greater (PTFE fiber disk).[2,3,21] Robotic systems are now available to automate extraction process and use either head pressure or vacuum to move liquids through the disks. These systems, in combination with the sorbent disk format, seem to have potential for improved applications; however, the technology is yet in its infancy. The interrelationship of flow rate, disk geometry, and analyte recovery are important factors to be considered when choosing the best method for passing liquid through these devices. Manufacturers of the disks do recognize that, occasionally, samples will contain insoluble proteins, lipids, and natural precipitates that can plug the pores of the disk and adversely affect flow.[21,29] Centrifugation, filtration, or precipitation to clear the sample have been used to circumvent this annoyance and reap the benefits of disk technology.

D. WASHING DISK OF INTERFERENCES

A wash solution is applied to the disk to remove residual specimens after the sample passes and to desorb compounds that could potentially interfere in the subsequent test analysis. For reversed-phase sorbent applications, water, buffers, organic solvents, or mixtures of the these liquids have been used for this step; sometimes, a series of washes may be required to achieve the desired selec-

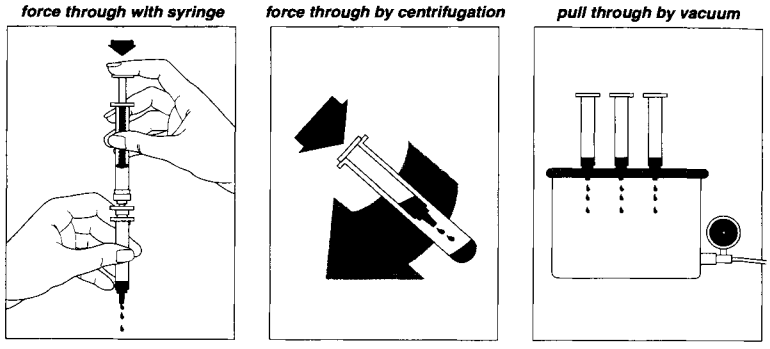

FIGURE 8–4. Techniques used to pass liquid through SPE disks.

tivity.[16] With normal-phase sorbents, the wash solutions are usually nonpolar solvents supplemented with polar modifiers at concentrations necessary to achieve the desired selectivity.

E. ELUTION OF ANALYTE

The unique geometry of SPE disks contributes to an efficient desorption of retained analytes in a small volume of eluant. Usually, the eluting solution can be added to the disk immediately after the wash solution(s) passes. Sometimes, however, the disk may need to be dried before elution to achieve quantitative recovery of analytes within a relatively small volume of solvent. Compatibility of eluting solution with the previously applied wash solution will guide this decision. To obtain reproducible results when the eluting solution is immiscible with the wash solution, dry the disk by forcing air or another gas through.

A variety of techniques have been reported for desorbing analytes from disks containing reversed-phase sorbents. Frequently, HPLC mobile phases or other solutions compatible with the analytical technique will efficiently desorb analytes to get highly concentrated eluates ready for direct injection onto an HPLC column or for application to other analytical systems.[13,14] A two-step elution process, wherein a strong solvent (such as acetonitrile) is applied first, immediately followed by water (or buffer), is yet another way to achieve a concentrated eluate in a small volume ready for direct analysis.[16,17] The steroid extraction procedure listed in Table 1 is one example of a two-step elution that effectively maintains quantitative recovery in a small volume of eluate that is compatible with the respective HPLC system. Solvents (methanol, acetonitrile, buffers, etc.) that are effective eluants, but not compatible with analytical systems, may require evaporation/concentration/reconstitution techniques to achieve necessary compatibility or adequate analytical sensitivity.[20] Disks containing ion-exchange sorbents have special requirements for elution. Herein, the eluting solution must be of a higher ionic strength, compared with the solution in which the analytes were applied and the pH needs to be adjusted to a value above the pK_a of cations or below the pK_a of anions. With normal-phase sorbents, the eluant must be more polar than the solution in which the analyte was applied. Nonpolar solvents (e.g., hexane supplemented with polar modifiers [such as alcohols, ethyl acetate, etc.]) at concentrations to effect displacement of analyte from sorbent are frequently used for elution. Again, the compatibility of the eluate with the analytical system will determine if direct testing of the eluate is possible or whether evaporation/concentration/reconstitution is required.

IV. OPTIMIZATION CHARTS

The method development process can be seen as a marriage (or compatibility) of sample preparation and analytical process (i.e., chromatography or other detection system). An understanding of the prevailing mechanisms of separation and the influence of test parameters on separation processes can be useful information in the design of optimal conditions for the disk extraction of an analyte. Each step of the extraction process requires close scrutiny to avoid haphazard choice of conditions. Methods based on sound chemical principles and good laboratory practice are more likely to be successful and "robust." Selection of appropriate and practically useful statistical procedures to evaluate a new method and to control it long-term contribute to method stability.[37]

Construction of optimization charts is one way to evaluate and document systematically each step of an extraction.[14,16–18] Through this process, the interrelationship of parameters and the rationale used to choose optimal conditions can be unequivocally established and easily communicated to others. The charts can also play a significant role in troubleshooting when problems do occur. Examples of optimization charts constructed for the disk extraction/HPLC analysis of the antiarrhythmic drugs mexiletine, flecainide, and verapamil from serum are illustrated in Figure 8–5.[18] Individual parameters of the extraction were studied; conditions for a respective parameter were varied sequentially and the affect of change on recovery calculated. Final selection of optimal con-

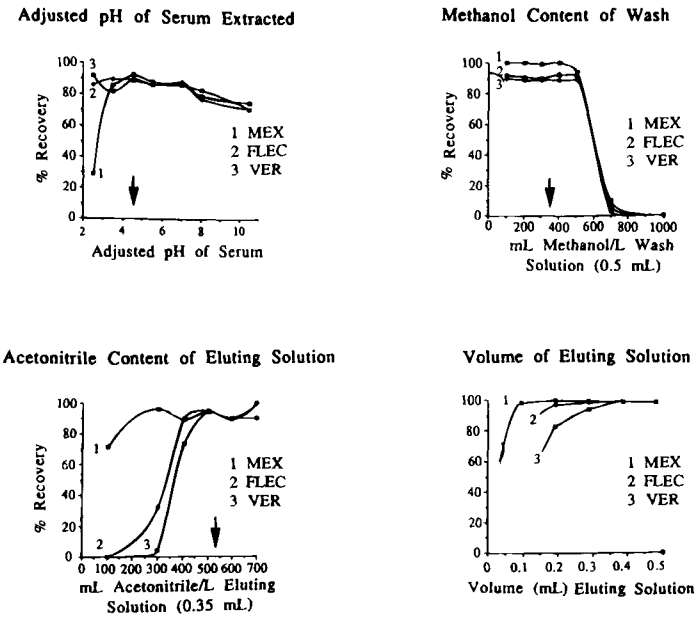

FIGURE 8–5. Optimization charts constructed during the development of disk extraction/HPLC analysis for mexiletine (MEX), flecainide (FLEC), and verapamil (VER) in serum. Optimal parameter for each chart is indicated by an arrow. (From Lensmeyer, G., et al., *Ther. Drug Monit.*, 14, 408, 1992. With permission.)

ditions (designated by the arrow in Figure 8–5) for adjusted pH of serum extraction, methanol content in the wash solution, acetonitrile content in the eluting solution, and the smallest volume of eluting solution necessary for complete recovery of analytes from the disk were established.

V. COMPARISON OF EXTRACTION DISKS AND TRADITIONAL PACKED SORBENTS

Formal studies in which the performance of the disks and traditional packed columns were compared side-by-side have been reported. Blevins and Schultheis[21] extracted a metabolite of tetrahydrocannabinol from urine using the glass fiber disk and conventional packed column. They report that, with the disk, fewer processing steps were required, and solvent volume was decreased by 88%.[23] Low-bed mass and low-void volume were listed as advantages of the disk. Wells et al.[15] extracted antiarrhythmic drugs from serum using 5 mm diameter PTFE C_8 disks and 100 mg C_8 packed columns. Both units contained sorbent particles from the same manufacturer. Linearity and recovery were equivalent with both devices. However, the disk extraction improved precision by a factor of 2, required 3.5 times less solvent volume for elution, reduced total solvent consumption by almost threefold, and was four times more efficient than the packed column because of increased capacity per sorbent mass of the disk. Chromatograms (Figure 8–6) of the antiarrhythmic drugs eluted in the smallest volume possible from the two devices demonstrate the improved analytical sensitivity with the disk over the packed column. The lower limit of detection was decreased by fivefold when the disk was used.[15]

Elution profile graphs (analyte recovery vs. fractional volume of eluting solvent) have been used to demonstrate quantitative recovery of analytes retained on various SPE devices.[14–18] In one report, investigators confirm the diverse patterns of elution for antidepressant drugs extracted with the disk and conventional packed column (Figure 8–7).[14] For the disk, quantitative recovery of the drugs was achieved with 0.7 to 1.0 ml eluting solution (HPLC mobile phase); 3.3 to 3.5 ml was required for the packed column. Moreover, the drugs eluted from the disk in essentially a single band, whereas with the column, multiple overlapping bands (chromatography) of the drugs were observed. When eluates from both devices were injected onto the HPLC column, a 3.5-fold greater analytical sensitiv-

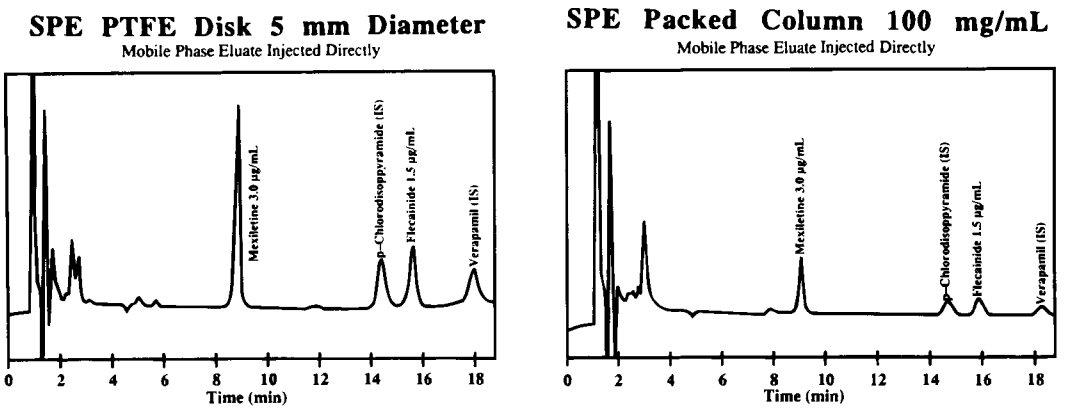

FIGURE 8–6. Comparison of chromatograms for antiarrhythmic drugs eluted from PTFE C_8 disk (5 mm diameter) cartridge and a C_8 (100 mg) packed column. The smallest volume of elution solvent (HPLC mobile phase) required for quantitative recovery was used, and the eluate was injected directly onto the HPLC column. IS, internal standard. (From Wells, D., et al., *J. Chromatogr.*, 33, 386, 1995. With permission.)

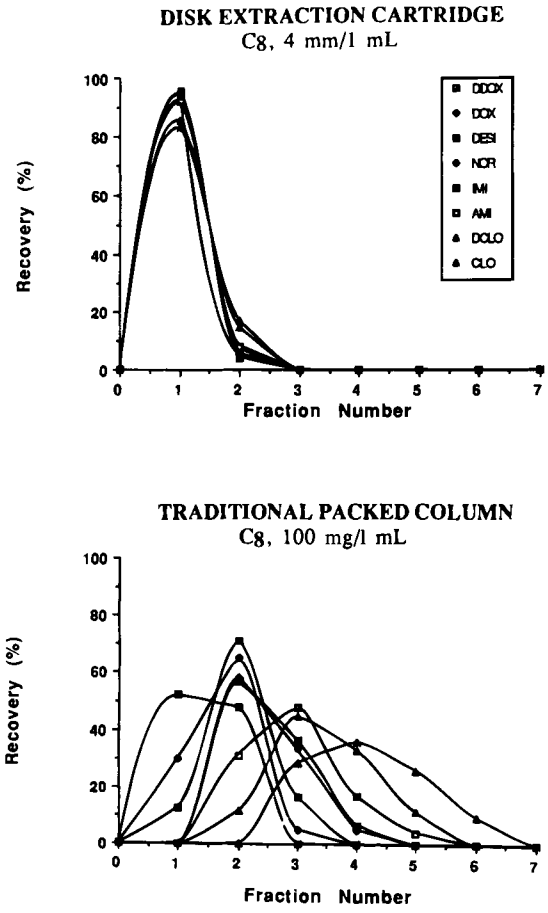

FIGURE 8–7. Comparison of elution volumes required to recover completely tricyclic antidepressant drugs retained on C_8 PTFE disk and C_8 packed column. Successive 0.5-ml aliquots of mobile phase eluting solution were applied, collected from each sorbent, and chromatographed. DDOX, desmethyldoxepin; DOX, doxepin; DESI, desipramin; NOR, nortriptyline; IMI, imipramine; AMI, amitriptyline; DCLO, desmethylclomipramine; CLO, clomipramine. (From Lensmeyer, G., et al., *Ther. Drug Monit.*, 29, 444, 1991. With permission.)

ity was achieved with the disk extraction. Most importantly, the disk was able to produce a more concentrated eluate than has been possible with traditional SPE materials.

VI. CONCLUSIONS

Second-generation SPE devices in the form of highly efficient disks have improved upon the limited performance of conventional packed-bed SPE columns and liquid/liquid techniques. The disk has demonstrated less tendency to channel, smaller void volume, decreased binding of interferences, and improved method performance characteristics. Emerging applications have launched the disk technology into the field of analytical toxicology with anticipation of new ways to use the disk. Perhaps the full potential of the disk will be realized through application of its unique properties in automated processes of the future.[38]

ACKNOWLEDGMENTS

I am grateful to my colleagues, Dr. Donald A. Wiebe, Richard Miller, and Thomas Doran, for their suggestions and comments. I also thank Dr. David Wells of the 3M Company and Dr. Dennis Blevins of ANSYS, Inc., for sharing information about extraction disk technology.

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Chapter 9

AUTOMATION, DIRECT-SAMPLE ANALYSIS, AND MICROCOLUMN LIQUID CHROMATOGRAPHY

Steven H. Y. Wong

CONTENTS

I. INTRODUCTION

Advances in liquid chromatographic column technology, instrumentation, automation, and supportive software have greatly enhanced the applications of liquid chromatography (LC) for the clinical, pharmaceutical, and research laboratories.[1–3] The current concern about health care delivery with increasingly limited resources affects both the available capital for instrumentation purchases and maintenance, as well as the support for personnel. In short, the clinical laboratory, as in other parts of the health care delivery system, is undergoing a vigorous process of cost-cutting through streamlining and downsizing. Automation may (as emphasized by Sasaki,[5] Felder,[6] Markin,[7] and Hoover[8]) be one of the most important measures.

This chapter examines three related advances in LC that have been or may be readily applied to offer cost-effective and sensitive analysis for drugs and poisons, complementary to immunoassays. Recent evidence indicates extensive use of automation in LC for biomedical analysis, such as the 2.3 million screenings of hemoglobinopathies as shown by Lorey et al.[9] and the monitoring of polymerase chain reaction (PCR) products by Zellinger et al.[10] This latter application may be further extended into toxicology for DNA fingerprinting, therapeutic drug monitoring (TDM) for genotyping, and possibly in environmental toxicology. Direct-sample analysis (DSA)/automation for drug assay was readily achieved by commercially available analyzers such as REMEDi for dedicated drug analysis,[11–14] and PrepStation™/HPLC,[15,16] and ASPEC[17–19] and ASTED for general drug analysis.

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Another major advance is the application of microcolumns for drug analysis. Wong[1] previously reviewed the principles of microcolumn chromatography according to Scott.[20] Since that review, increasing applications of microcolumns and availability of instrumentation have reaffirmed the potential efficacy of this methodology for toxicology and TDM. As seen in all areas of chromatography and further demonstrated by the concept of unified chromatography recently shown by Schurig et al., a microcolumn was used for the chiral analysis of hexobarbital in gas chromatography (GC), high-performance liquid chromatography (HPLC), supercritical fluid chromatography (SFC), and capillary electrochromatography (CEC), with enhanced sensitivity. One of the more practical advantages is the reduced cost associated with reduction in solvent consumption and disposal, but consensus is lacking. However, microcolumns interfaced with mass spectrometry (MS) are being increasingly applied for clinical drug analysis, Heida et al.[22,23] The feasibility of applying these three related advances to clinical practice would depend on the clinical demand, and the need, the interest, and the resources of the toxicology laboratory. For that purpose, this chapter would address (1) the current status of automation in LC and the various approaches of DSA, followed by published examples; and (2) the principle of microcolumn chromatography and selected clinical examples for toxicology and TDM.

II. AUTOMATION AND DSA/LC

Automation in the clinical laboratory, as proposed by Sasaki[5] and others[6–8] is increasingly vital to the survival of a cost-effective laboratory. For the clinical laboratory performing toxicology and TDM drug assays, immunoassays with high throughput analyzers would always account for the majority of the procedure.[24] However, chromatography, including HPLC and GC, is used for assays not readily performed by immunoassay either because of the lack of commercially available kits for newly introduced drugs or for not being cost-effective. Furthermore, GC/MS provides definitive confirmation and sometimes screening analysis for steroids, as indicated by Bowers.[25] Inherent to the time needed for partitioning and transfer processes of analytes in chromatographic systems and sample preparation, the total analysis time ranged from 20 to 60 min—longer than those of the immunoassays. As compared with immunoassay analyzer, chromatographic procedures demand more personnel involvement and expertise, and this may be readily overcome with modern DSA and automated chromatographic systems, such as REMEDi,[11–14] PrepStation™,[15,16] and others.[26] Furthermore, these chromatographic approaches may be used for a larger range of drugs/metabolites. Wong proposed DSA/LC with sample preparation minimized, eliminated, or automated.[2] Thus, the various DSAs may be incorporated into the toxicology laboratory and are complementary to immunoassays within the overall clinical laboratory.

A. AUTOMATION IN CLINICAL AND TOXICOLOGY LABORATORIES

As proposed by Sasaki,[3] automation has already been initiated in selected areas, including specimen processing, clinical chemistry, high-volume TDM and clinical toxicology, urine analysis, hematology, and coagulation. As shown in Figure 9–1, the automation process starts with the entry of the sample into the clinical laboratory. For easy identification, bar codes may be used for labeling sample tubes and later scanned for test order and patient information. Then, a robot or robotic station performs the appropriate aliquoting for subsequent testing. These aliquots, with appropriate labeling, are then transported in the conveyor to the appropriate test station. Upon arrival at the test station, a robotic arm then transfers the sample tube to the sample compartment for testing by the analyzers. The test results are then automatically transferred to the laboratory information system and may be further downloaded into the hospital information system. For toxicology and TDM assays such as theophylline and ethanol when performed by high throughput analyzers, automation offers efficiency and cost-effectiveness. Many of the currently available analyzers may be readily arranged into an integrated configuration for total automation. Currently, chromatographs have not

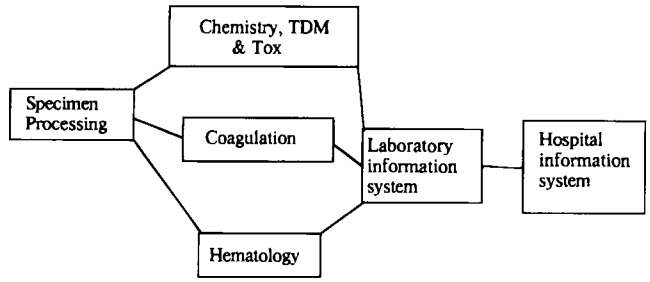

FIGURE 9–1. Laboratory automation.

been configured into the overall automation scheme, but may be in the future. The following automated chromatographic systems and DSA approaches potentially fulfill this requirement.

B. DSA AND AUTOMATED LIQUID CHROMATOGRAPHIC SYSTEMS

The term automation has been applied, rather indiscriminately, to describe the applications using automated, modular HPLC preceded by manual sample preparation to the currently commercially available and totally integrated systems. These latter systems offer total automation involving preparation, HPLC analysis, and subsequent data reduction. To aid in better appreciation of the degree of automation, and to offer a rational basis for comparison, a classification of automation level is proposed in Table 9–1. In considering DSA and automated LC for clinical drug analysis, three proposed levels of automation, as shown by Table 9–1, may be based on the integration of automated sample preparation, liquid chromatographic analysis, data reduction, and potential for interfacing with the laboratory computer for patient data transfer later on. Level I automation includes sample preparation by a large variety of automated diluter/dispenser stations. Typically, the station pipettes sample urine or serum into designated tubes/vials for subsequent steps. These steps include addition of internal standard(s) before further liquid/liquid or solid-phase extractions. Liquid transfer is guided by a liquid sensor. Vortexing, phase separation, axial centrifugation, filtration, evaporation, reconstitution, and weighing steps may be programmed as part of the procedure. Some workstations, such as Zymate, also perform centrifugation. Final extraction steps may include evaporation and reconstitution. In short, the station is capable of performing repetitive procedures usually executed by the laboratory technologist. Then, the samples are transferred for subsequent chromatographic analysis. Currently, most HPLC may offer Level I automation, capable of performing automated analysis from sequential injection to chromatographic data reduction for identification and estimation of drug peak(s). Thus, by combining Level I sample preparation and

TABLE 9–1
Proposed Automation Levels in Liquid Chromatographic Drug Analysis

Level	Capabilities
I	Column switching Automated sample preparation with diluter/dispenser, liquid transfer, weighing, vortex, and others controlled by computer Automated HPLC for automated injection and/or column switching, analysis, and data reduction
II	On-line sample extraction similar to above Level I with added robotics and/or column switching, limited data reduction, and integration
III	Chemometrically integrated for drug identification/quantitation, with ease and reliability similar to autoimmunoanalyzers A. Column switching B. Robotic liquid transfer/solid-phase extraction C. Robotic liquid transfer/solid-phase extraction and microdialysis

HPLC analysis, the toxicology laboratory may be able to streamline drug analysis with some flexibility. Level II automation includes on-line sample preparation with chromatographic analysis. Some of the systems may offer added flexibility for liquid/liquid and solid-phase extractions with robotics and/or by column switching. Others use dedicated, automated solid-phase extraction systems with built-in extraction cartridge exchange. Level III may be considered as the most advanced system, with user-friendly software programming (chemometrics for integrated operation and/or drug identification/quantitation). With minimal training, the laboratory technologists may be able to perform Level III automated drug analysis with ease. These units are user-friendly and have reliability similar to that of an autoimmunoanalyzer. Furthermore, Level III automation may be achieved by combining an automated HPLC with selected chromatographic DSA column technologies.

Based on a previous review,[1] the merits of DSA for clinical drug analysis may be listed as: (1) alternative to immunoassay; (2) lower cost, compared with conventional chromatographic assays, and automation that makes it possible to achieve the whole "run" in a realistic time frame; (3) customized assays—viable alternative for assaying newly introduced drugs/metabolites; (4) reduced personnel exposure to infectious samples; (5) simultaneous monitoring of parent drug and metabolite(s); (6) possible resolution of interference from other drugs; (7) increased precision and accuracy with automation; (8) data suitable for forensic analysis; (9) various approaches offering a larger range of selectivity; (10) as in the case of microcolumn applications, most of these approaches allow analyses with a minute sample of 3 to 500 µl, usually smaller than the conventional chromatographic assays or immunoassay (limited sample-size analysis, such as neonatal and pediatric drug monitoring, may be readily performed); and (11) ideal for assay of light-sensitive drugs, such as nifedipine.

Table 9–2 shows the various DSA and their assigned automation level classifications, updated from previous reviews. The following section describes various DSAs with an ascending level of automation.

C. DSA WITHOUT AUTOMATION

Micellar chromatography is based on reactions among micelles, monomers, serum proteins, and drugs.[27,28] Serum protein micelle complexes elute after the solvent front, followed by the drug/micelle/monomers. Analysis of theophylline and carbamazepine were demonstrated, and with currently available HPLC, this may be readily automated to offer Level III capability.

Another major innovation in technology for DSA is the restricted access media (RAM). Two commercially available RAM are: the internal surface reversed phase (ISRP)[29] and the shielded hydrophobic phase (HISEP),[30] whereas another RAM, the dual zone media, was under development.[31] ISRP, pioneered by Pinkerton, is a bimodal column support consisting of small-pore 52 Å silica gel with the hydrophobic peptide, glycine-phenylalanine-phenylalanine (GFF), bound to the internal surface of the pore, as shown in Figure 9–2. The bimodal chromatographic processes are the exclusion chromatography of the protein molecules as a result of the pore size and the reversed-phase hydrophobic interaction of drug molecules with GFF after migrating inside the pores. Figure 9–3 showed the DSA of phenobarbital and carbamazepine in serum by using ISRP column[1] and other antiepileptics, such as phenobarbital and theophylline, may also be analyzed by ISRP. HISEP, another RAM for DSA, is also a bimodal column.[30] The column support consists of a polymeric hydrophobic network of bonded polyethylene oxide, capable of excluding protein macromolecules, while drug and lower molecular weight analytes penetrate the network for hydrophobic interaction with the phenyl moiety. Phenobarbital was readily analyzed by direct injection of patient serum samples after centrifugation. The results correlated highly with those obtained by fluorescence polarization immunoassay.[33]

D. DSA WITH AUTOMATION

1. Level I

Column switching for dedicated drug analysis has been one of the most persistent modes of automation in the pharmaceutical research and development laboratories, but seldomly used in the

TABLE 9–2
DSA Approaches by LC and Proposed Automation Levels

Approaches	Manufacturers
DSA without automation	
Microinjection	
Micellar chromatography	
Restricted Access Media (RAM)	
Internal surface reversed phase (ISRP) (Pinkerton column)	Regis Chemical
Shielded hydrophobic phase (HISEP)	Supleco
Dual zone media	Dow Corning
Electrochemical detection with photolytic derivatization	
Level I	
Column switching	
Level II	
Advanced Automated Sample Preparation (AASP)	Varian
OSP-2 (automated extraction cartridge exchange)	Merck
Solvent extraction	
Column switching	
Level III	
Automated column switching/multimodal chromatography with chemometrically enhanced data processing (REMEDi)	Bio-Rad
Automated extraction/multidimensional chromatography with chemometrically enhanced data processing (PrepStation™/LC, GC, and GC/MS)	Hewlett–Packard
Automated robotic liquid transfer and solid-phase extraction ASPEC, and with microdialysis, ASTED, and Zymate	Gilson, Zymark

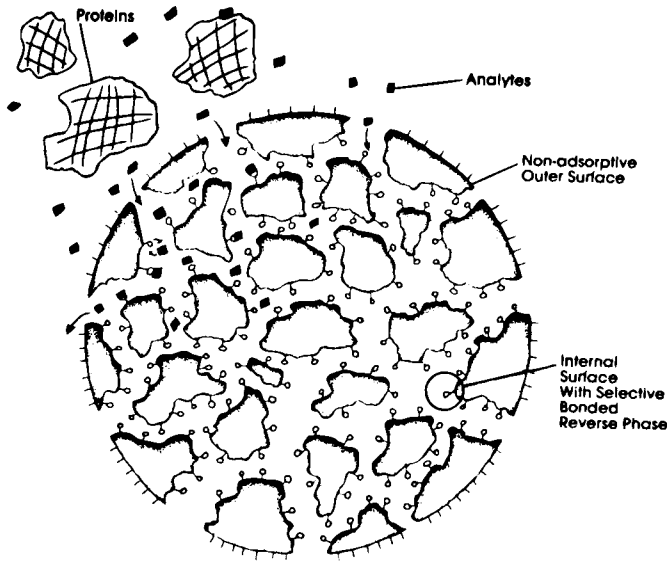

FIGURE 9–2. Internal surface reversed phase (ISRP) packing. (From Hagestam, I. H. and Pinkerton, T. C., *J. Chromatogr.*, 368, 77–84, 1986. With permission.)

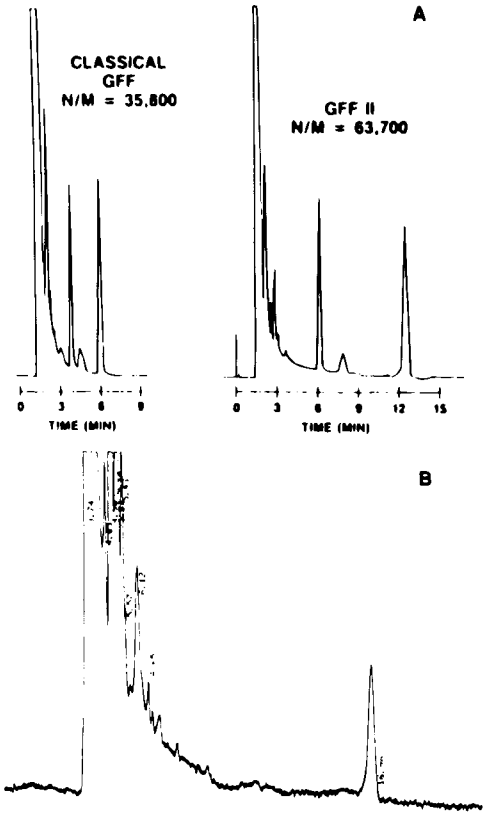

FIGURE 9–3. (A) The 10-µl serum-phenobarbital-carbamazepine test mix for the original GFF column and the new GFFII column. Mobile phase: buffer/ACN (8:2) at 0.6 m/min (Courtesy of Regis Chemicals), and (B) DSA chromatogram of the serum sample from a patient medicated with carbamazepine. (From Wong, S. H. Y., *Am. Assoc. Clin. Chem.*, 13, 7–24, 1992. With permission.)

clinical laboratory. This approach requires intimate hands-on experience of the analyst with both the column-switching systems, interconnection of multiple HPLC pumps, and computer programming.

Demotes-Mainard et al.[34] used automated column switching for extracting vancomycin in serum into two reversed-phase precolumns, followed by analysis with a C-18 column. Before LC analysis, aliquots (100 µL) of serum were mixed with an internal standard solution. After mixing, 30 µl of the supernatant was injected into one of the two precolumns connected to a six-port switching valve for further extraction for 1 min, followed by backflushing of the eluant into the analytical column. The precolumn was estimated to last for about 200 injections. Total analysis time was about 8 min. Detection limit was 2 µg/ml. The results were highly correlated to those of the enzyme multiplied immunoassay technique (EMIT) assay.

Zellinger et al.[10] analyzed PCR products of the c-erb-2 oncogene copy number of human breast carcinomas using an anion-exchange packing. Analysis time was 5 min per sample. The authors suggested it as a potential replacement for the Southern blot hybridization. This automated procedure may be used potentially for DNA fingerprinting for toxicology and genotyping for TDM.

Johansson et al.[35] performed chiral analysis of alprenolol, metoprolol, and propranol in blood and brain dialysates. Small aliquots, 8 µl, of brain dialysates or plasma extracts were injected into a cellulose column for chiral separation, followed by trapping in two small precolumns. By selective reversed elution of the precolumn through column switching, the pure enantiomer was eluted into a C-18 column for quantitative analysis. Detection limit was about 25 to 400 ng/ml.

2. Level II

Etienne et al.[36] analyzed *d,l*-folinic acid (*d,l*-FA), a vitaminic factor used in oncology, and 5-methyltetrahydrofolate (5MTHF), an active cofactor of *l*-FA, by performing a preliminary solid-phase extraction, using BondElut RP-18 of plasma, followed by a bimodal–on-line achiral and

chiral LC analyses using dual LC, as shown in Figure 9–4. The first LC (equipped with a microbore column, C-8, 119 × 2 mm i.d., and ultraviolet [UV] detector) was used to quantitative 5MTHF with sensitivity of 50 nmol/l and to elute *dl*-FA in a minimized elution volume of about 500 µl. The second interconnected LC used a chiral, human serum albumin column and an electrochemical detector to analyze *d,l*-FA at a dection limit of 20 nmol/l. Retention times for 5MTHF, *l*-FA and *d*-FA were 9 min in the first HPLC, and 6 and 12 min of the second HPLC, respectively. Total analysis time was 18 min. This assay was used for pharmacokinetic studies of cancer patients.

Bourque et al.[37] analyzed drugs of abuse using a roboticized arm for sample transfer into a injector containing a solid-phase reagent, 9-fluorenylacetate-activated ester. The ester, bound onto a polystyrenedivinylbenzene packing, reacted with the analytes in urine or plasma up to 100 injections with unaltered sensitivity. Amphetamine in urine was detected at a concentration of about 500 ppt or 0.5 ng/ml.

Chollet and Kunstner[38] demonstrated the automated analysis of cebaracetam, a new neotropic drug, in urine by an on-line solid-phase extraction system. Using OSP-2 by Merck, a commercially available, automated solid-phase extraction system with extraction cartridge exchange, urine loaded into a vial in an autosampler was injected into the preconditioned, regenerated C-18 cartridges. Extraction was sequentially performed by washing with phosphate and then acetonitrile (ACN)/diluted phosphoric acid (9:1). The total extraction time, including precolumn regeneration, was about 22 min. Regenerated precolumn was useful up to 27 times. The method was calibrated from 0.3 to 30 µg/ml.

Prados et al.[39] analyzed catecholamines by using an on-line, carboxylic resin extraction of 25 to 50 µl of plasma diluted with the buffer of an internal standard *N*-methyldopamine. The extracts were analyzed by reversed-phase column, followed by ethylenediamine derivatization and postcolumn peroxyoxalate chemiluminescence reaction detection using bis(4-nitro-2-[3,6,9-trixadecyloxycarbonyl]phenyl)oxalate and hydrogen peroxide. Detection limit was 1 fmol.

Wolf et al.[40] assayed, after microdialysis of rat brain, extracellular valproic acid by using an automated precolumn derivatiziation with bromomethylmethoxycoumarin, followed by LC analysis with fluorometric detection—excitation = 325 nm and emission = 398 nm. The extracellular level at 90 min was estimated to be 17 µg/ml. The authors suggested that microdialysis be useful if supplemented by a sensitive assay.

The Advanced Automated Sample Preparation (AASP) process was used by Ni et al.[41] to analyze tricyclic antidepressants. With conditioned C-8 cartridges, aliquots of plasma, internal standard solution, and phosphate were mixed and transferred into the cartridge. After clean-up with phos-

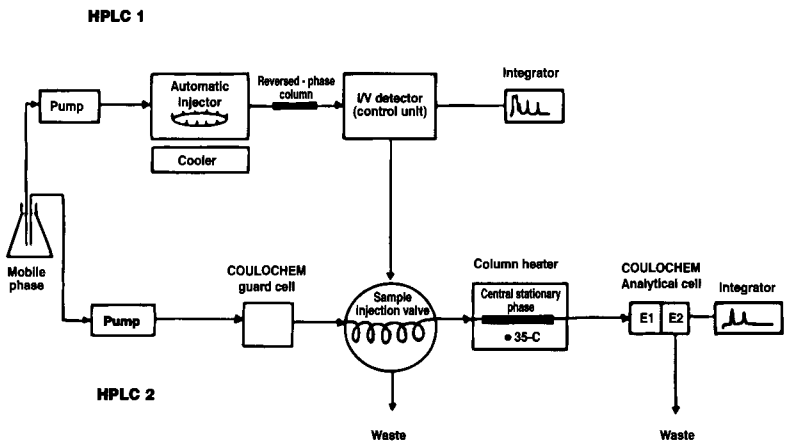

FIGURE 9–4. Chromatographic system for analyses for flinic acid diastereoisomers and 5 MTHF in plasma (From Etienne, M. C., et al., *Clin. Chem.*, 39, 82–86, 1993. With permission.)

phate, the cartridges were backflushed with mobile phase for analysis with a cyan column. Kabra et al.[42] used the AASP for the analysis of cyclosporine in whole blood, with prior manual protein precipitation with zinc sulfate.

3. Level III

Automated solvent extraction with "FAST-LC" was demonstrated by Bannister et al.,[43] based on Technicon's clinical chemistry analyzer technology. Serum aliquots were transferred sequentially for mixing and extraction in coils, and the extracts were injected directly into the HPLC. Antiepileptics and antidepressants analyses were performed with ease of operation comparable with some of the Level III systems; but, unfortunately, this was not introduced commercially.

Hsieh et al.[44] analyzed verlukast, a leukotriene D4 antagonist in plasma by using the robotic Zymate I for protein precipitation, followed by on-line injection for reversed-phase analysis and fluorescence detection. Figure 9–5 shows the schematic of the robot configuration used in the study. Aliquots, 200 µl of plasma, were mixed with internal standard. Then, it was vortex-mixed with acetonitrile for protein precipitation. After centrifugation, 400 µl was aspirated into a clean tube, and 50 µl was injected for HPLC analysis. The throughput was about 120 samples for 15 h. Chromatographic analysis time was 7.5 min. Calibration was linear from 0.1 to 5.0 µg/ml, with a detection limit 0.1 µg/ml. The automated method provided results that were highly correlated to those of a manual method.

With advances in HPLC instrumentation, totally automated, commercially available DSA analyzers would include: REMEDi, PrepStation/HPLC/GC/GC/MS, ASPEC, and ASTED and Zymate. Each of these instruments offers unique capabilities and versatility as shown by Table 9–3.

ASPEC and ASTED offer robotic liquid transfer and solid-phase extraction. Nichols et al.[17] used the ASPEC for automated extraction, analysis, and data reduction for fluoxetine and its metabolite, norfluoxetine. This was based on a previously published automated procedure for carbamazepine and its metabolites.[17] After manual mixing 1 ml of patient's plasma with an internal standard solution, the mixture was transferred into ASPEC for solid-phase extraction with a C-18 cartridge. The extracted drug was eluted and further extracted with heptane/isopropanol. After standing for phase separation, the upper heptane phase was backextracted with phosphoric acid. With further phase separation, the acidic extracts were injected into a C-8 column for sequential automated analysis. Mean recovery of fluoxetine and norfluoxetine were 62 and 70%, respectively. Results of this pro-

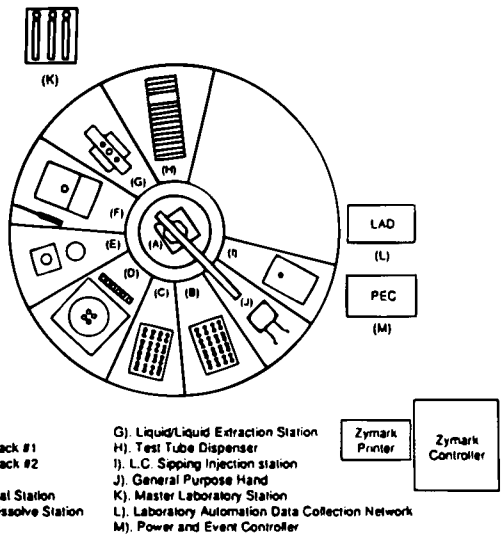

FIGURE 9–5. Schematic of the robot configuration used in the study. (From Hsieh, J. Y., et al., *J. Chromatogr. B*, 661, 307–312, 1994. With permission.)

TABLE 9–3
Comparison of Commercially Available Level III DSA

	ASPEC/ASTED	PrepStation™	REMEDi	Zymate
Manufacturer	Gilson	Hewlett–Packard	Bio-Rad	Zymark
Chromatographic systems	HPLC	HPLC, GC, GC/MS	HPLC	HPLC, GC, GC/MS
Program	Sampler manager HPLC system controller	Bench Supervisor	Paradise	System II controller
Sampling	Robotic pipet	Robotic pipet	Pipet	Robotic Pipet and others
Extraction modes	LL SPE PS	LL SPE PS Vortex Axial centrifugation	SPE	LL SPE PS Vortex centrifugation
Other functions	Add, dialysis	Add, evaporate		Add, evaporate, weigh
Chromatographic analysis	Batch	Batch or sequential	Sequential	Batch or sequential
Detection UV	Single, dual, scanning, photodiode array	Photodiode array	Scanning	N/A
Run time/sample	10–20 min	30–60 min	20 min	Variable (e.g., 15 h for 120)
Flexibility—relative rating	+	++	0	+++

Note: Abbreviations used are — LL, liquid/liquid; SPE, solid-phase extraction; PS, phase separation; N/A, not applicable.

cedure were highly correlated to a manual method. The authors suggested that backextractions obviated potential interferences. Swart et al.[19] used the ASPEC for automated analysis of acyclovir, used for the treatment of herpes, in plasma for a large number of samples in pharmacokinetic studies. The mixture of 0.5 ml of plasma and sodium octanesulfonate was loaded into the ASPEC for solid-phase extraction with C-18 columns, followed by automated sequential, quantitative analysis by a C-18 Nova-Pak column. Detection limit using 250 nm was 10 ng/ml, and recoveries ranged from 82 to 88%. Total extraction and analysis was 10 min. It was used for a pharmacokinetic study on 24-h-a-day basis. Hsyu et al.[45] analyzed (–)-2′-deoxy-3′-thiacytidine, a reversed transcriptase inhibitor for the treatment of acquired immune deficiency syndrome (AIDS), in rat dialysate by ASTED, an automated solid-phase extraction with SCX cation-exchange cartridges, followed by reversed-phase analysis with a C-18 column. Aliquots, 130 µl of samples, were loaded into the upper chamber of the dialyzer, followed by dialysis for 25 min. Then the dialysate was extracted into a cartridge, and the extract was injected into the HPLC by reversed elution for 2 min. Total extraction and analysis time were about 30 min. Calibration range was from 25 to 10,000 ng/ml. Detection limit was 25 ng/ml. Recovery was low at 27%, possibly caused by inefficient dialysis and other factors. This automated procedure, offering minimized exposure to the analyst, may be used for analysis of other anti-AIDS drugs in potentially infectious human serum and urine samples.

REMEDi performs drug identification in urine/plasma and other matrix by using column switching for extraction, followed by multicolumns, multimodal separations, and UV scanning for peak identification.[11–14] Chromatographic data are being processed chemometrically for drug identification.[11–12] Figure 9–6 shows the schematics. After mixing aliquots of urine or serum with the internal standards solution followed by filtration, the filtrates, loaded into the autosamplers, were injected sequentially into the extraction columns. By using column switching, the extracts were eluted into the analytic columns. Eluted peaks were then matched chemometricially to the stored

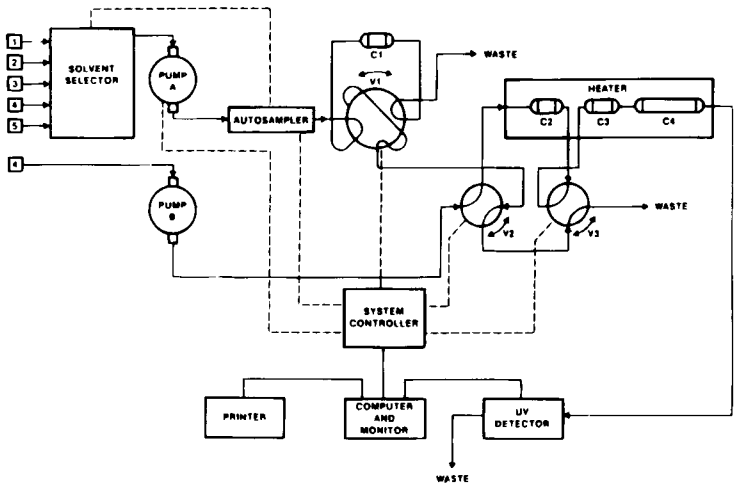

FIGURE 9–6. Experimental set-up for on-line analysis of basic drugs in urine according to the final procedures described in the text. (From Binder, S. R., et al., *J. Chromatogr.*, 473, 325–341, 1989. With permission.)

drug library for identification. Figures 9–7 and 9–8 showed the analyses of urine samples, identifying cocaine and benzoylecgonine, and desipramine and metabolites, respectively.[1]

TDM analysis, was demonstrated by Patelet et al.[46] for the measurement of disopyramide by REMEDi. Although the REMEDi results were highly correlated to those of EMIT, the REMEDi mean of 2.64 mg/l was significantly lower than the EMIT mean of 3.14 mg/l, possibly because of the incomplete recovery of the on-line sample extraction and assay calibration. However, the difference was not of clinical significance for TDM. Other potential analysis for TDM drugs included procainamide, *N*-acetylprocainamide, and quinidine.

Kalasinsky et al.[14] explored the forensic applications of REMEDi. They performed an off-line extraction of whole blood and tissue with butanol/ethyl acetate (2:8), followed by evaporation of the organic layer to 50 µl. This was reconstituted with saline for about 90 to 120 min. After addition of the internal standards solution, the mixtures were analyzed by REMEDi. Using the above protocol for 50 drug standards, the analytical performance and detection limits were established, ranging from 50 ng/ml for alprazolam and verapamil to 1,000 ng/ml for benzoylecognine, caffeine, phenyl-

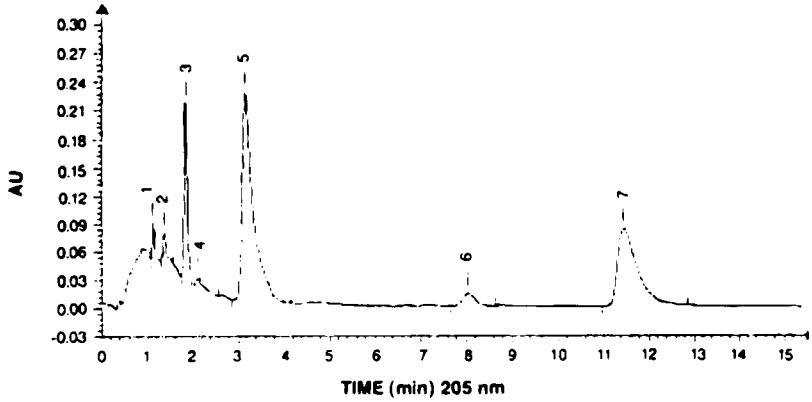

FIGURE 9–7. Chromatogram of the analysis of a patient's urine by REMEDi, showing the presence of benzoylecgonine (peak 3) and cocaine (peak 6). Peaks 5 and 7 are internal standards. (From Wong, S. H. Y., *Am. Assoc. Clin. Chem.*, 13, 17–24, 1992. With permission.)

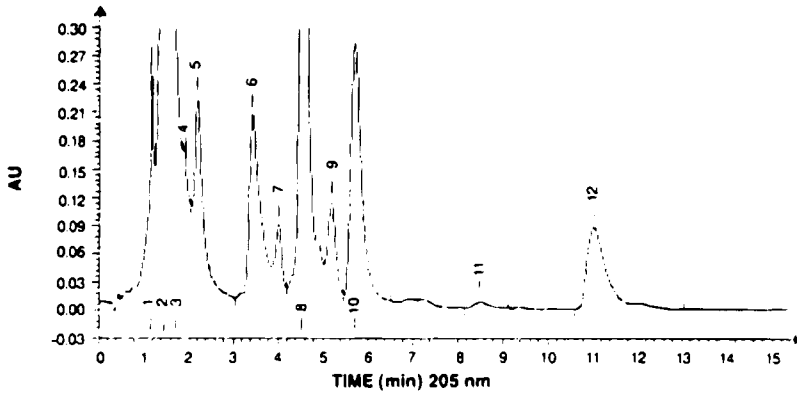

FIGURE 9–8. Chromatogram of the analysis of a patient's urine by REMEDi showing the presence of a despramine metabolite (peak 2), 2-hydroxy desipramine (peak 8), and desipramine (peak 10). Peaks 6 and 12 are the internal standards. (From Wong, S. H. Y., *Am. Assoc. Clin. Chem.*, 13, 17–24, 1992. With permission.)

propanolamine, and thioridazine. Cotinine was not detected by this protocol. Table 9–4 shows the comparison of seven drug/drug classes positives with fluorescence polarization immunoassay, GC/nitrogen–phosphorus detection, and GC/MS. Based on the findings of 50 cases from the medical examiner's office, the aforementioned protocol complemented other methods.

Another totally automated HPLC system is the PrepStation/HPLC. Wong et al.[16] compared the automated felbamate analysis to a previously developed manual liquid/liquid procedure. Using the computer program in Bench Supervisor™, the serum sample was deproteinized for assay by a liquid/liquid extraction with ACN containing an internal standard, followed by a hexane wash as shown in Figure 9–9. Table 9–5 shows the program for the assay. Extracts were sequentially ana-

TABLE 9–4
Summary of REMEDi Positives of Forensic Urine Samples Compared by Class with Fluorescene Polarization Immunoassay (FPIA), GC/Nitrogen–Phosphorus Detection (GC/NPD), and GC/MS Final Report of Forensic Cases. (From Kalasinsky, K.S., et al., J. Anal. Toxicol. With permission)

	Positives			
Class	FPIA	REMEDi	GC/NPD screening and GC/MS confirmation	GC/MS quantitiation
Phencyclidine	1	1	0	1
Amphetamine	2	11[a]	3	2
Amphetamine class (PE[b] and PPA[c])	20	19	24	25
Benzodiazepine	4	0	0	0
Phenytoin	3	0	0	3
Morphine (opiates)	6	6	5	6
Cocaine metabolite (benzoylecgonine)	3	3	0	3
Barbiturate	1	1	0	1

[a] REMEDi does not distinguish between amphetamine, phentermine, and phenethylamine.
[b] PE, Pseudoephedrine.
[c] PPA, Phenylpropanolamine.

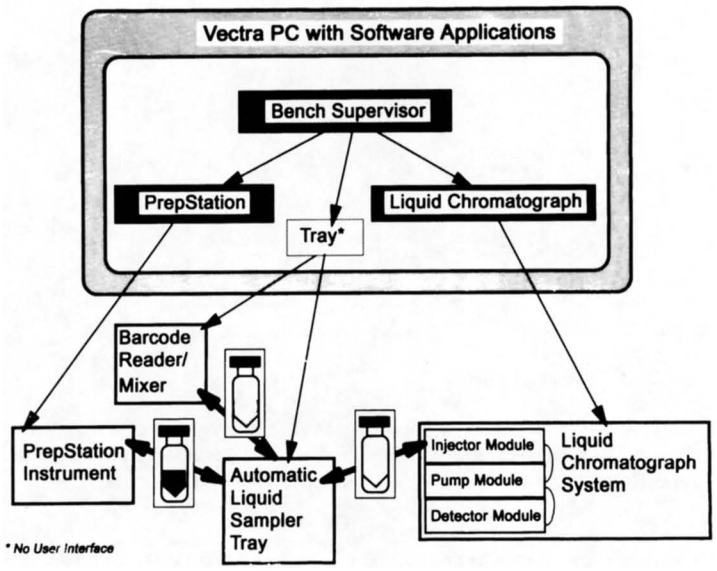

FIGURE 9–9. An outline of the totally automated PrepStation™ and liquid chromatograph. (From Wong, S. H. Y., et al., *Ther. Drug Monit.*, in press. With permission.)

TABLE 9–5
PrepStation™ Method for Felbamate. (From Wong, S. H. Y., et al., Ther. Drug Monit., in press. with permission)

Current Method = FELBAMATE.TSP

1. Liquid/liquid extraction of felbamate
2. Rinse system with 2.500 ml of ch3cn using aspirate flow path
   - Volume = 2.500 ml — Solvent = ch3cn
   - Draw speed = 10.0 ml/min — Station name = PS 1
   - Dispense speed = 5.0 ml/min — Flow path = aspirate
3. Transfer 0.600 ml from INT.STD to PATIENT
   - Volume in = 0.600 ml — Source vial = INT.STD
   - Volume out = 0.600 ml — Destination vial = PATIENT
   - Aspirate speed = 2.0 ml/min — Station name = PS 1
   - Dispense speed = 5.0 ml/min — Needle height = 1.00 mm
   - Presample air gap = 0.010 ml — Wait for fill = 5 sec
   - Overshoot = 0.60% — Prefill solvent path = No
   - Flow path = aspirate — Prefill solvent = deproteinizing/ISTD
4. Mix PATIENT at high speed for 3.00 min
   - Time = 3.0 min — Vial name = PATIENT
   - Speed = high
5. Wait for 5.00 min
6. Rinse system with 2.500 ml of IPA using aspirate flow path
   - Volume = 2.500 ml — Solvent = IPA
   - Draw speed = 10.0 ml/min — Station name = PS 1
   - Dispense speed = 5.0 ml/min — Flow path = aspirate
7. Rinse system with 2.500 ml of hexane using aspirate flow path
   - Volume = 2.500 ml — Solvent = Hexane
   - Draw speed = 10.0 ml/min — Station name = PS 1
   - Dispense speed = 5.0 ml/min — Flow path = aspirate
8. Dilute 0.400 ml from PATIENT to 0.800 ml with hexane into felbamate wash
   - Source volume = 0.400 ml — Source vial = PATIENT
   - Final volume = 0.800 ml — Destination vial = felbamate wash
   - Aspirate speed = 2.0 ml/min — Solvent = hexane

TABLE 9–5 *Continued*

	Draw speed = 10.0 ml/min	Station name = PS 1
	Dispense speed = 5.0 ml/min	Needle height = 2.00 mm
	Wait for fill = 5 sec	Overshoot = 0.60%
	Prefill solvent path = No	
9.	Rinse system with 2.500 ml of IPA using aspirate flow path	
	Volume = 2.500 ml	Solvent = IPA
	Draw speed = 10.0 ml/min	Station name = PS 1
	Dispense speed = 5.0 ml/min	Flow path = aspirate
10.	Rinse system with 2.500 ml of ch3cn using aspirate flow path	
	Volume = 2.500 ml	Solvent = ch3cn
	Draw speed = 10.0 ml/min	Station name = PS 1
	Dispense speed = 5.0 ml/min	Flow path = aspirate
11.	Dispense 0.200 ml of ch3cn into felbamate wash from sample loop	
	Volume = 0.200 ml	Solvent = ch3cn
	Draw speed = 10.0 ml/min	Vial name = felbamate wash
	Dispense speed = 5.0 ml/min	Needle height = 1.00 mm
	Overshoot = 0.60%	Station name = PS 1
	Flow path = aspirate	Prefill solvent path = No
12.	Mix felbamate wash at high speed for 0.50 min	
	Time = 0.5 min	Vial name = felbamate wash
	Speed = high	
13.	Transfer 0.100 ml from felbamate wash to felbamate injection vial	
	Volume in = 0.100 ml	Source vial = felbamate wash
	Volume out = 0.100 ml	Destination vial = felbamate injection vial
	Aspirate speed = 2.0 ml/min	Station name = PS 1
	Dispense speed = 5.0 ml/min	Needle height = 0.00 mm
	Presample air gap = 0.010 ml	Wait for fill = 5 sec
	Overshoot = 0.60%	Prefill solvent path = No
	Flow path = aspirate	Prefill solvent = deproteinizing/ISTD
14.	Sample—ready felbamate injection vial	
	— END —	

Vial/Cartridge Information Table

Name	Type	No. of Uses
Felbamate injection vial	Empty vial	1
Felbamate wash	Empty vial	1
INT.STD	Reagent	3
PATIENT	Sample	N/A

Solvent Information

PS 1 SPE module 2.5-ml syringe

Station	Port	Solvent	Size
PS 1	1	Deproteinizing/ISTD	500.0
PS 1	2	Hexane	500.0
PS 1	3	ch3cn	500.0
PS 1	4	IPA	500.0
PS 1	5	Saline	0.0
PS 1	—	Air	N/A

Note: Abbreviations used are—ch3cn, acetonitride; INT.STD, internal standard; ISTD, internal standard; IPA, isopropyl alcohol; N/A, not applicable.

lyzed by using a 15-cm μBondapak C-18 column and UV detection at 214 nm as shown in Figure 9–10. This procedure may be readily used for automated assay of other antiepileptics, and a solid-phase extraction protocol was developed for tricyclic antidepressants by McQuire et al.[15]

From a previous review and reaffirmed by recent studies, the following guidelines for DSA were proposed[2]:

1. Establish a column life by noting the injection volume and number.
2. Limit the analysis of a single group of drugs to a particular column and mobile-phase composition. This would enhance equilibration and extend column life.
3. As a result of possible system variance, such as injection volume, it is strongly suggested that duplicated injections should be made for standard, quality control and patients samples. The peak height or peak area value should be within 10% of each other. Furthermore, standards and quality controls should be placed at random positions to check on system performance.
4. Because of possible multidrug therapy, patient samples may contain several drugs and metabolites. To ascertain that the interested drug peak is not co-eluting with another drug/metabolite/endogenous substance, patient samples should be analyzed twice and in random order. If possible, photodiode array UV detection should be used to establish peak purity.
5. As a result of DSA, automation may be easily achieved to minimize exposure of the analysts. This may be followed by containment design, "closed systems" such as those used in the nuclear industry.

III. MICROCOLUMN LC

A. PRINCIPLES OF MICROCOLUMN LC

Horvath et al.[47] studied microcolumns packed with pellicular particles. Scott[20] investigated the theoretical, practical, technical, and experimental details. Recent reviews[1,2,48–52] indicated the field of microcolumn has steadily advanced and matured on a conservative and applications-driven basis.

Scott proposed that the minimum detectable mass (m):

$$m = VpC/2, \tag{1}$$

where C = minimum detectable concentration and Vp = peak width of solute at the peak base.

It may be rearranged to show that:

$$m = 2\pi r^2 lw(l + k)C/(N)^{1/2}, \tag{2}$$

where r = column radius, l = column length, w = fraction of mobile phase-occupied volume, k' = capacity factor of the solute, and N = column efficiency in theoretical plates.

Thus, low detection limit may be achieved by using small column radius. For classification of microcolumns, the internal diameter of a microbore column is arbitrarily set at about 1 mm. In com-

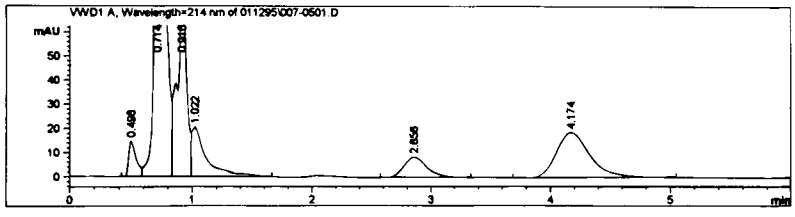

FIGURE 9–10. Chromatogram of extracts of a 50-mg/l standard and a patient with 32 mg/l of felbamate. (From Wong, S. H. Y., et al., *Ther. Drug Monit.*, in press. With permission.)

parison to the conventional 4.6-mm i.d. HPLC column, a theoretical 20-fold increase in sensitivity may be achievable. Figure 9–11 shows major categories of microcolumns: open-tubular capillary column, with an i.d. of 15 to 50 μm, a major category for SFC, and the packed capillary column, with an i.d. of 40 to 80 μm.[48] These latter microcolumns are now commercially available columns, with accompanying hardwares.

The chromatographs used for microcolumn LC consist of a syringe pump to deliver a low and pulseless flow rate of 1 to 100 or so μl/min, whereas other conventional LCs (e.g., Hewlett–Packard with reciprocating pumps) may also be used for 2-mm i.d. columns. Readers should attempt microcolumn separations with available HPLC before purchasing a dedicated instrument.

Capillary LC would require further modifications or additions to existing HPLC. Davis and Lee,[53] based on the analysis of peptide, offered a practical guide for achieving capillary LC analysis. Capillary columns, with internal diameter ranging from 0.01 to 0.5 mm, may be constructed from recycled supports and fused-silica columns. Conventional LC pumps and detection may be readily modified for flow rates of 0.5 to 50 μl/min. Flurer and Novotny[54] demonstrated capillary immunoaffinity LC using a 30 cm × 250 μm i.d. fused-silica microcolumns packed with C-8 with or without saturation with immunoglobulin G for the analysis of human plasma proteins, such as albumin, transferrin and, α_1-antitrypsin. Battersby et al.[55] demonstrated, as shown in Figure 9–12, the capillary HPLC system modified from an available HPLC.

Table 9–6 shows selected clinical or potential drug analyses using microcolumns. Sample size may be readily reduced for a 1-mm i.d. column, with flow rate ranging from 60 to 100 μl/min and adequate detection limits. These were limited by the small injection volume of 0.5 μl. Other advantages would include reduced solvent consumption and generated liquid waste. Different from the theoretical considerations, the clinical applications of 1- and 2-mm microcolumn analyses showed that the enhanced mass sensitivity was not, in general, an achievable and practical advantage. This is offset by the low drug concentrations in clinical samples, such as cyclosporine and rapamycin, typically in low microgram per liter ranges. Thus, a larger sample size is needed for sample extraction/preconcentration, so that the drug concentration would be within the detection limit. However, for selected clinical applications, such as the analyses of chloramphenicol and cyclosporine, a small sample of 5 or 500 μl may be used, which is ideally suitable for a limited sample-size analysis, such as for neonatal and pediatric drug monitoring. Furthermore, because 2-mm columns may be con-

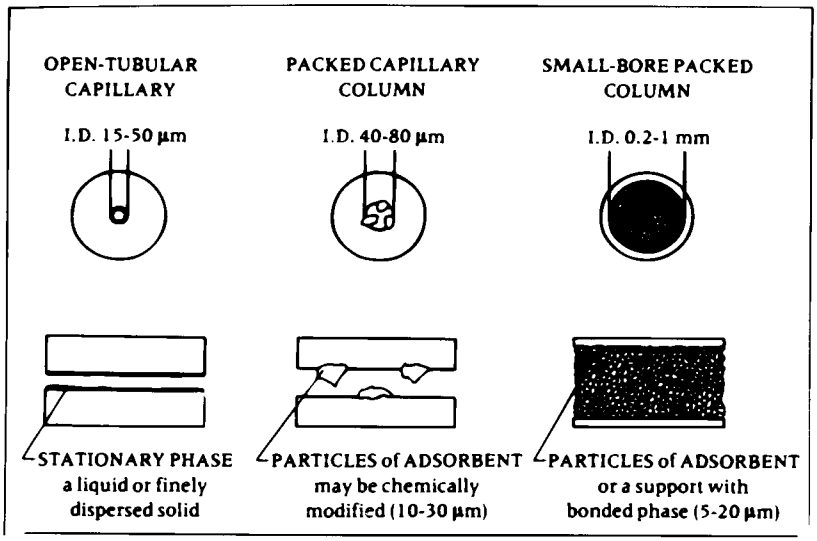

FIGURE 9–11. Types of microcolumns used in HPLC. (From Novotny, M. V. and Ishii, D., *J. Chromatogr. Libr.*, 30, 1–336, 1985. With permission.)

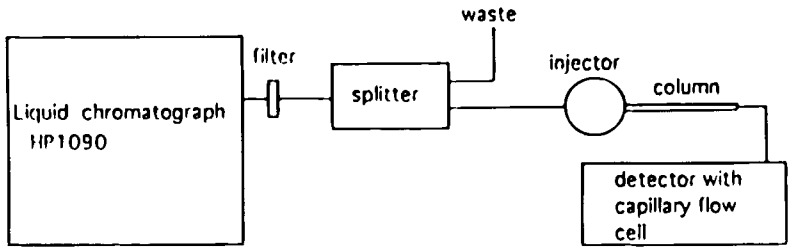

FIGURE 9–12. Arrangement of a capillary HPLC. (From Battersby, J. E., et al., *J. Chromatogr. B*, 662, 335–342, 1994. With permission.)

nected to conventional HPLC, published studies supported the minimized mobile phase consumption and generated waste. These discrete advantages may warrant further investigations of microcolumns.

B. MICROCOLUMN APPLICATIONS

1. Capillary Column Analyses

Sato et al.[56] demonstrated sensitive, semiquantitative, on-line analysis of a cephalosporin, cefaclor in serum by a capillary LC/fast atom bombardment-MS (FAB-MS).[56] This technique was advantageous for analysis, without derivatization, of polar, thermolabile compounds with a high molecular weight. With column switching for injection and concentration, samples of 100 to 500 µl were analyzed by using a C-18 capillary column, 150 × 0.3 mm i.d., interfaced to FAB-MS. The mobile phase was water/methanol (MeOH)/acetic acid/glycerol (69:30:0.5:0.5) at 4 µl/min.

TABLE 9–6
Illustrative Comparison of Microcolumns for Clinical Drug Analysis

Drugs	Sample volume (µl)	Injection volume (µl)	Flow rate (µl/min)	Detection limit (mg/l)	Mobile phase volume (ml)
Capillary 0.3-mm i.d. column analyses					
Theophylline, theobromine, and caffeine	200–1000	10–100	4	0.005	0.12
Cefaclor	100–500	—	4	0.05–0.1	—
Microbore 1-mm i.d. column analyses					
Theophylline and caffeine	20	0.5	80	1.0 1.5	0.8
Procainamide, *N*-acetylprocainamide	250	0.5	100	0.3 0.2	1.0
Chloramphenicol	5	0.5	60	3	0.9
5-Fluorouracil	(40–200 mg)	—	60	3 ng/g	2.4
Narrowbore 2-mm i.d. column analyses					
Cyclosporine	200–500	20	250	0.003	0.3
Tacrolimus (FK 506)	1000	20	100	0.0002	0.4
Rapamycin	3000	70	350	0.001	12.3
Flecainide	100	20	500	0.02	5.0
Carbamazepine, 10,11-epoxide, 10,11-transdiol	200–1000	10	500	0.025 0.010	5.0

Detection limits ranged from 50 to 100 ng/ml. The authors suggested that, with added isotopic internal standard, quantitation may be achieved.

Heida et al.[22,23] demonstrated the capillary LC/FAB-MS analysis of theophylline, theobromine, and caffeine in 0.2 to 1 ml of plasma and urine. After solid-phase extraction with Extrelut columns, all of the extracts (10 to 100 µl) were injected into the two capillary columns before MS analysis. The capillary column was packed with 5-µm C-18, 150 × 0.3 mm i.d. Analysis was performed by using acetic acid/glyercol/MeOH/water as mobile phase at a flow rate of 4 µl/min. Total analysis time was 30 min. Figure 9–13 shows the UV and mass chromatograms.[23] Detection limit was 5 ng/ml. Furthermore, theophylline in hair was identified in patients on daily 400-mg doses for more than 2 years. The authors suggested the potential clinical and forensic applications of this protocol.

Battersby et al.[55] analyzed recombinant DNA-derived human growth hormone by using capillary LC column, 10 cm × 320 µm i.d., and 214 nm detection with a capillary Z-shaped flow cell. A microflow processor was used to deliver 3.5 µl/min. Reversed-phase LC was interfaced with a triple quadrupole MS for peptides identification. Sensitivity was increased about 100 times, compared with the conventional LC method. This would be of interest to doping control in sports medicine.

By using an open-capillary column coating mono-6-*O*-octamethylenepermethyl-β-cyclodextrin to demethylpolysiloxane, Schurig et al.[21] demonstrated unified enantioselective capillary chromatography—GC, SFC, LC, and CEC analysis of hexobarbital. Miniaturization offered the following advantages: low flow rate enhancing LC/MS interface, low pressure drop, minimized packing and mobile phase utilization, enhanced temperature programming, and mass sensitivity.

2. 1-mm Microcolumn Analyses

The author previously demonstrated analyses of theophylline, caffeine, procainamide, and *N*-acetylprocainamide and chloramphenicol.[1,57] Figures 9–14 shows the chloramphenicol analysis by using a, high carbon load C-18 column packing for increased interaction of the analytes with the functional groups. This was needed to resolve the prodrug esters from chloramphenicol. Sample size was about 5 µl, ideal for neonatal drug monitoring.

Jochheim et al.[58] determined 5-fluorouracil (5FU) in tissue, serum, and plasma samples by using a microbore, Nucleosil-120 column, 125 × 1.6 mm i.d., and fluorescence detection—excitation =

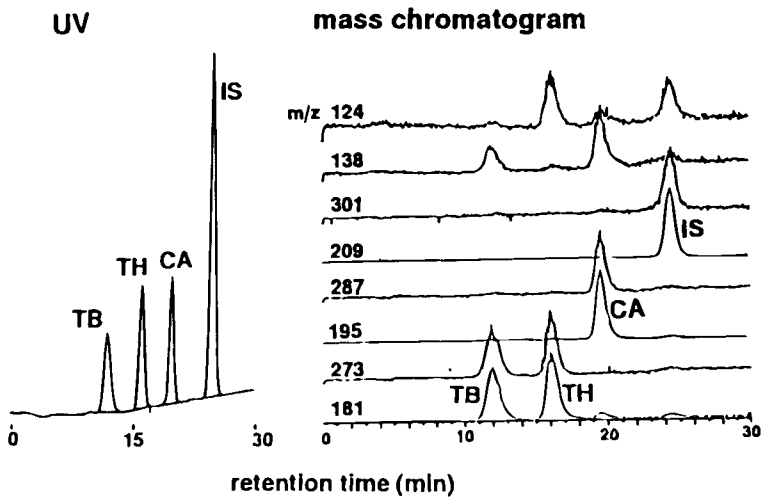

FIGURE 9–13. LC/UV (273 nm) chromatogram (left), and mass chromatogram (right) of the standard solution of theophylline (TH), theobromine (TB), and caffeine (CA): 60 ng. IS, internal standard (100 ng). (From Hieda, Y., et al., *J. Chromatogr. B*, 667, 241–246, 1995. With permission.)

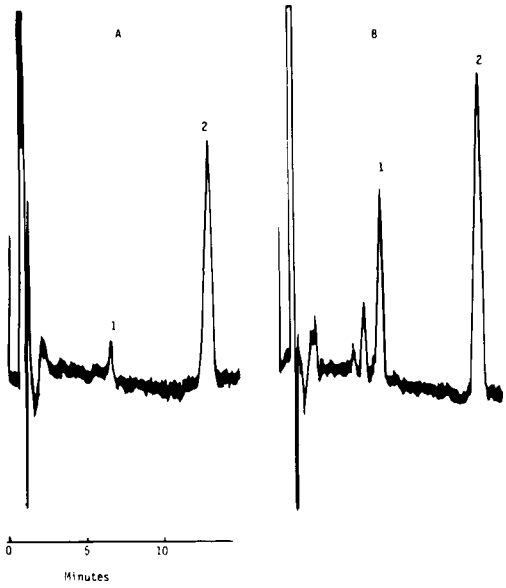

FIGURE 9–14. Microbore liquid chromatograms of 0.5-µl aliquots of supernatant from procedure B. Chromatogram A shows 6 mg/l of chloramphenicol after oral administration of chloramphenicol palmitate, and chromatogram B shows 21 mg/l of chloramphenicol after intravenous administration of chloramphenicol succinate. Peak identification: 1, chloramphenicol; 2, internal standard. (From Wong, S. H. Y., et al., *J. Liq. Chromatogr.*, 11, 1143–1158, 1988. With permission.)

305 nm and emission = 407 nm, with precolumn derivatization with 4-bromomethyl-7-methoxycoumarin. Mobile phase was ACN/MeOH/water (30:15:50), at 60 µl/min. A retention time of 5FU was 27 min, and total analysis time was 40 min. Detection limit was 3 ng/g of tissue, with sample size ranging from 40 to 200 mg. The authors found drug concentration in the tissue up to 1,000 times higher than the surrounding tissue.

Mays et al.[59] studied the photolysis products of sulfiram, used for topical treatment of scabies, by using microbore LC/MS and –MS/MS for the structural elucidation. Sulfiram, when exposed to fluorescent room light, would form eight products, including disulfiram (DS) at a conversion rate of 0.7%/hr. Aliquots, 1 to 2 µl of the photolysis product, was injected into a C-18 column, 150 × 1 mm, and analyzed by gradient elution with water/ACN at 50 µl/min. Retention times of sulfiram and DS were 15.4 and 16.6 min, respectively. The eluant, at a split ratio of 1:20, was introduced into the MS. Because DS is used for aversion therapy for alcoholism, the photoconversion of sulfiram to DS may account for the adverse reaction to ethanol ingestion. Benson et al.[60] determined the structure of photochemical products of disulfiram using microbore LC-continuous-flow liquid secondary ion MS. Aliquots, 2 µl of the product solution, were analyzed by a C-18 column, 150 × 1 mm i.d., with ACN/water/glycerol (50:48:2) as the mobile phase at a flow rate of 59 µl/min. An identified sulfine compound was shown to inhibit aldehyde dehydrogenase.

Kiang et al.[61] determined enprostil acid, a prostaglandin analog, in 1 to 2 ml of human plasma by using phenyl solid-phase extraction and precolumn reaction with 2-bromoacetyl-6-methoxynaphthalene. After a conventional LC elution into a guard column, injection through the guard column was subsequently directed to a microbore LC with detection by a 325 nm helium–cadmium laser for excitation and fluorescence emission at 450 nm. The column was 150 × 1 mm i.d., with MeOH/water (4:6) as the mobile phase at 50 µl/min. Total analysis time was 60 min. Detection limit was 5 pg/ml of plasma.

Fujiwara et al.[62] studied the *in vitro* metabolism of chloroacetanilide, a herbicide, by using microbore LC/FAB-MS and LC/thermospray-MS for structural elucidation. Microbore LC/FAB was used for molecular formula elucidation, whereas LC/thermospray provided structural information for both polar and less polar metabolites. A C-18 microbore column, 250 × 1 mm i.d., was used with 0.1% trifluoroacetic acid water/ACN gradient (20 to 100%) at 40 µl/min. After incubation of the herbicide with S-9 rat liver enzyme, aliquots (100 µL) were mixed with MeOH to terminate the *in vitro* metabolism. Centrifugation of the methanolic supernatant was ensured, followed by evapo-

ration before LC/MS analysis. Of seven identified metabolites, four were conjugated with glutathione. This application may be used for environmental toxicology.

3. 2-mm Microcolumn Analyses

Analyses with 2-mm i.d. columns generally use larger sample sizes and injection volumes with higher sensitivity limits of 0.003 to 0.02 mg/l. This is readily performed by conventional HPLC instrumentation.

Etienne et al.,[36] as shown in the automation section, used a microbore column to analyze 5MTHF and *d,l*-FA. The microbore column was selected to deliver a minimized elution volume of 500 µl for chiral separation later on.

Kearns et al.[63] characterized a cephalosporin, cefpirome disposition in lactating females by extracting a small amount of breast milk, serum, or urine (50 µl) with isopropanol, followed by microbore LC analysis. Analysis was performed with a C-18 column, 100 × 2-mm i.d., with MeOH/triethylamine (TEA) (1:9) as the mobile phase. Flow rate was 0.5 ml/min. Retention time was 2.7 min, with a total analysis time of 6 min per sample. Calibration was established for 0.6 to 500 mg/l. The protocol may be readily used for pharmacokinetic studies.

Yeleswaram et al.[64] analyzed labetalol in biological fluids of pregnant sheep. After extraction of 250 µl of fluids with ethyl acetate and backextraction with phosphoric acid, analysis was performed by injecting about 60 µl of sample extract into a C-18 column, 200 × 2.1 mm i.d., with phosphate/ACN (56:44) as the mobile phase at a flow rate of 0.5 mL/min, followed by fluorescence detection. Retention time was 4 min, and total analysis time was 8 min. Detection limit was 1.6 ng/ml.

Kuhlenkamp et al.[65] established two forms of human liver cytosolic tocopherol binding protein (TBP), with a molecular weight of 36.6 kDa. Gel filtration of hepatic cyctosol was subjected to Affi-Gel Blue affinity chromatography. After further purification with FPLC, TBP fraction was eluted at pH 5.9, and further purification by microbore LC showed two closely eluting peaks: two forms of TBP. Microcolumn analysis was performed by using a C-18 column, 150 × 2.1 mm. i.d., with gradient elution using TEA/ACN at 75 µl/min. Total analysis time was 60 min. The two forms of TBP reacted with polyclonal rabbit anti-rat TBP. Identification of TBP would be useful for future physiological studies.

Cheng et al.[66] determined the concentration of serotonin, catecholamines, and metabolites in plasma of depressed patients treated with moclobemide and fluoxetine. After ultrafiltration, microbore LC analysis and a dual electrochemical detection. Detection limits were 0.2 to 0.5 pg per injection. The authors advocated the use of the protocol for multiple blood collection and pharmacokinetic study.

Taylor et al.[67] demonstrated a sensitive and specific LC/MS assay of tacrolimus (FK 506) using a microbore C-18 column, 300 × 2.1 mm i.d., and MeOH/acetate buffer (8:2) as the mobile phase at 100 µl/min. A solid-phase extraction with 1 ml of whole blood was performed with a C-18 cartridge, followed by washing with water, MeOH/water and then heptane. Elution was performed with heptane/isopropyl alcohol. After evaporation and reconstitution with the mobile phase, 100 µl was injected for LC/MS analysis. Twenty samples were extracted in 1 h. The mass spectrometer was an atmospheric pressure ionization triple quadrupole instrument with a pneumatically assisted electrospray. The product ion of 768.4 was detected from the third quadrupole. Analysis time was only 4 min. The assay was linear from 0.2 to 100 µg/l. In a comparison study for 175 specimens, the results of this protocol were highly correlated to an enzyme-linked immunosorbent assay (ELISA) method. Overestimation by the ELISA method was attributed to metabolites cross-reactivity with the murine monoclonal antitacrolimus antibody.

Yatscoff et al.[68] described an HPLC assay of rapamycin, a new immunosuppressant, by extraction 5 ml of whole blood with diethyl ether. The supernatant was transferred, evaporated, and reconstituted with the mobile phase. This was subjected to a hexane wash, and 100 µl was injected into a 5 µm, C-8 column, 250 × 2.1 mm i.d., with water and methanol as the mobile phase. Analysis parameters were: flow rate, 0.35 ml/min., temperature, 45°C., and detection at 278 nm. Retention time of rapamycin was 30 min and total analysis time, 35 min. Detection limit was 1ng/ml.

IV. CONCLUSIONS

Recent advances in HPLC software, hardware-column technologies, and integrated sample preparation have greatly enhanced the current and potential applications of HPLC. Automation/DSA is gaining acceptance in clinical toxicology laboratories, offering greater efficiency and minimized personnel exposure for handling potentially infectious samples, almost at the same level as in pharmaceutical research and development laboratories. Their applications were directed to the currently used therapeutic and illicit drugs, for which immunoassays may not be available. Furthermore, novel applications have been demonstrated for newer drugs. Automated HPLC for PCR products beckons the potential application in DNA and other forensic analysis, whereas the use of automated HPLC for 2.3 million hemoglobinopathies screening certainly affirmed a most important application in biomedical sciences. Future areas of improvement would most likely include faster robotic sample preparation, interfacing with supercritical fluid extraction, integrated automation and patient data transfer downstream to the laboratory information system and subsequently to the hospital information system, and increasingly "friendly" software. These innovations also apply to other chromatography: GC, GC/MS, and CEC. The other major advances in microcolumn are used on a practical level without major "retooling" of the chromatography. Advantages include enhanced sensitivity for limited sample-size application in neonatal drug monitoring, and biotechnology analysis, solvent and waste reduction, and ready interfacing with MS, especially for capillary HPLC. With the aforementioned technologies, the analysts in the clinical toxicology and TDM laboratories are well equipped to advance the frontiers in collaboration with colleagues in other clinical specialties.

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Chapter 10

COUPLED MASS SPECTROMETIC—CHROMATOGRAPHIC SYSTEMS

Larry D. Bowers

CONTENTS

I. INTRODUCTION

Mass spectrometry (MS) continues to play an important role in toxicological analyses. It has been used in two modes: as a means of increasing confidence in the identity of an unknown agent and as a means of achieving selective, trace-level quantitation. The structural information content of the electronic ionization spectrum has been widely recognized, and refinement of library searching routines,[1] such as probability-based matching, dot-product, and Euclidean distance, as well as the continued expansion of libraries of spectra, has improved the toxicologist's ability to match unknown to library spectra. At the same time, several spectral interpretation programs, such as STIRS, can be of assistance in interpreting unknown spectra. Other computer-based tools allow the prediction of fragmentation patterns or allow rapid screening of possible fragment ions. All of these advances allow the toxicologist to present conclusions in new and better ways.

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Similar breakthroughs in the instrumental approaches to produce and analyze ions have greatly increased the ability of the toxicologist to obtain mass spectral information about unknown compounds. This proliferation of techniques makes selection of the correct mass spectrometric "tool" a difficult task. A complete review of MS, or even xenobiotic-related analyses using MS, is beyond the scope of this chapter. I will summarize the fundamental advances in coupling column chromatographic systems to mass spectrometers that impact clinical and forensic toxicology. Examples extracted from the literature serve to illustrate the strength, or limitations, of various techniques. Although this chapter will focus on developments in on-line interfacing of column chromatographic techniques, thin-layer chromatography (TLC) can also be interfaced to a mass spectrometer.[2,3] Given the recent advances in instrumental high-performance TLC,[4] it is possible that, in the future, applications of planar chromatography-MS may find their way into the toxicology laboratory.

II. INSTRUMENTATION

The quadrupole mass filter (QMF) has become a common tool in almost all toxicology laboratories. Although gas chromatography (GC)/MS has matured, developments that improve detection limits and cost-effectiveness have been plentiful. Refinements in technology, such as the introduction of monolithic filters by Hewlett–Packard in the 5971 and 5972 Mass Selective Detector (MSD), have resulted in the relatively inexpensive production of very reliable bench-top units. Continued improvements in production techniques have given rise to inexpensive limited function detectors, such as the Hewlett–Packard GCD. Competition has resulted in "research-grade" features—such as positive chemical ionization (PCI) and negative chemical ionization (NCI), improved mass resolution, and even MS/MS—being available on instruments costing less than $60,000. In the future, MS instruments will need to accommodate even narrower "fast GC" peaks and broad mass scanning ranges for macromolecular applications.

Just as important have been the advances in ancillary technology. Improvements in GC injection port technology should allow injection of up to 25 μl of sample (Varian 1078 temperature programmable split/splitless injector), eliminate septum bleed (Merlin Microseal), and improve injector inertness (Restek Silcosteel coatings). The development of new, low-bleed capillary GC stationary phases using polysilphenylene-siloxane bonding chemistries (J&W DB-5ms and XLB and SGE BPx series) has resulted in lower background currents. Improved detector performance of channel electron multipliers (Galileo 5770/5778 and K&M Ceramic CEMs), active film multipliers (ETP Scientific), and high-energy dynodes have reduced background noise and drift, improved signal amplification, and increased detector life. This list of examples is not meant to be comprehensive, but serves to illustrate the importance of instrument refinements on the toxicologist's ability to detect lower concentrations of toxins. An excellent overview of some important factors in improving the detection limits in GC/MS has been published.[5,6] In addition to these instrumental "refinements," exciting developments in both mass isolation/manipulation techniques and interface technology continue to appear.

A. QUADRUPOLE ION TRAP (QIT)

Paul and Steinwedel[7] described both the QMF and the QIT (Figure 10–1) in work published in 1953. Although QMF devices were rapidly assimilated into analytical laboratories, quadrupole ion storage devices were relegated to a relatively minor research role. (For more details about the theory and practical applications of the QIT, see March and Hughes[8] and March and Todd.[9,10]) Interest in QITs increased with the reports by Stafford et al. that mass spectra could be obtained using a radiofrequency (RF)-only storage and mass scanning technique[11], and that sensitivity and mass resolution were improved when helium was used as a bath gas.[12] The potential use as a GC detector was obvious. As originally implemented by Finnegan in the ITD700 and ITD800, the GC effluent entered the QIT, and compounds were ionized *in situ* by passing an electron beam through the trap.

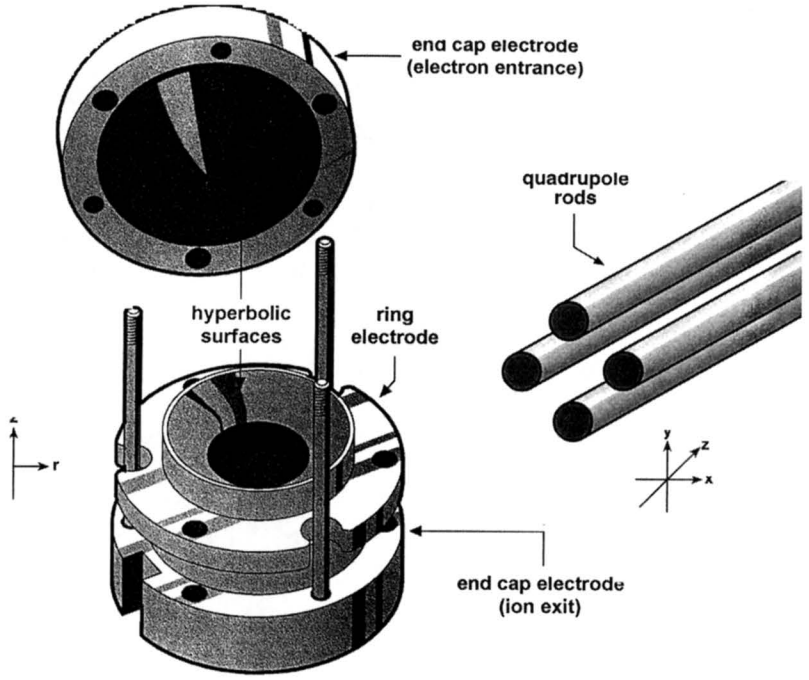


FIGURE 10–1. Schematic diagram of a QIT (left) and a QMF (right). The similarity of operation of the QIT and QMF can be envisioned by passing an axis through the y axis of the QMF and rotating the rods to form a solid "ring" and two "end cap" electrodes. In the QMF, direct current (DC) and radiofrequency (RF) voltages are applied to opposite rods to produce an electrostatic field that provides stable oscillations to ions with a specific *m/z* and unstable oscillations for all others. In the QIT, an RF voltage is applied to the ring electrode to produce a three-dimensional electrostatic field for trappng all ions. When the RF voltage is ramped, ions are ejected in order of increasing *m/z*. (From Varian Associates, Palo Alto, CA, 1996. With permission).

At high ion concentrations in the trap, the ion cloud distorted the applied fields and resulted in mass misassignment. This problem, called space charge, was addressed by a feedback-controlled attenuation of the ionization time known as automatic gain control (AGC). Further improvements in mass resolution and detection limits were achieved by application of a voltage to the end-cap electrodes during scanning (axial modulation). Because of their ability to store ions and thus potentially lower the limits of detection, particularly for the analysis of dilute protein solutions, ion trap technology has been the subject of increased interest in the MS community.

From the perspective of analytical toxicology, QITs present several appealing advantages. In addition to the increased sensitivity inherent in a storage device, the ability to store and manipulate ions has begun to translate into inexpensive MS/MS instrumentation and better control over ionization techniques. Because operation of the QIT is controlled by the specific waveforms applied to the ring electrode and end caps, it is, in theory, possible to have a single instrument that incorporates computer-selectable high mass resolution,[13–15] rapid scanning, and MS/MS and MS/MS/MS functions. Improved commercial GC/MS instruments with changes in hardware design (Finnegan GCQ) and software (Tool Kit for MS/MS, Varian Saturn III), two liquid chromatography (LC)/MS instruments (Finnegan LCQ, Bruker Esquire), and a glow discharge instrument (Teledyne Electronic Technologies 3DQ) based on QIT technology have been introduced to the market in the last 3 years. The Bruker Esquire is particularly intriguing, with a mass resolution of 0.2 units and a mass-to-charge (*m/z*) range of 6000.

Although QITs have many potential advantages, ion–molecule reactions during the ion storage period do present problems. These self-induced chemical ionization (CI) reactions frequently result

in excessive $[M+1]^+/M^+$ ratios for compounds containing nitrogen or oxygen. The abundance of the $(M+1)^+$ ion at *m/z* 304 in the QIT spectrum of cocaine (Figure 10–2A) would make calculation of the molecular formula, as required for manual interpretation[16] of unknown spectra, difficult. The increased abundance does not, however, preclude spectral matching, especially if the reference spectra are obtained on an ion trap or if the matching criteria in the searching algorithms are modified to take into account the unusual features of QIT spectra. Similar problems were encountered in the early days of QMF use when comparing spectra with those obtained on magnetic sector instruments, but these problems have been resolved. With the introduction of the GCQ, Finnegan has attempted to solve the problem of "nonclassical spectra" through the use of an external ion source (Figure 10–2B).

Wu et al.[17] concluded that the ion trap mass spectrometer permitted full-scan identification of derivatized amphetamine and metamphetamine, codeine and morphine, 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol, benzoylecgonine (BE), and phencyclidine while maintaining analytical limits of quantitation below the National Institute of Drug Abuse guidelines. The use of selected ion monitoring (SIM) yielded detection limits at least as good as QMFs. Our experience with the QIT suggests that its operation requires systematic exploration of a number of parameters that can affect

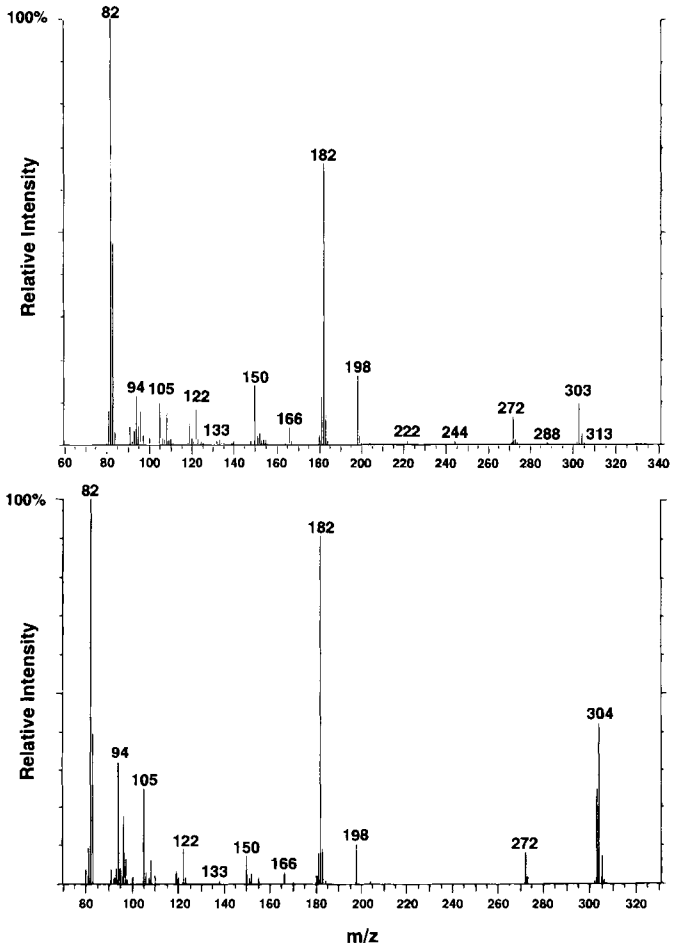


FIGURE 10–2. Mass spectrum of cocaine. Spectrum (top) obtained from the original QIT (Finnegan ITD40) with *in situ* ionization. Note prominent (M+H)+ peak formed by proton exchange reactions in the trap. Spectrum (bottom) obtained from QIT with external ionization source (Finnegan GCQ). (From Cairns T., unpublished data, 1996).

the quantitative and qualitative nature of the results. It is difficult to use expertise obtained from either QMF or magnetic sector mass analyzers to aid in the selection of the correct experimental conditions for the QIT. Subtle differences in waveforms can result in substantial differences in performance. With the proprietary nature of some of the waveforms, direct comparison of various trap technologies may be difficult.

1. Selective Ion Storage (SIS)

As previously mentioned, one of the limitations of the QIT is that a limited number of ions can be present in the trap. Although AGC controls the number of ions in the trap, this is a nondiscriminating process that decreases all ions present in the GC effluent. It would be much more effective to store only the ions of interest and selectively eject ions, such as those from column bleed, that do not provide analytical information. Because the operation of the QIT is controlled by the waveforms applied to the ring and end-cap electrodes, selective storage (or ejection) of ions in the ion trap can be accomplished by several methods, including swept frequency techniques; broad band excitation, such as specific stored waveform inverse Fourier transforms,[18] filtered noise,[19] field-modulated selective ejection,[20] and multistep isolation routines.[21]

One of the potential advantages of the QIT occurs when multiple ions are monitored in the SIM mode. Because the QMF must sequentially focus the ions of interest on the detector, the fraction of the time spent on any one ion is small as the number of ions monitored increases. Because multiple ion ranges can be stored simultaneously in the QIT (Varian Selected Ion Storage),[22,23] an improvement in signal-to-noise ratio would be expected. As shown in Figure 10–3, this is observed with a crossover at about four ions stored. Other methods of selectively storing ions, such as alternate scan selective storage, may not achieve this duty cycle enhancement.

The importance of ion storage can be further illustrated by the report that, although the QIT had superior sensitivity in the scan mode for several derivatives of the anabolic steroid nandrolone, a QMF had lower limits of detection in the SIM mode when the drug was isolated from a urine

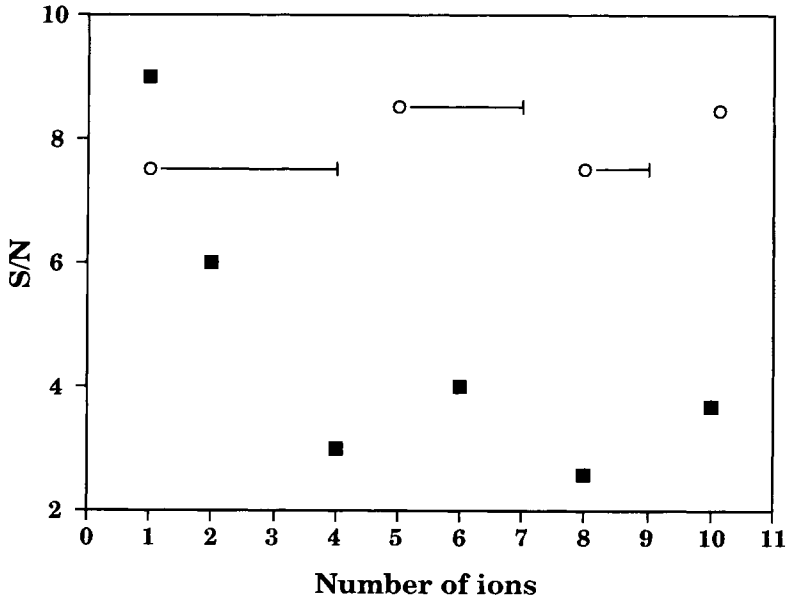


FIGURE 10–3. Comparison of the QMF and QIT in the selected-ion isolation mode. A varying number of ions from the isotropic clusters were collected after injection of 25 pg tetrachlorobenzene (TCB). Signal-to-noise ratio (S/N) for the *m/z* 216 ion was computed as a function of the number of ions observed in the SIM or SIS collection. The horizontal lines are used to indicate that no changes in conditions was required in the ion trap to observe the ions in the isotropic cluster. Dwell times on the OMF were adjusted to acquire 12–19 data points across the peak. (From Bowers, L.D. and Borts, D.J., unpublished data, 1996.)

matrix.[24] We were able to confirm this observation. Using the same hardware, but with updated software for ion storage, however, we were able to demonstrate that the limits of detection for the same compounds are at least 10-fold better on the QIT relative to the QMF when selected ion storage was used. The difference is caused by the elimination of matrix background ions in the SIS mode, which allows the QIT to be filled with the ions of interest.

a. Chemical Ionization

The QIT has some unique advantages in CI relative to normal QMF techniques. When methane is used as a reagent gas, both CH_5^+ and $C_2H_5^+$ are produced in approximately equal amounts. The $C_2H_5^+$ can participate in both proton exchange and hydride abstraction reactions.[25] Because the QIT can store or eject ions, selective storage of the CH_5^+ is, in principle, readily accomplished, simplifying the CI reaction chemistry. In addition to the selective storage of CI species, the relatively long reaction times available in the QIT relative to the dynamics of CI in an external source allow the use of very low concentrations of CI reagent gas. This, in turn, allows the use of less volatile compounds, as CI reagents. For example, acetonitrile (CH_4CN^+) can be used as the CI reagent for cocaine (Figure 10–4). A vial of liquid acetonitrile is attached to the vacuum system of the instrument, and sufficient reagent gas is available to produce analytically useful ion currents. Greater fragmentation is sometimes observed for CI on the QIT because of internal energy acquired from collisions with the bath gas. This could potentially be a positive finding for identification of drugs using CI, although no systematic studies have been done. Furthermore, electron capture negative CI cannot be conducted in an internal ionization QIT because of the elimination of the thermal electrons by the RF field. Selective storage of hydride abstraction systems may overcome this limitation.

2. Tandem Mass Spectrometry

The ability to isolate a molecular species selectively on the basis of *m/z* ratio and subsequently fragment it was a major advance in MS. Since the description of the RF-only quadrupole collision cell by Yost and Enke in 1972, MS/MS has played a significant role in trace-level detection.[26] In

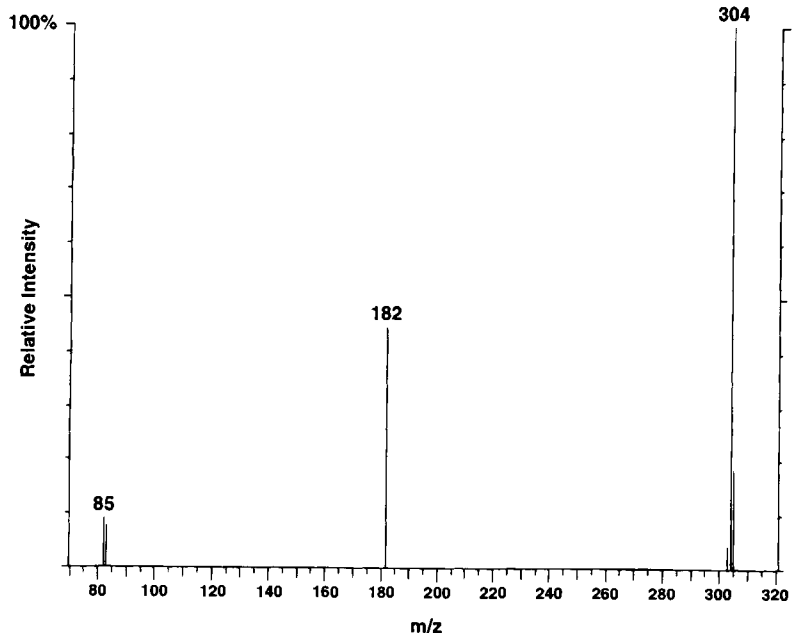


FIGURE 10–4. Chemical ionization mass spectrum of cocaine using acetonitrile as the reagent gas on an ion trap mass spectrometer. (From Bowers, L. D., unpublished data, 1996.)

MS/MS, an ion of interest is isolated in the mass analyzer and provided sufficient internal energy through collisions with a reagent gas to fragment bonds. The product ions are scanned to obtain structural information. A technique analogous to SIM, called selected reaction monitoring (SRM), can be used to improve detection limits for quantitation. The combination of chromatographic retention time, precursor ion isolation, and collision-induced dissociation (CID) to specific product ions results in a noise-free, and therefore sensitive, analytical tool. Some have suggested that MS/MS is as effective as high-resolution MS when very low limits of detection are required, but little hard data comparing the two techniques are available. In environmental testing of dioxans, a tandem QMF instrument had limits of detection a factor of 5 higher than a high-resolution MS operated with a resolution of 10,000. The criteria for compound identification were ±25% for ion ratios, although no consensus on the requirements for ion ratio precision has been achieved for MS/MS. Despite the power of the mass spectrometer, there are circumstances, such as the quantitation of the isobaric native and ring-open forms of rapamycin, wherein chromatographic separation is crucial. Although advances continue to be made in QMF MS/MS,[27] some of the most interesting advances are the result of the application of QIT technology.

a. Resonant Excitation

Ions trapped in the QIT oscillate at a secular frequency determined by the frequency and amplitude of the RF voltage applied to the ring electrode, the radius of the trap, and the mass of the ion. If an oscillating voltage in resonance with the secular frequency of a particular mass ion is applied to the end caps, those ions will absorb the energy most efficiently. The increase in ion energy results in more energetic collisions with the bath gas and, when sufficient energy is present, results in CID. The product ions can then be trapped and the MS/MS spectrum scanned. With knowledge of the QIT operating characteristics, the secular frequency of an ion of any mass can be calculated, and the appropriate selective excitation frequency applied. This is known as *resonant excitation.*

From a practical perspective, space charging and nonideality of the trap geometry can result in slight shifts in the secular frequencies. The calculated frequency would thus be different than the actual frequency, resulting in inefficient excitation. Several approaches have been described to deal with this problem.[28–31] Multifrequency irradiation applies several frequencies over a 1 to 2 KHz range to the end-cap electrodes to excite a band of masses and thus ensure that any frequency shifts are taken into account. Another approach is to modulate the RF voltage applied to the ring electrode that, in turn, varies the secular frequency of the ion so that it will correspond to the calculated frequency. The result of either of these approaches is to increase the conversion efficiency observed for CID.

b. Nonresonant Excitation

An alternative approach to the resonant excitation process is to isolate the ion of interest selectively, then excite all ions in the trap. This can be done by modulating the trapping voltage, which moves all ions away from the center of the trap.[32] When the voltage is removed, the ions rapidly relax to the center of the trap. Collisions occurring with the bath gas convert the kinetic energy into internal energy and can result in CID. This process is repeated for a number of cycles. One unique feature of nonresonant excitation is that product ions formed in the first cycle will be excited in the subsequent cycles and may undergo secondary CID. The benefit (or detriment) of this byproduct of the nonresonant excitation process has not been established. In Figure 10–5A, the precursor ion, which was the molecular ion (*m/z* 432) of the di-trimethylsilyl (TMS) derivative of testosterone, was isolated and fragmented to the product ions shown. Because the product ions remain in the QIT, it is relatively easy to repeat the CID process with good efficiency, resulting in an MS/MS/MS (MS^3) spectrum. In Figure 10–5B, the molecular ion was isolated and fragmented, and the $(M\text{-}15)^+$ product ion was isolated and subjected to CID conditions, again resulting in the spectrum shown. A commercial instrument (Finnegan LCQ) advertises the ability to perform MS.[10] The analytical utility of sequential fragementation beyond MS/MS/MS has yet to be determined clearly.

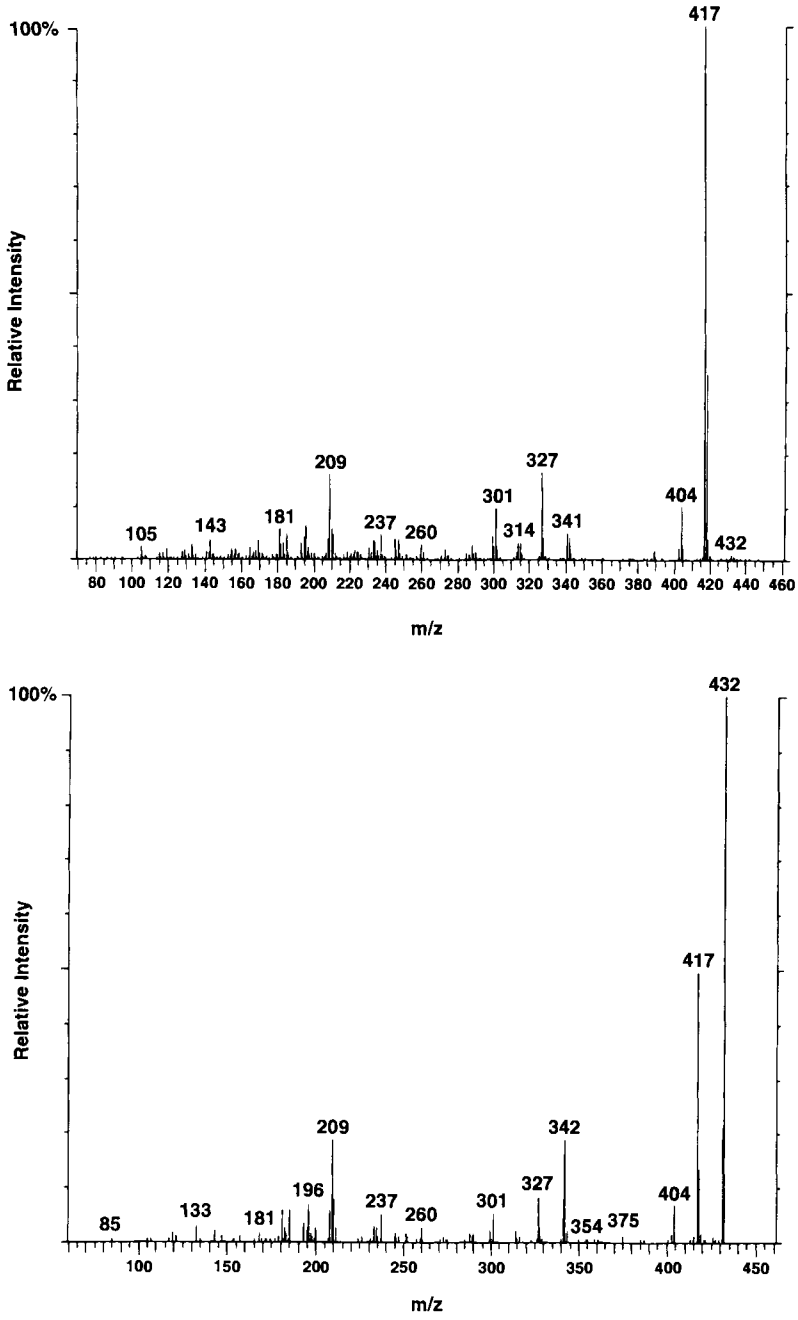


FIGURE 10–5. MS^2 (top) and MS^3 (bottom) spectra of testosterone. In the MS/MS spectrum, the molecular ion (*m/z* 432) was isolated and fragmented to the product ions shown. In the MS/MS/MS spectrum, the molecular ion was isolated and fragmented, and the $(M\text{-}15)^+$ ion was isolated and subjected to CID conditions, again resulting in the spectrum shown.

B. LIQUID INTRODUCTION INTERFACES

Although it has been 22 years since the first direct coupling of a liquid chromatographic effluent to a mass spectrometer,[33] most of the advances have taken place within the last decade.[34] It has become clear that no single liquid interface will solve all analytical problems. Thus, the development of interfaces that can handle liquid samples has been one of the most dynamic areas in MS.

Thermospray is generally recognized as the first successful high-performance LC (HPLC)/MS interface, although it has been overshadowed by recent developments. Continuous flow-fast atom bombardment (CF-FAB) has also been used successfully, especially for introduction of macromolecular species at very low flow rates.

1. Atmospheric Pressure Chemical Ionization (APCI)

Horning et al.[35] first described APCI in 1973. In the positive-ion mode, electrons from an electron source impact the components of ambient air, which in turn form $H_3O^+[H_2O]_n$ ions. The source of electrons for ionization was originally ^{63}Ni, but has generally been a corona discharge generated by a direct current.[36] In the negative-ion mode, an electron capture mechanism by electronegative species, such as oxygen, results in formation of the superoxide ion, $O_2^-[H_2O]_n$. When used as a liquid interface, the effluent from a chromatographic system is desolvated. The resulting gas phase molecules are ionized by proton transfer or charge transfer reactions that occur between the reagent ion and the compound of interest. Because the ion produced from this reaction may contain solvent ions, the interface between the atmospheric ionization source and the mass spectrometer (Figure 10–6) must have a means of declustering the ions formed.

Most commercial instruments now offer APCI interfaces. In many cases, it is possible to detect compounds at the picogram level. More recently, low-power microwave-induced plasmas,[37] glow discharges,[38] and RF plasmas[39] have been used to achieve higher electron densities that result in limits of detection in the femtogram range and linear dynamic ranges of 10^4 in concentration.

The advantage of APCI to the toxicologist lies in the area of ionization of neutral molecules, such as steroids and relatively polar, low-molecular weight species. For these molecules, APCI produces mainly protonated molecular ions that can be monitored directly or fragmented either in the interface region or in a collision cell (SRM). The majority of applications of APCI have been for selective quantitative analysis, because there are no libraries available for matching MS/MS spectra, nor are there clearly delineated rules for interpretation that exist for electron ionization (EI). Henion et al.[36] described a robust "heated pneumatic nebulizer" that allowed direct coupling of standard HPLC columns to a mass spectrometer. A co-axial stream of air assists in the nebulization of the the HPLC effluent, and the solvent is removed during passage through a large diameter, heated quartz tube. The ions are transported through the interface region (Figure 10–6), wherein declustering of the solvent molecules occurs, and into the mass spectrometer. This approach has been used

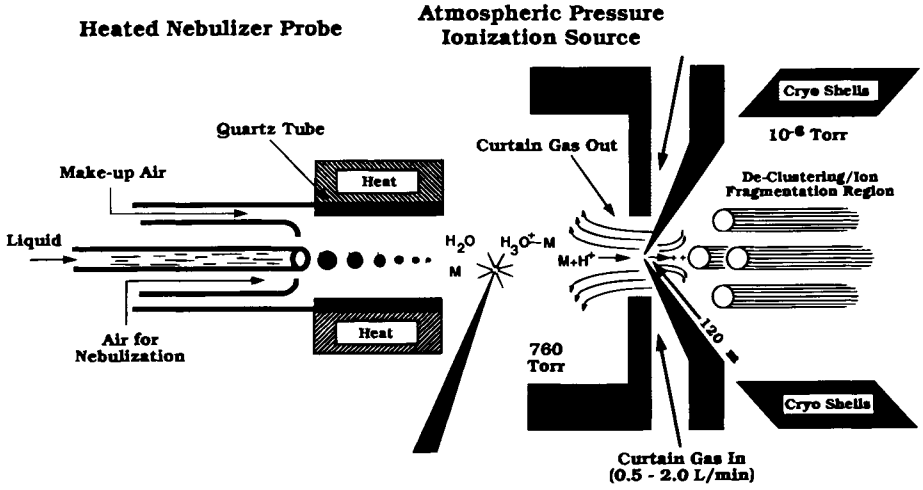


FIGURE 10–6. Schematic diagram of APCI interface used in the Sciex API III^{Plus} HPLC/MS/MS system. (From PE Sciex, 1996. With permission.)

routinely for analysis of hundreds of samples per day in the pharmaceutical industry for pharmacokinetic studies and metabolite identification. APCI is a useful adjunct to electrospray (ES) ionization for addressing a wide spectrum of problems.

2. Particle Beam Interface (PBI)

Willoughby and Browner[40] originally developed the PBI as a means to obtain a monodisperse aerosol to improve the transport efficiency of molecules from the liquid to a gas state. The original design used pneumatic nebulization, followed by two stages of momentum separation to remove the solvent so that the molecules of interest are delivered to a high-vacuum mass spectrometer source. Later designs used ultrasonic- or heat-assisted (Figure 10–7) nebulization to facilitate the use of mobile phases with large mole fractions of water. The PBI remains the only liquid interface that can produce traditional EI spectra that can be searched with standard libraries. Recent modifications of the interface can obtain full-scan spectra on 50 to 100 ng quantities of analyte, which are comparable with bench-top GC/MSDs. It has been reported that the mobile phase composition, interface design, presence of co-eluting "carrier" substances, and the concentration and volatility of the compound of interest all affect the quantitative nature of the PBI.[41,42] The recent finding that electrostatic charging during the nebulization process decreases transmission to the mass spectrometer should further improve limits of detection.[43] Although a number of manufacturers have offered PBIs, the Waters Integrity HPLC-diode array spectrophotometer-mass spectrometer instrument offers the first integrated approach to identification of compounds after HPLC separation. The most frequent use of PBI/MS has been in the environmental and residue analysis areas, wherein identification, as well as quantitation, is required.

The fact that the compounds of interest are transported in the gas phase into an ion source allows the application of both PCI and NCI techniques. This can substantially reduce the detection limits for quantitative studies. Christians et al.[44] reported limits of detection and quantitation of 25 and 250 pg for NCI of the immunosuppressive agent tacrolimus and its metabolites. A number of studies using negative ionization PBI/MS to detect antibiotics and other residues in meat and milk have been reported.

3. Electrospray

The emergence of ES and matrix-assisted laser desorption ionization has revolutionized the use of MS in the analysis of biological macromolecules. Whitehouse et al.[45] first described an

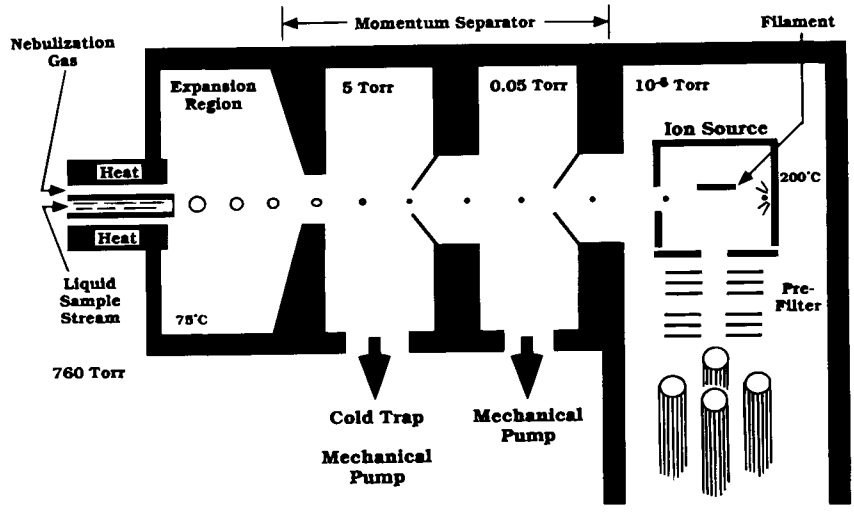


FIGURE 10–7. Schematic diagram of a PBI.

HPLC/MS interface based on ES as a result of their interest in the pioneering work of Dole. Nebulization of the liquid in ES occurs because of the imposition of a large (3 to 5000 V) electrostatic field on a metal capillary containing the HPLC effluent. The partial charge separation between the liquid and the metal tube results in instability in the liquid, which in turn results in the expulsion of a series of charged droplets from a Taylor cone that forms at the tip of the metal capillary. The droplets evaporate as they drift through an atmospheric pressure region, expelling smaller droplets as the charge-to-volume ratio exceeds the Raleigh instability limit. The mechanism of ion formation is still under debate, but surface desorption of the ion from the highly charged droplet surface seems to be gaining support over the solid desorption model.[46,47] The protonated molecular ions produced, which may be associated with solvent molecules, are then declustered and transported to the mass spectrometer (Figure 10–8).[48]

The revolutionary aspect of ES is that multiply charged ions could be efficiently formed from polymers and biological molecules with masses greater than 100,000 Da. If 20 charges exist on a molecule of mass 10,000, the *m/z* would be 500, well within the *m/z* range of quadrupole mass analyzers. The fact that a charge distribution is generally observed on these macromolecules gives the analyst multiple estimates of its molecular mass, resulting in mass estimates with precision better than 1%. Thus, it is easy to detect the difference between recombinant human growth hormone and native growth hormone (molecular weight = 22,120 vs. 22,260), which differ by a single methionine residue or the microheterogeneity of glycopeptides differing by a single monosaccharide. Although the analysis of proteins provides new opportunities for the toxicologist in understanding mechanisms of toxicity, the major impact of ES is in the efficient, gentle ionization of labile small molecules. Because ion formation occurs from the liquid state, the best response is observed for ionic compounds. Adjustment of the solution pH can produce positive ions for carboxylates and even sulfates. It is not unusual to observe adducts with ammonia, sodium, or potassium if these ions are present in the mobile phase.

In its original form, ES was limited to flow rates of less than 10 µl/min and was relatively inefficient at producing ions from solutions with high conductivity or high surface tension. Bruins et al.[49] introduced an HPLC/MS interface (Ion Spray™), which included pneumatically assisted nebulization that allowed introduction of liquid flow rates of up to 200 µl/min, with an optimum of about 50 µl/min. Development of a subsequent liquid shield allowed direct spraying of liquids at rates of 1 to 2 ml/min, although most of the liquid condenses on the shield and is discarded; in effect, a gas phase "split" introduction.[50] Other modifications of the ES interface have diminished some of

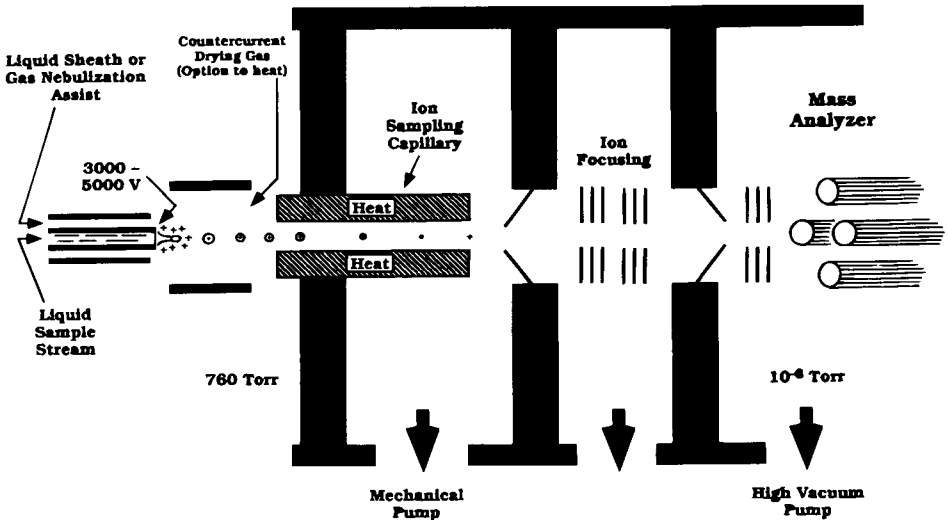


FIGURE 10–8. Schematic diagram of an ESI source.

its original limitations.[51,52] Interestingly, a decrease in flow rate does not result in the expected diminution of response, probably because of an increase in ionization efficiency. As a result, the liquid flow can be split and two detectors used in parallel with little or no loss in sensitivity. Hopfgartner et al.[50] found the ion spray MS interface to be a mass flow sensitive detector, indicating that, for their interface, the chromatographic peak height is dependent on flow rate, whereas peak area is much less so. Careful characterization of most interface designs with respect to their effect on quantitative chromatographic data are not available.

III. APPLICATIONS IN GC/MS

The primary reason for the popularity of GC/MS in drugs of abuse and forensic testing is its widespread acceptance as a definitive identification technique by the legal community.[53] The application of SIM to improve limits of detection and ion ratio standards of ±10%[54] or ±20%[55] for compound identification has been widely accepted. The availability of a GC/MS instrument is not a guarantee of success, however, because subtle problems can arise. Variations in the ion ratios for the pentafluoropropyl derivative of BE have been observed over a 3-month period to have a coefficient of variation of 50%.[56] Joern[57] has suggested that ion ratio variability is related to laboratory environment factors, such as temperature control. Ionization efficiency can also be affected by co-eluting species, as observed for the TMS derivative of BE in the presence of a 20-fold excess of fluconazole.[58] Although both of these observations were made on the Hewlett–Packard 5970B MSD, source design on other instruments may either increase or decrease the severity of the problem. These cases are the exception, and ion ratios within 20% of a standard in the same run can be routinely obtained. Nevertheless, the consistency of the ionization and fragmentation processes should not be taken for granted. Having only SIM data, therefore, can be misleading and acquisition of a spectrum to rule out the presence of interfering substances is highly recommended.

The use of a deuterium-labeled internal standard can compensate for variations in the ionization and fragmentation process. Bodor et al.[56] noted that correction of the ion ratios with those from a co-eluting deuterated internal standard reduced the variability to less than 15%.[57] Wu et al.[58] observed a consistent ratio for d_0 and d_3 quantitation ions for BE, even though the absolute signal intensity was reduced 5-fold by co-eluting fluconazole. Simply using a deuterated internal standard, however, does not guarantee success. The use of silylated derivatives in conjunction with an internal standard containing three (or fewer) deuterium atoms can result in nonlinearity of the assay at high concentration, because the stable isotopes of silicon in the unlabeled compound ($^{30}Si^{13}C$) can contribute to the internal standard signal.[59] The reported conversion of several derivatives of ephedrine and pseudoephedrine to methamphetamine in the temperature port under excessive heat also will not be detected by stable isotope internal standards.[60]

Although many laboratories question the cost-effectiveness of GC/MS confirmation of drug screens in clinical or emergency room toxicology, increasing numbers of test results from clinical laboratories are being used in the legal arena. Drug test results on meconium, for example, may be used by individuals who may not appreciate the need for screening and confimatory tests. A second factor in the decision to use GC/MS screening for emergency room toxicology is the trend among immunoassay kit manufacturers toward specific assays targeted for workplace drug testing. The use of an immunoassay specific for *S*-(+)-amphetamine/methamphetamine precludes detection of other stimulants or their metabolites, such as ephedrine and phenylpropanolamine arising from ingestion of methcathinone. Workplace testing in the hospital setting should involve more, for example, than simply testing for the standard opiates morphine and codeine, because the intelligent user with access to prescription drugs can simply switch to another opiate. It is important for the toxicologist to tailor the program to the needs of the client organization, and the availability of mass spectrometric methods makes customization possible. Although instrumentation is becoming less expensive, it still requires significant expertise to operate and maintain a GC/MS system. In the present climate of health care economics, the lack of expertise may be the limiting factor in making this

valuable technology available in the clinical setting. Nevertheless, competent mass spectrometric analysis should be required of any laboratory involved in testing that may result in legal action.

A. DERIVATIZATION CHEMISTRY

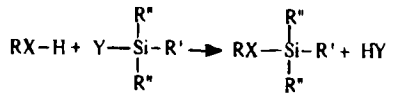
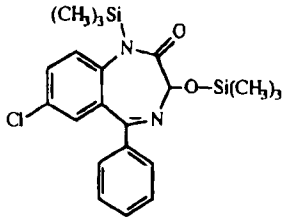
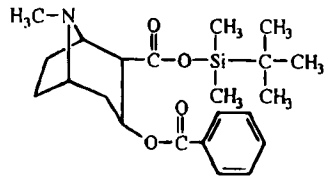
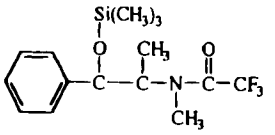
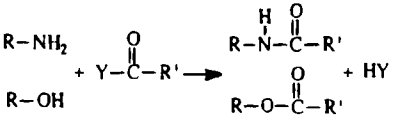
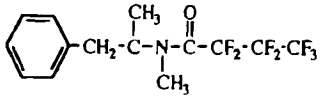
Derivatization techniques remain an important aspect of xenobiotic detection by GC/MS. Because many drug molecules are not volatile, formation of a derivative can improve chromatographic performance and may either improve or degrade the information content of the mass spectrum. For example, the formation of TMS derivatives frequently decreases the presence of structurally informative ion in favor of losses of methyl and silyl groups. Three excellent books on derivatization provide both theoretical background and examples of derivatization reactions.[61–63] Several useful derivatizing reagents and reactions are listed in Table 10–1. (Refs. 64–70 are contained therein.)

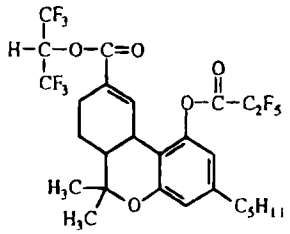
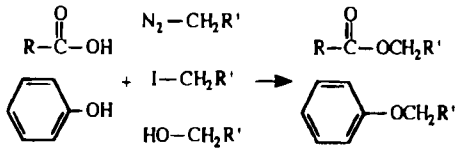
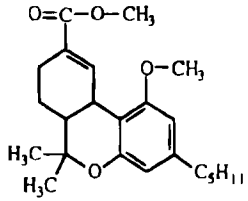
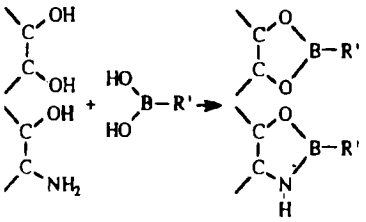
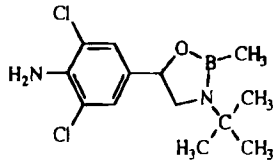
In an attempt to improve the efficiency of derivatization, Thompson and Dasgupta[71] used microwave heating to decrease derivatization times for trifluoroacetyl, pentafluoropropionyl, and heptafluorobutyryl derivatives of amphetamines to less than 6 min. It is important to consider the reaction solvent, as well as the derivatizing reagent, when conducting a derivatization. For example, Crouch et al.[66] reported the CI GC/MS analysis of cocaine metabolites, including norcocaine, after derivatization with *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide in ethyl acetate. Underivatized norcocaine was observed in the chromatogram. If the same reagent is used with acetonitrile as the solvent, MTBS derivatives of norcocaine, norcocaethylene, and norbenzoylecognine can be obtained.[72]

The separation of chiral compounds has become a more important topic in recent years. The differentiation of *S*-(+)-methamphetamine, a stimulant, from *R*-(–)-methamphetamine, a decongestant, (Figure 10–9) or dextrorphan (a metabolite of the antitussive dextromethorphan) from levorphanol, an opiate, is important for both workplace and athletic drug testing. In addition, metabolism of drugs such as selegiline to regulated substances such as *R*-(–)-amphetamine and *R*-(–)-methamphetamine can have serious consequences if not properly identified. Although chiral capillary GC columns are available, most methods for chiral separation in GC/MS involve chiral derivatization. The principle behind this separation is the reaction of the chiral compounds with an optically pure derivatizing reagent, which results in the formation of a diastereomer. Because of the two chiral centers, the three-dimensional structure of the derivatized compounds are sufficiently different to be separated on an achiral column. It is important to note that this approach requires that a derivatizable functional group is close to the chiral center (≤3 bonds), that both enantiomers react with the derivatizing agent at the same rate or achieve the same equilibrium, and that no differences in reaction occur as a result of different amounts of enantiomer in the reaction mixture. For example, the distance between the derivatizable function (—OH) and the chiral center precludes separation of dextrorphan and levorphanol by this approach. The three principal reagents used for chiral derivatization are listed in Table 10–1. Cody[70] has reviewed enantiomeric separations of methamphetamine and documented that *S*-(–)-*N*-(trifluoroacetyl)prolyl chloride is the most popular reagent. Early reports of difficulties with this reagent were the result of racemization of the derivatizing agent by triethylamine, which was used as a proton sink for the reaction products. LaBelle et al.[73] used (*R*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPA; Mosher's reagent) to resolve the enantiomers of methamphetamine, ephedrine, pseudoephedrine, and methcathinone. We have also used MTPA in conjunction with isothermal capillary GC/MS on a DB-17 column to resolve the enantiomers of amphetamine.

A relatively new application of GC/MS is the detection of metals after derivatization with a volatile organic ligand, such as acetylacetone, diethyldithiocarbamate, or bis(trifluoroethyl)dithiocarbamate. The advantages of GC/MS for metal analysis include the ability to speciate metals, the use of isotopic internal standards to improve accuracy and precision, and low limits of detection. The drawbacks of the technique include selection of a complexing agent with appropriate volatility and memory effects. Aggarwal et al.[74] have reported on the quantitation of arsenic, cadmium, cobalt,

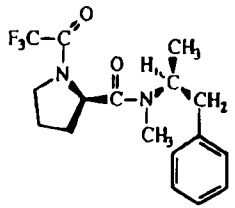
TABLE 10–1
Derivatization Reactions

Typical reaction	Derivatization reagents	Example derivatives	Comments
Silylation			
	BSTFA; BSA		BSTFA used to derivatize benzodiazepines before NCI GC/MS confirmation[64]
	MTBSTFA		Loss of *tert*-butyl group (I M-57 I +) gives intense ion in mass spectrum; amines much harder to derivatize than —OH; has been successfully used with amphetamine; BE[65]; tetrahydrocannabinol; steroids; useful for MS/MS precursor ion
	MSTFA		Sequential addition of MSTFA to silylate the OH group, followed by addition of MBTFA to acylate the NH function, gives a mixed derivative for *N, O* compounds like ephedrine[66]
Acylation			
	HFB anhydride		HFB derivatives of amphetamine/phenteramine and methamphetamine/ephedrine can be separated on a DB-5 5%-phenylmethylsilicon capillary column

	Pentafluoropropionyl anhydride and pentafluoropropanol		Use of pentafluoropropionyl anhydride and pentafluoropropanol gives acylation of—OH and alkylation of phenol to form the mixed derivative[67]
Alkylation			
	Alcohols; diazocompounds; organic iodides		Extractive alkylation with CH_3I in toluene with THAH catalyst provides single-step extraction and derivatization; THAH has to be removed before injection to achieve good detection limits and column life[68]
Cyclic boronation			
	Methylboronic acid; butylboronic acid; phenylboronic acid		Cyclic boronate derivative of clenbuterol gives intense ions with good structural information content;[69] other derivatives give rise to almost exclusively *m/z* 86 because of α-cleavage

(*Continued*)

TABLE 10–1 *Continued*

Typical reaction	Derivatization reagents	Example derivatives	Comments
Chiral Derivatizing Agents			
See acylating reactions above	LTPC; (–)-methyl chloroformate; MTPA		The TP derivative of *R*(–)- and *S*(+)-methamphetamine is the most widely used[70]

Note: Abbreviations used are: BSTFA, *N,O*-bis-TMS-trifluoroacetamide; BSA, *N,O*-bis-TMS-acetamide; MTBSTFA, *N*-methyl-*tert*-butyldimethylsilyl-trifluoroacetamide; MSTFA, *N*-methyl-TMS-trifluoroacetamide; MBTFA, *N*-methyl-bis(trifluorocetamide); HFB, heptafluorobutyryl; THAH, tetrahexylammonium hydroxide; LTPC, (–)-*N*-(trifluoroacetyl)prolyl chloride; MTPA, (–)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride; TPC, *N*-(Trifluoroacetyl)prolyl.

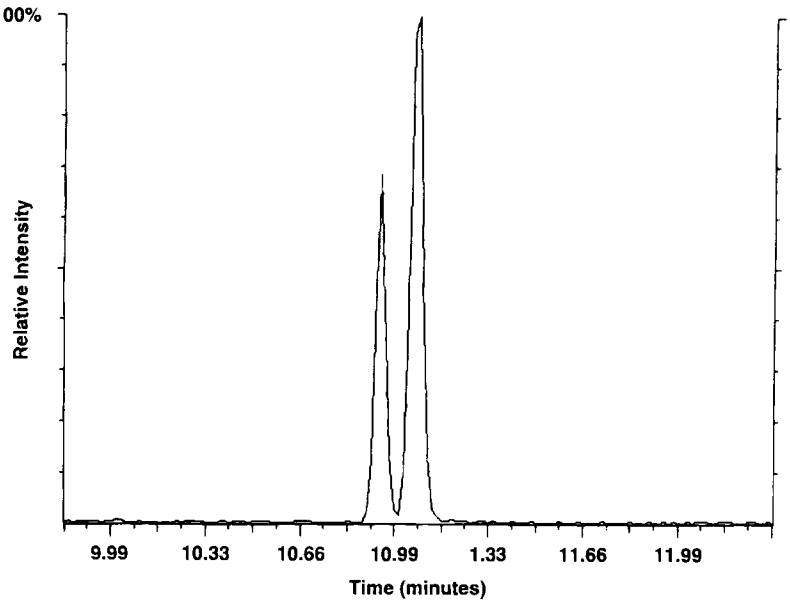


FIGURE 10–9. Chromatographic separation of *R*-(–) and *S*(+)-methamphetamine as the α-methoxy-α-(trifluoromethyl)phenylacetate derivatives.

chromium, copper, mercury, nickel, lead, platinum, selenium, tellurium, and zinc.[74] This group has reported on a GC/MS NCI method for blood lead using ^{204}Pb as an internal standard.[75] The lead is first extracted from blood as the pyrrolidine dithiocarbamate chelate, then derivatized with 4-fluorophenylmagnesium bromide to produce $Pb(C_6H_4F)_4$, which is then analyzed. The technique has a quantitation limit of 30 μg/l, well below the new 100 μg/l threshold for concern issued by the Centers for Disease Control, with precision less than 5% at 100 μg/l and good accuracy.

B. CHEMICAL IONIZATION

CI has become more widely used in the toxicology community to achieve lower limits of detection. Over 50 papers have appeared in the last 5 years using CI in pharmacokinetic and drug safety studies. The basic chemistry of CI has been reviewed by Harrison.[25] The design of the ion source is critical in achieving ultimate limits of detection, and some source designs compromise CI efficiency for simplicity in conversion from EI operation. In these cases, careful optimization of EI conditions can give detection limits similar to PCI caused by the inefficiency of the ionization process. Ishikawa et al.[76] found that the EI-SIM limits of detection for phenothiazines in plasma or urine were lower than either PCI or NCI. In general, however, NCI has shown improved detection limits. This seems to be caused by the decrease in background a result of the selective electron capture mechanism that gives rise to the ions observed. Papac and Foltz[77] were able to analyze LSD in urine or plasma by GC/NCI/MS after conversion to its *N*-trifluoroacetyl derivative. A linear response from 0.1 to 3.0 μg/l was observed in plasma. Fitzgerald et al.[64] selected NCI for analysis of the TMS derivatives of nordiazepam, oxazepam, temazepam, lorazepam, *N*-1-hydroxyethylflurazepam, α-hydroxyalprazolam, and α-hydroxytriazolam, because the limits of detection (50 μg/l) were up to several thousand times better than either PCI or EI.

As previously mentioned, the availability of improved commercial instrumentation has been a major factor behind the appearance of CI applications in the last 5 years. Shaw et al.[78] reported a factor of 6 improvement in limits of detection (80 ng/l) for the trifluoroacetate derivative of tetrahydrocannabinol from plasma by using a high-energy dynode in combination with negative ionization. De Jong et al.[79] reported 1 to 10 ng limits of detection for stimulants in urine using CI on a QIT, but

how much improvement might be achieved with selective storage algorithms that have appeared since this report is not clear.

C. ISOTOPE RATIO MS (IRMS)

The use of the specialized technique of IRMS requires the breakdown of organic compounds in the GC effluent to CO_2 or N_2 so that accurate measurement of ^{13}C, ^{15}N, or ^{18}O enrichment can be made. For ^{13}C, this has been done with an 850°C combustion interface containing copper and platinum, followed by removal of the H_2O produced, and passage of the CO_2 to the isotope ratio mass spectrometer.[80] The isotopic abundance of the unknown is then continuously compared with a reference gas via a dual inlet source and a magnetic sector MS with parallel detectors to improve the precision of the measurement. Preliminary data suggest that exogenous testosterone use can be detected by comparing the ^{13}C enrichment of testosterone to that of steroid precursor molecules.[81] The difficulty for applications to biological uses is the limit of detection. Browne et al.[82] report the detection limits of 0.46 to 2.62 μg/ml of $^{15}N_2$ or ^{15}C phenobarbital using a commercial interface. Using uniformly labeled ^{13}C-stearic acid, Goodman and Brenna[80] report precision of $^{13}C/^{12}C$ ratios of 0.001 with 30 pg of fatty acid. Note that detection of isotope enrichment is a more difficult problem than detection of a stable isotope-labeled drug.

Abramson and Coworkers[84–86] have advanced the use of a microwave plasma to provide a chemical reaction interface mass spectrometer technique that can detect ^{2}H-, ^{13}C-, ^{14}C-, ^{15}N-, S-, P-, Cl-, Se-, and Br-containing compounds. They have reported an interface to a bench-top MSD[84] and to an HPLC/CRIMS system.[85,86] Detection limits have been improved to 60 pg of $^{2}H_8$-phenylalanine and 200 pg of L-methionine with the latter interface.[83] The advantage of this approach is that the instrument can be returned to normal MS operation by turning off the microwave plasma. In addition, with the coupling of the technique to HPLC, detection of Phase II metabolites can be achieved without hydrolysis.[85] To date, this technique is about an order of magnitude less precise than IRMS because of the mass analyzers used.

The need for a dedicated instrument and the past difficulty in operation of the combustion interface have limited the routine use of GC/IRMS. The future applications of this technique in drug monitoring will depend on alleviating these limitations.

IV. APPLICATIONS IN HPLC/MS

Biological and environmental applications of HPLC interfaced directly to a mass spectrometer have been recently reviewed.[87,88] Although many of the advances in HPLC/MS have been focused on the ability to characterize proteins and peptides, the robustness of some interfaces, such as the heated pneumatic nebulizer interface, has made this technique routine in pharmaceutical analysis. The regulatory requirement for more complete data promises to continue this trend toward HPLC/MS characterization of new xenobiotics. The implementation of HPLC/MS in toxicological analysis has lagged because of the expense of the instrumentation, the inability of many of the interface techniques to provide qualitative structural data, and the absence of clearly documented advantages over the presently used technique of GC/MS in the clinical and forensic toxicology arenas. With the decrease in the price of instrumentation, this should change, provided the expertise is available to capitalize on this improved technology.

Acquiring structural information is frequently difficult because of the "soft" ionization that occurs in most liquid interfaces. This generally requires MS/MS analysis to obtain structural information, and no libraries exist to assist in spectral matching. Clearly, the major impact of HPLC/MS has been in the area of selective quantitation of compounds that are labile or difficult to prepare for GC/MS analysis. One of the first applications of HPLC/MS was xanthine analysis, and, for a time, caffeine was the primary compound for evaluating HPLC/MS interfaces. Similarly, the initial report of an APCI interface involved quantitation of labile sulfa drugs. As mentioned above, no single interface technique is optimal for analysis of all compounds. Pleasance et al. have compared several

HPLC/MS interfaces for measurement of N-methyl carbamate pesticides.[89] In the case of carbaryl in SIM mode, APCI had the lowest limit of detection at 50 pg, followed by ES at 500 pg, thermospray at 800 pg, and PBI at 10 ng. The same pattern followed for seven other pesticides studied. Although these compounds are not typical compounds for toxicological analysis, they illustrate the point that nonionic, low molecular weight compounds are best ionized by APCI, as summarized by Niessen and Tinke.[34] Moving belt CI, thermospray, PBI, APCI, and ES were used in an attempt to analyze the labile oxidation products of paralytic shellfish toxins.[90] ES was the only interface to generate analytically useful ions.

HPLC/MS has received wide acceptance in the testing of meat and milk. Two groups[91,92] have reported on the analysis of β-adrenergic antagonists in meat and achieved limits of detection in the 100 pg on-column range with APCI, which is about 50-fold lower than the limits of detection observed for thermospray detection. A variety of workers have reported on detection of antibiotics in meat and milk using both PBI[93] and ES.[94]

A few applications of HPLC/MS to forensic toxicology have appeared. Cocaine, BE, methylecgonine, and norcocaine were quantified after solid-phase extraction from urine using APCI.[95] Rule and Henion[96] have reported on the on-line protein G immunoaffinity extraction of LSD from urine, followed by reversed-phase HPLC/MS. A column switch arrangement allowed direct transfer to the HPLC column, avoiding sample cleanup. The limits of detection for LSD was 500 ng/l using ES. A further study applied the same technology to analysis of crude potato extract for analysis of the pesticide carbofuran.

Anabolic steroid analysis has also benefited from application of HPLC/MS techniques. Methandrostenolone (Dianabol) and its 17-epimethandrostenolone metabolite were quantified using positive-ion APCI HPLC/MS/MS. The detection limit in a urine matrix was 180 ng/l, with a precision of 3% at the 16 μg/l level, and a linear dynamic range was at least three orders of magnitude.[97] Stanozolol and several of its hydroxylated and dihydroxylated metabolites could be quantified by positive-ion APCI SRM after enzymatic hydrolysis of their glucuronide conjugates.[98] The direct detection of intact sulfoconjugated hydroxy metabolites in human urine was also achieved. Quantitation of the sulfoconjugate of boldenone in urine was accomplished by negative-ion pneumatically assisted ES. Using SIM of the molecular anion, the limit of detection was 10 pg on-column and with a linear dynamic range of over 500 in the concentration range.[99] Note again that ES ionization provides excellent detection limits for ionic compounds, but that APCI is more effective for nonionic species. Negative-ion MS/MS of the sulfoconjugates yields almost exclusively sulfate ion, and thus no structural information for confirmation of identity is provided as would be available with positive ionization. Kobayashi et al.[100] analyzed 60 steroids by APCI and found that fragment ion correlated with their substituents in the 3- and 4-positions. We have also observed differences with ES in NH_4^+ adduct formation between steroids with a 3- vs. a 17-hydroxyl group, which may be helpful in grouping steroids in screening.[101]

We have been investigating the direct detection of testosterone and epitestosterone conjugates in urine (Figure 10–10). Positive-ion SIM for epitestosterone glucuronide gives detection limits of 25 pg on-column when packed capillary HPLC columns are used, and lower detection limits for epitestosterone sulfate, testosterone sulfate, and testosterone glucuronide.[101] Interestingly, we found that the epitestosterone glucuronide partially fragmented in the interface region before entering the mass spectrometer, giving rise to the protonated aglycone. Because the mass of the intact molecule was monitored, this resulted in an apparent decrease in response for epitestosterone glucuronide. The glucuronide conjugate of epitestosterone was much more labile than that of testosterone, even though the two compounds are epimers. Thus, careful investigation of the operating parameters of the HPLC/MS system is imperative to achieving success in HPLC/MS.

The application of HPLC/MS techniques to macrolide and peptide drugs has played an important role in determining both therapeutic concentrations and the role of metabolites in toxicity. Crowther et al.[102] described the analysis of a pentapeptide using deuterated internal standard in the concentration range of 0.2 to 20 μg/l. We have demonstrated the importance of the selective quan-

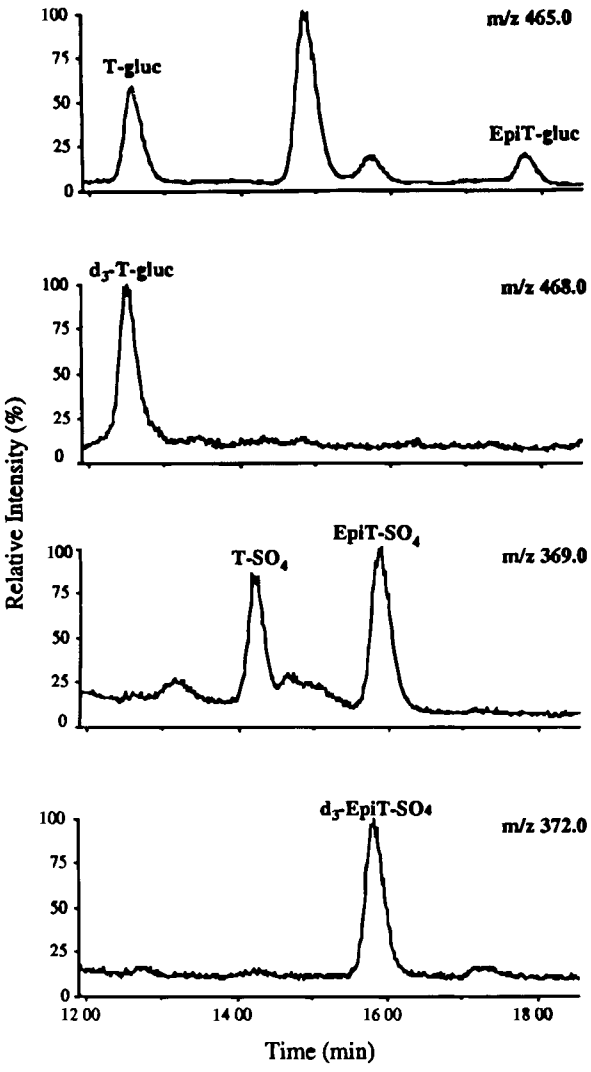


FIGURE 10–10. HPLC/MS separation of glucuronide (T-gluc) epitestosterone glucuronide (Epi T-gluc) and sulfate conjugates extracted from urine. [16,16,17-$^2H_3$] Testosterone glucuronide (d_3-T-gluc)] and [16,16,17-$^2H_3$] epitestosterone sulfate (d_3-Epi T-SO_4) were used as internal standards. (From Sanaullah and Bowers, *J. Steroid Biochem. Molec. Biol.*, in press. With permission.)

titation of cyclosporine metabolites in renal and liver biopsy tissues for estimating their potential role in toxicity.[103] The limit of detection in tissue samples was 500 fg because of the specific detection available through SIM.

A. CHARACTERIZATION OF DRUG METABOLITES

The role of MS in identifying the products of Phase II metabolism has recently been reviewed. The soft ionization is ideal for producing protonated molecular ions of glucuronide, glutathione, sulfate, and phosphate conjugates of xenobiotics. Sinz and Remmel[104] were able to determine a unique quaternary amine glucuronide conjugate of the anticonvulsant lamotrigine 2-*N*-glucuronide using ES MS/MS.

An explanation for the metabolic epimerization of a number of 17α-methyl anabolic steroids was achieved using HPLC/MS. After administration of methandrostenolone, 17-epimethandrostenolone, the parent steroid, and a sulfuric acid conjugate of the parent were isolated and identified by their daughter-ion mass spectra in the urine of both man and horse.[105] Spontaneous hydrolysis of methandrostenolone-17-sulfate gave 17-epimethandrostenolone and several dehydration products. This reaction had a half-life of 16 min in equine urine at 27°C. Thus, although the sulfate conjugate could be found in the bladder, normal urine retention resulted in the appearance of the

epimer in urine collections. A number of other hydroxylated metabolites were also identified. Not only did this finding explain the metabolism of the 17α-methyl steroids, but also lead to a facile method for synthesis of 17-epimer standard materials via a sulfate intermediate.

Another interesting investigation facilitated by HPLC/MS was the study of the stereospecificity of metabolism of *N*-methyl-dextrorphan and *N*-methyl-levorphanol.[106] Using stable isotope-labeled compounds, the glutathione and glucuronide conjugates of *N*-methyl-dextrorphan and the glucuronide conjugate of dextrorphan were found in bile. In contrast, the sulfate, glutathione, and glucuronide conjugates of *N*-methyl-levorphanol and the glucuronide conjugate of hydroxylevorphanol were found in bile. This remarkable stereospecific Phase II metabolism would not have been detected with standard analytical techniques.

Poon et al.[107] studied the metabolism of the chemotherapeutic agent 4-hydroxyandrostendione. In breast cancer patients, 4-hydroxyandrostendione glucuronide, 4-hydroxyandrostendione sulfate, 5β-androstan-3α-ol-4, 17-dione sulfate, and 5β-androstan-3α, 17-diol-4-one sulfate were found in urine after an oral dose. In prostatic cancer patients given the drug intramuscularly, no 4-hydroxyandrostendione sulfate was observed. Whether this was the result of the route of administration or the sex difference in the patients was not clear. A number of 5α-reductase inhibitors have been analyzed by APCI, with detection limits on the order of 200 ng/l.[108,109] The metabolic pattern of taxol in plasma and urine was monitored using APCI.[110] Speciation of platinum-containing antitumor drugs in tissues using HPLC combined with inductively coupled plasma MS is of growing importance in understanding the kinetics of these drugs.

B. DIRECT MEASUREMENT OF DRUG–PROTEIN INTERACTIONS

Most of the interest in ES/MS has been the result of application to protein characterization. An example relevant to toxicology is direct HPLC/MS determination of the structures of covalent adducts formed between a drug and protein involved in toxicity. One interesting example is the modification of human serum albumin by tolmetin glucuronide.[111] Enzymatic digests of the protein can be analyzed by HPLC/MS and modified peptides identified. In the case of tolmetin, some of the modified peptides contained tolmetin linked covalently via a glucuronic acid to a protein lysine group via Schiff base formation. Similar approaches have been described for adduct formation with DNA and other macromolecules.

The recognition that noncovalent interactions between proteins and small molecules could be studied with ES/MS has opened new areas for study. Receptor-ligand, enzyme-substrate, enzyme-inhibitor, and enzyme-product complexes can be studied directly by measuring the relative amounts of free ligand or substrate, free receptor or enzyme, and the complex. Noncovalent complexes of FK506, dihydro-FK506, and FK520 with albumin have been observed with pneumatically assisted ES/MS.[112] No complexation between cyclosporine and albumin was detected, although this could be caused by dissociation during ion formation. Ganem et al.[113] demonstrated that competition between drugs, or their metabolites, for binding to their receptor can be studied directly with ES/MS. In the presence of a slight molar excess of tacrolimus, virtually quantitative formation of the tacrolimus–FK-binding protein (FKBP) complex was observed. Competition between equimolar amounts of sirolimus and tacrolimus for FKBP resulted in the appearance of a 2:1 ratio of sirolimus-FKBP tacrolimus-FKBP complexes, in good agreement with the binding constants measured by other techniques. Cyclosporine did not form a complex with FKBP, as expected. Henion et al.[114] have reviewed recent applications of positive- and negative-ion MS and MS/MS for DNA adducts, heme–globin complexes, and antibiotic-binding proteins. The opportunity to understand toxicity better through direct MS analysis of drug–protein interactions is an exciting new area of application.

V. APPLICATIONS IN CAPILLARY ELECTROPHORESIS (CE)/MS

Development of an interface between CE instruments and mass spectrometers has been complicated by the difficulties of working with the nanoliter per minute flows associated with CE and

maintaining fidelity of the chromatographic resolution.[115] Two interfaces have been described for CE, involving a co-axial sheath flow[116] or a liquid junction[117] to provide sufficient liquid flow for an ES interface. Pleasance et al.[118] compared the two interface approaches, and found sheath flow to be more robust and reproducible. An interface without sheath flow using 10 μm i.d. capillaries has been reported.[119] Interfacing of CE via CF-FAB has been reported for the analysis of peptides and proteins. A second problem for the CE/MS interface is pH adjustment when, for example, an alkaline pH is required for the separation of anions, but an acidic pH is necessary to provide a protonated species for mass analysis. Another issue, which impacts primarily protein and peptide work, is the time required to acquire a complete mass scan over a 1,000-Da range within the half-width of the CE peak (typically 1 to 3 sec). For this reason, QITs and Fourier transform MS are becoming more popular, because they can scan at greater than 5,000 Da/sec. Nevertheless, CE/MS has been used for analysis of xenobiotics and given the proliferation of reports on CE; one would anticipate increased interest in CE/MS as well.

A number of applications of CE have been covered in another chapter,[120] but the specific issues associated with CE/MS will be discussed herein. Johansson et al.[121] first described CE/MS analysis of sulfamethazine and benzodiazepines using effluents containing 15 to 20% methanol. Detection limits were on the order of 2 pmol on-column. Tomlinson et al.[122,123] have extended this work to nonaqueous solutions for CE/MS separation of the metabolites of haloperidol and mifentidine, both of which are hydrophobic. Methanol has a number of positive effects: reducing the electroendosmotic flow; decreasing solute interactions with the capillary surface; decreasing the current flow to levels comparable with the ES interface; and improving the efficiency of the ion evaporative process. Although CE/MS results in very narrow peaks for haloperidol and its metabolites, near-baseline separation of the same ten compounds has been reported in a reversed-phase HPLC/thermospray MS system.[124] The elution order of the compounds was different, as would be expected, but total run time in both systems is about 15 min. Limits of detection are hard to compare between these studies, especially because different interface techniques were used.

Sheppard et al.[125] described the chiral capillary electrophoretic separation of terbutaline and ephedrine isomers using β-cyclodextrin as a mobile phase modifier. Using MS detection, the protonated noncovalent guest–host inclusion complex (*m/z* 1557), β-cyclodextrin (*m/z* 1330), and terbutaline (*m/z* 226) were all observed. Using SIM, a spiked urine sample containing 27 mg/l terbutaline could be detected with a signal-to-noise ratio of 25, an improvement of 100-fold over ultraviolet detection. Urine terbutaline concentrations are reported in the 1 to 100 μg/l range. This example illustrates the difficulty in performing trace analysis with MS in the presence of a large excess of mobile phase modifier. Unfortunately, many of the CE separations reported in the literature involve micellular electrokinetic capillary chromatography, which would fall in this category.

VI. CONCLUSIONS

Numerous advances in MS and in its interfacing with various chromatographic techniques have broadened the capabilities of toxicologists. As commercialization of the new ion storage device capabilities continues, GC/MS/MS will become a widespread analytical tool. Although GC/MS continues to dominate routine toxicological analysis, the sensitivity of HPLC/MS and HPLC/MS/MS, particularly for Phase II metabolites, will increase its appeal. Several exciting approaches to decreasing sample preparation in combination with selective mass spectrometric detection may impact both screening and confirmation assays.

Although the analytical capabilities available to the toxicologist will increase, it is important to remember that chromatography-mass spectrometric systems are just tools. As illustrated herein, the chemistry of the compound and of its derivatives can play a major role in the success or failure of the analysis. In an era of laboratory reengineering and downsizing, MS will remain an area where continuing staff education and experience are an imperative.

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Chapter 11

PENTAMER FORMATION ASSAY BASED ON DRUG FUNCTIONAL MECHANISMS

Masakazu Kobayashi

CONTENTS

I. INTRODUCTION

Pentamer formation assay (PFA) is a mechanism-based method to assay immunosuppressive compounds, such as tacrolimus (FK506) and cyclosporin A (CsA). Tacrolimus (FK506) is a 23-membered ring macrolide and CsA **2** is a cyclic undecapeptide (Figure 11–1), respectively, but they have been proven to act at the same point of signal transduction in T cells after T-cell receptor stimulation by antigen presented on major histocompatibility complex-II molecules of antigen-presenting cells. Tacrolimus was found in 1984 by Kino et al.[1] of Fujisawa Pharmaceutical Company, Japan in a fermentation broth of *Streptomyces tsukubaensis* No. 9993. This was isolated from the soil of Mt. Tsukuba, north of Tokyo. FK506 is unique because of its potent immunosuppressive activity and its discovery by intentional screening methods in their laboratories. Kino et al. screened compounds that possess antiproliferation activity after T-cell stimulation signal, but without cytotoxicity. Thus, it was theoretically anticipated that tacrolimus inhibits stimulated T-cell proliferation, while remaining nontoxic to lymphocytes and other organs. The adverse events directly associated with tacrolimus should be reversible to the cells, and irreversible damage is considered to be through indirect mechanism.

Tacrolimus binds to an immunophilin superfamily of FK506 binding proteins (FKBPs). Five members have been reported for FKBP: FKBP-12,[2] FKBP-12.6,[3] FKBP-13,[4] FKBP-25,[5] and FKBP-52.[6] FKBP-12 is considered to mediate the immunosuppresive activity of tacrolimus. Although all of these immunophilins retain peptidyl prolyl *cis-trans*-isomerase (PPIase) activity

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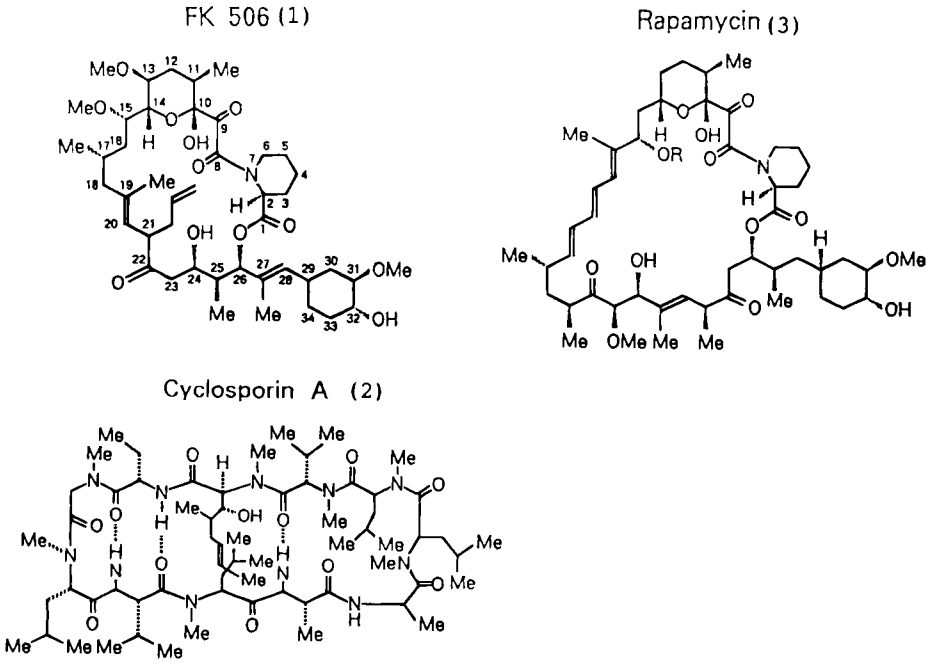

FIGURE 11–1. Structure of tacrolimus (FK506; **1**), CsA (**2**), and sirolimus (rapamycin; **3**).

inhibited by tacrolimus, PPIase activity contributes minimally to the drug's immunosuppressive activity.[7,8] Tacrolimus penetrates into cells and exerts its immunosuppressive activity in T cells by binding to cytosolic receptor FKBP-12 to form a binary complex. This further binds activated protein phosphatase 2B class, calcineurin.[9] Calcineurin is allosterically activated by Ca^{2+} and calmodulin, and dephosphorylates phosphate groups attached to serine and threonine residues in the protein. In T-cell signaling, the key substrate for calcineurin is NFATc/p/x (cytosolic component of nuclear factor of activated T cells), as shown in Figure 11–2.[10–12] The complex of tacrolimus, FKBP-12, Ca^{2+}, calmodulin, and calcineurin is called *pentamer complex.*

CsA binds to its corresponding immunophilin members, called cyclophilins (CyPs): CyP-A,[13–15] CyP-B,[16] CyP-C,[17] CyP-D,[18] CyP-40,[19] and CyP-160.[20] CyP-A, CyP-B, and CyP-C, together with CsA, bind with an activated calcineurin, inhibiting its phosphatase activity (similar to that of tacrolimus/FKBP-12[21]). The immunosuppressive activity of tacrolimus and CsA for various species is summarized in Table 11–1. In humans and rodents, tacrolimus is 10- to 100-fold more potent in the *in vitro* assays than CsA.[1] This difference may be explained by the difference in the drugs' affinity to their respective immunophilins and the difference in the ability for further pentameric complex formation of the binary complexes with activated calcineurin.

II. PRINCIPLE

Enzyme-linked immunosorbent assay (ELISA)-based method is available for tacrolimus (our original ELISA,[22] INCstar's Pro-Trac, and Abbott's IMx[23]) and for CsA (INCstar's Cyclo-Trac, Abbott's TDx, and Syva's Emit). The high-performance liquid chromatography (HPLC)-ultraviolet method is used only for CsA,[24] and HPLC-mass spectrometry (MS) has been used only in limited research studies of tacrolimus.[25] Initially, many questions were centered around the clinical efficacy of tacrolimus immunoassay. The difference existed between immunosuppressive activities and the immunoassay results for the plasma of tacrolimus-treated patients.[26] The result of specific HLPC-MS differed from that of immunoassay results.[25] The difference also existed between extraction methods for the drug (solid-phase extraction vs. liquid phase extraction), claiming the possible pres-

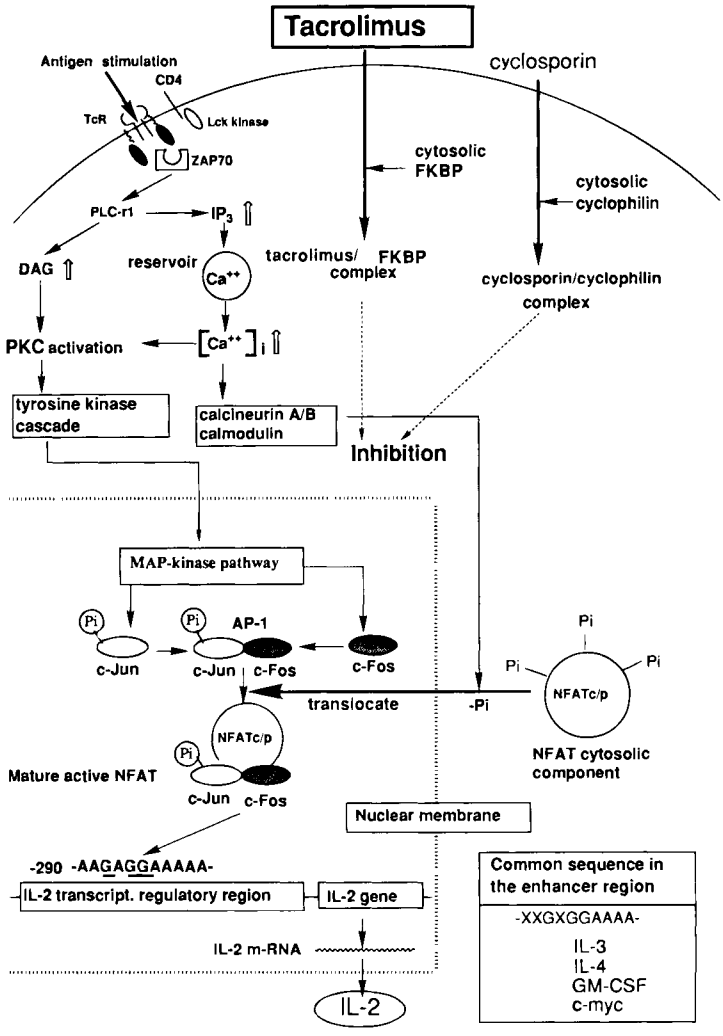

FIGURE 11–2. Signal transduction mechanism in T cell triggered by antigen presented on the major histocompatibility complex-II of antigen-presenting cells, and the functional point of tacrolimus and CsA (cyclosporin A). TcR, T Cell receptor; Lck, lymphocyte cytoplasmic kinase; IP_3, Inositol-(1,4,5)-triphosphate; DAG, diacyl glycerol; PKC, protein kinase C; MAP, mitogen-activated protein kinase; Pi, phosphate; IL-2, interleukin 2; IL-3, interleukin 3; IL-4, interleukin 4; GM-CSF, glanulocyte-monocyte colony stimulating factor; c-myc, cellular counterpart of myelocytomatosis virus oncogene product.

ence of metabolites. These may be extracted by solid extraction, but not by liquid extraction.[27] On the contrary, selected metabolites with immunosuppressive activity may not bind to an anti-FK506 monoclonal antibody.[27]

Both Fujisawa's original ELISA[22] and Pro-Trac are competitive immunoassays. Patient-originated tacrolimus and its metabolites and tacrolimus–reporter conjugate competitively bind to the

TABLE 11–1
***In Vitro* Immunosuppressive Activities of Tacrolimus (1) and CsA (2) to Lymphocytes from Various Species**

	Human	Baboon	Rat	Mouse	Dog
Tacrolimus	0.29[a]	3.85[a]	0.33[a]	0.3[b]	0.59[a]
CsA	50.5[a]	212[a]	45.4[a]	40[b]	40.8[a]

Note: Numbers are in ng/ml.

[a] From Eiras, G., et al., *Transplantation*, 49, 1172, 1990.
[b] Kino, T., et al., *J. Antibiot.*, 40, 1249, 1987.

anti-tacrolimus monoclonal antibody. As previously reported, tacrolimus circulates in plasma bound by α_1-acid glycoprotein.[28] However, Kobayashi et al.[29] showed that α_1-acid glycoprotein does not interfere with the tacrolimus immunoassay, but the corresponding immunophilin does interfere. FKBP-12[30] and possibly CyPs are circulating in plasma, and their concentration varies from patient to patient. The source of these plasma immunophilins is yet to be determined. If the removal of these immunophilins from patient's specimens is not complete, they may bind to both extracted drugs (tacrolimus, CsA, and their metabolites) and drug–reporter conjugates (reporter can be either enzyme or isotope) in the assay well. Immunophilin binding to the drug–reporter conjugate results in the inhibition of binding of the drug–reporter conjugate to the coated anti-drug antibody, thus decreasing the reporter activity (enzyme or isotope) captured in the competitive assay. If the calibrators are prepared in the assay buffer and the extraction is not interfered in a same rate as for patient specimens, decreased reporter activity for patient specimens falsely leads to an apparent high concentration of the drug. Thus, if the drug dose is to be adjusted based on the concentration, the dose would be reduced with a corresponding decrease in immunosuppression. This may be fatal for patients with organ transplants. Thus, proper monitoring of the immunosuppressive state would avoid over- or underimmunosuppression, which is the desired rationale of therapeutic drug monitoring.

Because good correlation has been confirmed by Winkler et al.[27] and Gonshior et al.[31] between these immunoassays and the specific HPLC-MS for tacrolimus, this implies the lack of a significant amount of immunoreactive metabolites in blood. However, the question of therapeutic window has been left unanswered in terms of immunosuppressive state of patients. The question of immunoassays to the therapeutic window is especially important for monitoring an immunosuppressant with many metabolites, irrespective of biological activity for CsA.[32]

PFA has been established based on the functional mechanism of tacrolimus and CsA to answer to these questions.[33]

III. SIGNIFICANCE OF PFA TO ANALYTICAL TOXICOLOGY AND ITS APPLICATIONAL RATIONALE

The major concerns for immunosuppressants for organ transplantation and autoimmune diseases is overimmunosuppression and the potential unknown toxicity of metabolites in relation to their immunosuppressive activities. The clinical efficacy of the drugs in suppressing interleukin-2 mRNA transcription would be defined by the following factors:

1. Drug concentration in lymphocytes
2. FKBP-12 (CyPs) concentration in plasma
3. FKBP-12 (CyPs) concentration in lymphocytes
4. Calcineurin concentration in lymphocytes.

Then, the toxicity would be defined by the following factors:

1. Drug concentration/distribution in tissues/organs
2. FKBP-12 (CyPs) concentration in the cell of the organs
3. FKBP-12 (CyPs) localization in the cell of the organs
4. Importance of intrinsic FKBP-12 (CyPs) function in the cell of the organs
5. Calcineurin concentration in the cell of the organs
6. Calcineurin localization in the cell of the organs
7. Importance of intrinsic calcineurin function in the cell of the organs.

Calcineurin is a serine/threonine phosphatase that acts downstream in T-cell receptor signal transduction pathways. The physiological significance of calcineurin has been the subject of lengthy

investigations, lagging behind the progress of protein tyrosine kinase (PTK) and protein tyrosine phosphatase (PTP). It is simply because PTK and PTP act at the direct signal (or upstream signal) after receptor activation and easiness of approach. Currently, many substrates for calcineurin have been reported, such as GAP43,[34] sinapsin-1,[35] τ-protein,[36] Ca^{2+} channel,[37] nitric oxide synthase,[38] PKA,[39] NFAT/c/p/x,[10–12] dopamine/cAMP-regulated phosphoprotein,[40] etc. This universal inhibition of calcineurin by tacrolimus or CsA via binary complex formation with their respective immunophilins may cause disruption of various cellular activities, thus causing disruption of homeostasis.

Therapeutic range of tacrolimus has not been established, because there is insufficient information on the concentration of FKBP-12 or CyPs, calcineurin in lymphocytes, and the concentration and toxicity of tacrolimus metabolites in blood.

IV. SAMPLING PRECAUTIONS FOR VARIOUS SPECIMENS

The assay structure is depicted in Figure 11–3, wherein calcineurin bound to FK506/FKBP-12 is detected by anticalcineurin antibody. Before performing PFA, it would be important to recognize the presence of immunophilins circulating in the patient's plasma[30] and blood cells.[41,42] In performing the PFA, as in the case for other immunoassays,[29] extracts of blood containing tacrolimus or CsA should be free of endogenous immunophilins which, if present, might interfere with the assay. Endogenous immunophilins from the patient sample would be also subject to competitive binding of the drug(s), with the exogenous assay immunophilins for complex formation. These exogenous immunophilins are bound on the solid phase of the microtiter plates. Methanol is the most commonly used organic solvent that denatures immunophilins in plasma and blood cells, extracting the immunosuppressant and its metabolites from various assay matrices at the same time.[43] The extract is concentrated to dryness. The residue is dissolved in the assay buffer, avoiding denaturing of other protein components of the assay reagents.

V. ANALYSES

Seven metabolites, whose structures are shown in Figure 11–4, have been isolated by an *in vitro* metabolizing system using rat hepatocyte microsome and have been chemically identified.[44,45] A similar metabolizing pattern has been observed in the human system *in vitro*.[46] These metabolites were assayed by the PFA and compared with mixed lymphocyte reaction (MLR) suppressive activity. Good correlation was confirmed as shown in Table 11–2.[33]

VI. INTERPRETATION

The PFA does not titrate the concentration of unchanged tacrolimus or CsA and their metabolites separately, but rather titrates the total immunosuppressive activity ascribable to tacrolimus or CsA in patient's blood. The primary concern with this type of assay is again interference by metabolites that have FKBP-12 binding activity, but no pentamer formation activity. A typical example is **M-3** (C15-demethyl-tacrolimus) (Table 11–2). The presence of **M-3** competes with tacrolimus and other pentamer-formable metabolites (immunosuppressive metabolites) in the first step of pentamer formation. Thus, its presence may underestimate the concentration of pentamer-formable compounds, leading to potential overdosage as a result. However, it was not the case, as shown in Figures 11–5 and 11–6.[33] To compete for 20 n*M* tacrolimus, several thousand-fold of **M-3** was required. This suggests that the pentamer complex is far more stable than the corresponding binary complex. That is, **M-3** in the binary complex is easily replaced with unchanged tacrolimus and other pentamer-formable metabolites when activated calcineurin is present. This finding is very important. This indicates that a total immunosuppressive activity ascribable to tacrolimus (or CsA) in patient's blood is properly assayed without being interfered with by nonimmunosuppressive

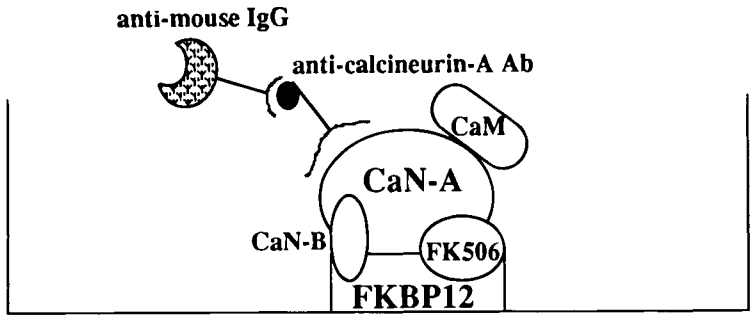

FIGURE 11–3. Structure of PFA complex for tacrolimus (FK506). IgG, immunoglobulin G; Ab, antibody; CaM, calmodulin; CaN-A, calcineurin A chain; CaN-B, calcineurin B chain; FKBP12, tacrolimus (12 kDa).

metabolites having significant FKBP-12 (or CyP) binding activity. It can be speculated that this mechanism-based assay of PFA is applicable for sirolimus (rapamycin) in combination with FKBP-12 and FKBP-12 rapamycin-associating protein (FRAP).[47] Tacrolimus and sirolimus in the mixture can be separately assayed using activated calcineurin for the former and FRAP for the latter, as shown in Figure 11–7.

VII. METHOD COMPARISONS

Currently, two assay kits are marketed for tacrolimus: one is a manual-type ELISA, Pro-Trac from INCstar Corp. (Stillwater, MN), and the other is an automated IMx kit from Abbott Diagnostics (Abbott Park, IL). INCstar's Pro-Trac is basically the same as Fujisawa's original ELISA.[22] These immunoassays use the same monoclonal antibody against tacrolimus.[22,23,29] Another mechanism-based receptor assay using FKBP-12 was reported by Murthy et al.[48] Table

TABLE 11–2
Comparison of Assay Methods for Tacrolimus Using Isolated Metabolites

	Characterization of FK506 metabolites (%)			
	FKBP-12 binding activity	**Complex formation assay**	**MLR suppression**	**Reactivity to FK506 McAb**
M-1	9.6	13.1	6.4	0
M-2	14.2	79.7	100	70–109
M-3	116.0	0	0	90.5
M-4	1.6	6.4	3.5	0
M-5	20.0	0	0	92.3
M-6	1.3	7.7	1.3	0
M-7	2.3	0	0	0
FK506	100	100	100	100
		$r = 0.336$	$r = 0.988$	
		$r = 0.302$		

Note: Values are calculated as relative activities of FK506. The methods examined were FKBP-12 receptor binding assay, PFA, MLR immunosuppressive assay, and immunocross-reactive assay to anti-FK506 monoclonal antibody (McAb).

Source: From Tamura, K., et al., *Biochem. Biophys. Res. Commun.*, 202, 437, 1994. With permission.

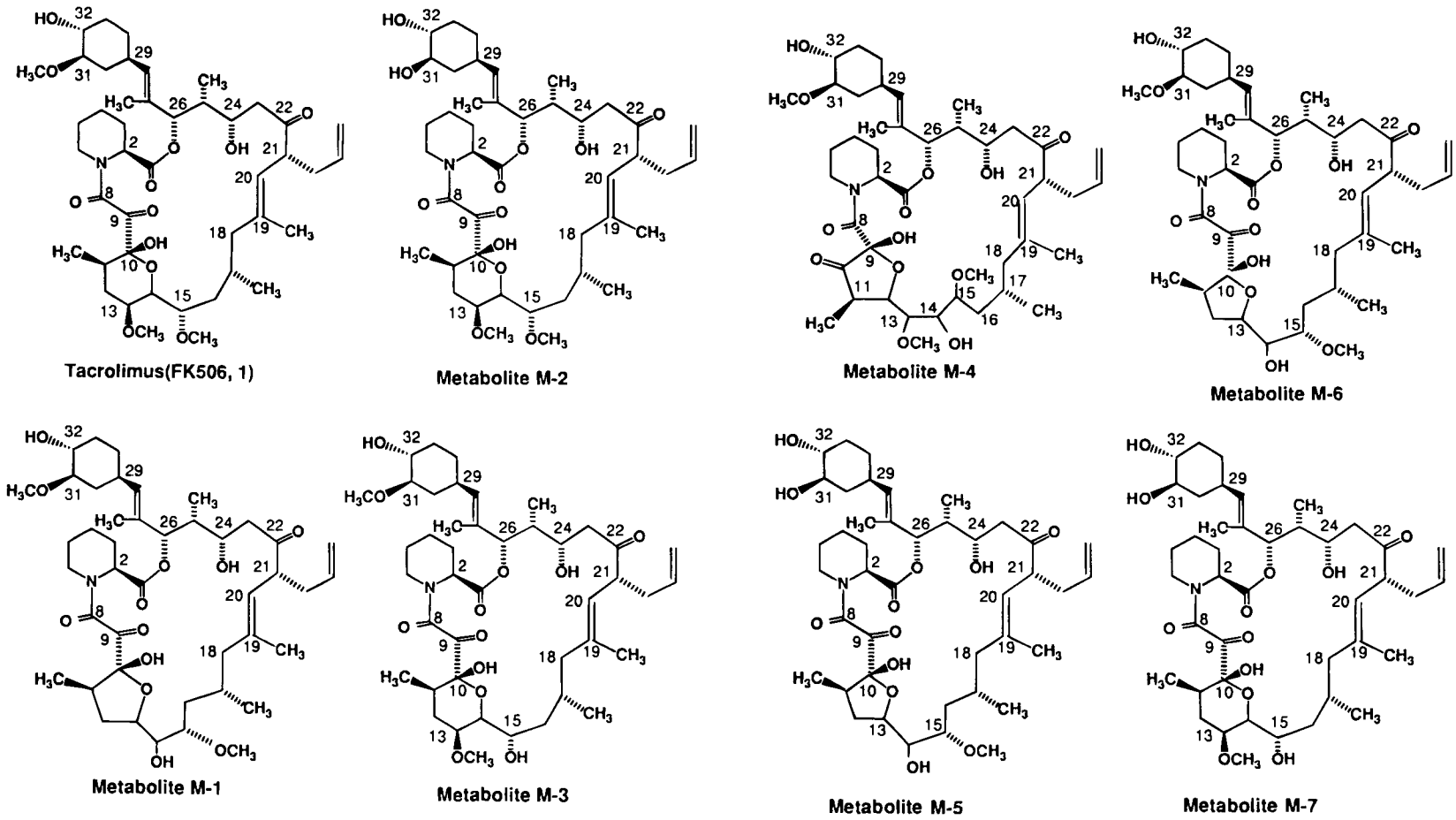

FIGURE 11–4. Structure of tacrolimus metabolites.

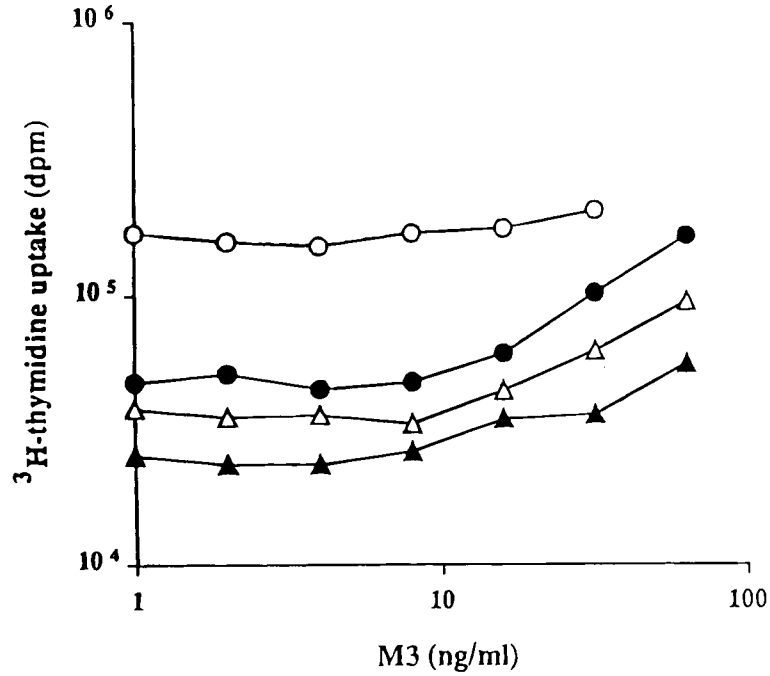

FIGURE 11–5. Interference of PFA by one of the tacrolimus metabolites, **M-3**, which possesses immunoreactivity to the antitacrolimus monoclonal antibody, but does not have immunosuppressive activity. Inhibition of concaravalin A stimulation by FK506 was measured in the presence of serially diluted **M-3** in the assay buffer. ○, FK506, 0 ng/ml; ●, FK506, 0.25 ng/ml; △, FK506, 0.5 ng/ml; ▲, FK506, 1.0 ng/ml. (From Tamura, K. et al., *Biochem. Biophys. Res. Commun.*, 202, 437, 1994. With permission.)

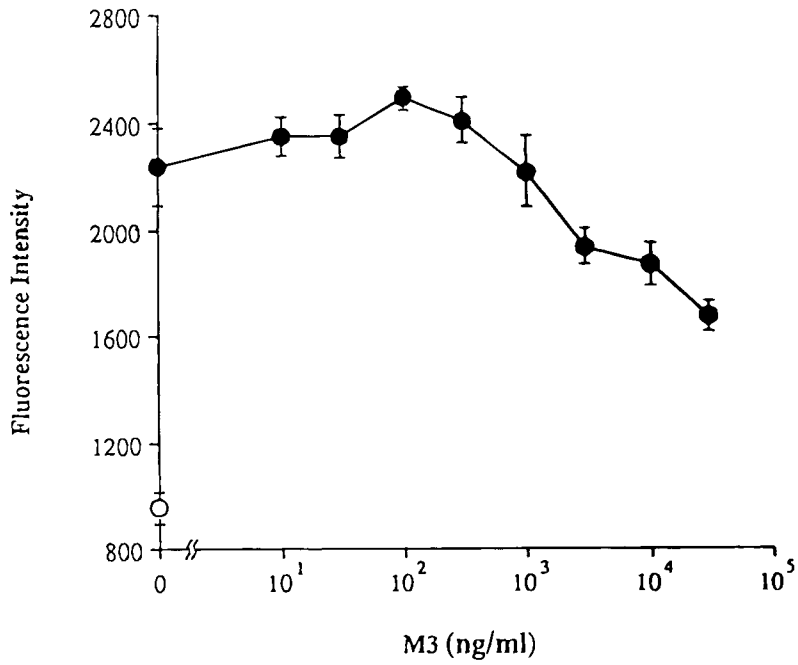

FIGURE 11–6. Interference of PFA by one of the tacrolimus metabolites, **M-3**, that possesses immunoreactivity to the antitacrolimus monoclonal antibody, but does not have immunosuppressive activity. Inhibition of FK506 pantamer formation (FK506, 40 ng/ml) was measured in the presence of serially diluted **M-3** in the assay buffer. (From Tamura, K. et al., *Biochem. Biophys. Res. Commun.*, 202, 437, 1994. With permission.)

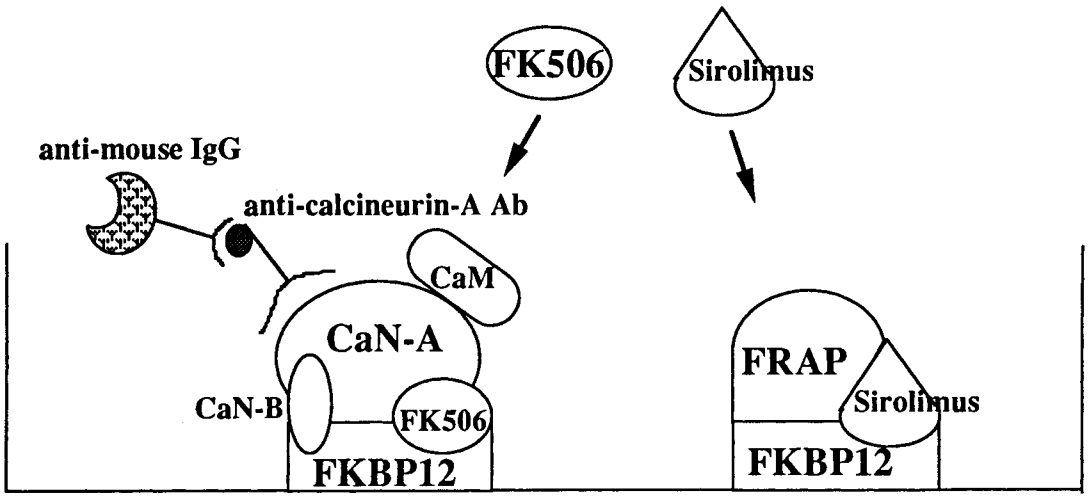

FIGURE 11–7. Simultaneous complex assay of PFA for tacrolimus (FK506) and trimer assay for sirolimus (rapamycin). IgG, immunoglobulin G; Ab, antibody; CaM, calmodulin; CaN-A, calcineurin A chain; CaN-B, calcineurin B chain.

11–2 shows the result of comparative assay studies for metabolites, isolated *in vitro*, for FKBP-12 receptor (binding) assay, PFA, MLR immunosuppressive assay, and an immunocross-reactive assay.[33] Metabolite **M-3** binds FKBP-12 more strongly than the parent tacrolimus, but does not have both pentameric complex-forming and immunosuppressive activities. On the contrary, metabolite **M-2** binds FKBP-12 less strongly than the parent (ca. 14% activity of tacrolimus), but still retains both pentameric complex-forming and immunosuppressive activities, comparable with the parent tacrolimus. This result indicates the inappropriateness of a simple receptor binding assay, proposed by Murthy et al.[48] for tacrolimus and CsA. Inconsistency of immunophilin binding and immunosuppressive activities of CyP was reported for the CsA derivative (MeAla-6)-CsA, which binds to CyP-A, but has no MLR immunosuppressive activity.[49,50] Thus, the PFA correlated well with the MLR immunosuppressive assay. FKBP-12 receptor assay is no better than the immunoassay using anti-tacrolimus monoclonal antibody.

Figure 11–8 shows the result of a comparative study of the PFA with the IMx and more specific HPLC-MS for patients' whole blood specimens.[51] Good correlation was obtained among these three assay methods. This supports the result of Winkler et al.[27] and Gonshior et al.,[31] and also strongly suggests that the quantity of tacrolimus immunoreactive metabolites is not significant in whole blood. However, this does not exclude the possible presence of immunoreactive metabolites in specific patients, for example, patients with cholestasis as suggested by Winkler et al.[27]

VIII. A RECOMMENDED PROTOCOL

One hundred microliters of FKBP-12 (5 μg/ml in phosphate-buffered saline [PBS]) is incubated overnight in immunoplate at 4°C. The plate is washed three times with PBS. The residual binding sites are blocked by being incubated with 300 μl of *0.5% bovine serum albumin (BSA) containing Tris-Triton buffer* (Assay buffer) at room temperature for 15 min. All incubations are done on the gentle plate shaker or rotator. The immunoplate is washed three times with *Tris-Triton buffer* (Wash buffer). One hundred microliters of extracted/reconstituted solution in assay buffer from serially diluted tacrolimus in normal human whole blood or from patients' specimens is added to each well, followed by an addition of 50 μl of calcineurin (33.3 μg/ml in Assay buffer; Sigma Chemical Co., St. Louis, MO; Catalog No. C-1907) and 50 μl of calmodulin (6.1 μg/ml in Assay buffer; Sigma, Catalog No. C-2277). The plate is incubated at room temperature for 1 h to complete the pentamer

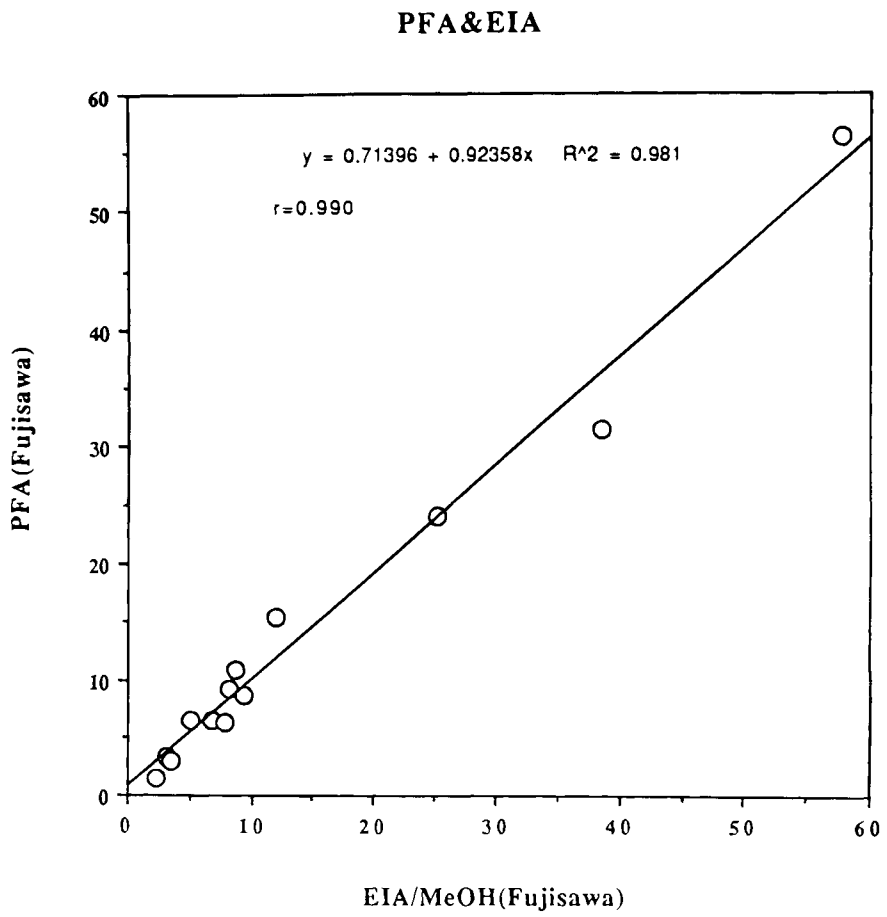

FIGURE 11–8. Correlation of PFA and manual ELISA for whole-blood specimens from liver transplant patients treated with FK506. EIA, enzyme immunoassay; MeOH, methanol.

formation and is washed four times with Wash buffer. Then, 100 µl of anti-calcineurin monoclonal mouse antibody (1 µg/ml in Assay buffer; Upstate Biotechnology Institute; Catalog No. 05-187) is added. After incubation at room temperature for 1 h the plate is washed again four times with Wash buffer. One hundred microliters of alkaline phosphatase-labeled anti-mouse immunoglobulin G (IgG) (1000-fold dilution in Assay buffer; horse; Vector Labs., Burlingame, CA; Catalog No. AP-2000) is added to each well and incubated at room temperature for 1 h. The plate is again washed four times with wash buffer. Two hundred microliters of 4-methylumbelliferyl phosphate (1 m*M* in assay buffer; Sigma, Catalog No. M-8833) is added and incubated at room temperature for 20 min. Generated 4-methylumbelliferone is titrated by fluorescent immunoplate reader (with 460 nm for emission and 360 nm for excitation). The assay result is calculated from the regression line generated on four-parameter logistics. Typical dynamic curves for tacrolimus is shown in Figure 11–9.

1. PBS (pH 7.4): NaCl (8.0 g), KCl (0.2 g), Na_2HPO_4 (0.15 g), and KH_2PO_4 (0.2 g) in distilled H_2O (total volume of 1 l).
2. Wash buffer (Tris-Triton buffer): 0.1% Triton X-100, 0.5 mM dithiothreitol, and 1 m*M* $CaCl_2$ in 50 m*M* Tris · HCl buffer (pH 7.5).
3. Assay buffer: 5 mg/ml of BSA in Wash buffer.
4. Calcineurin stock solution: Bovine calcineurin from Sigma (Catalog No. C-1907) is dissolved in distilled H_2O to prepare a 500 µg/ml solution. This is kept in a freezer as a stock solution. On the day of the assay, the stock solution is further diluted 15-fold by the Assay buffer.

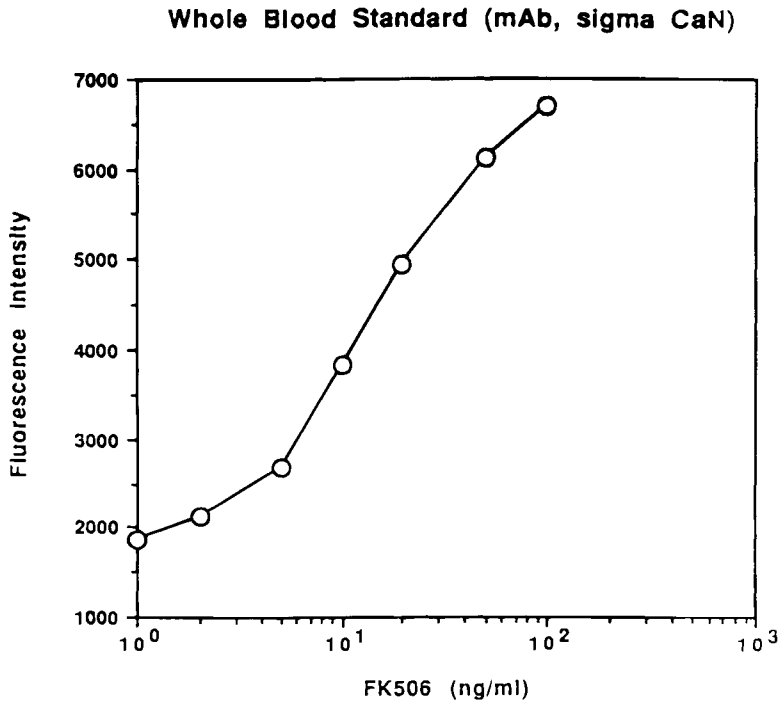

FIGURE 11–9. Dynamic curve of PFA for FK506 spiked in normal human whole blood. mAb, monoclonal antibody; CaN, calcineurin.

5. Calmodulin: Bovine calmodulin from Sigma (Catalog No. C-2277) is dissolved in H_2O (ca. 2 ml) to prepare a 243 μg/ml solution. This is kept in the freezer as a stock solution. On the day of the assay, the stock solution is further diluted 40-fold by the Assay buffer.
6. Anticalcineurin antibody: Anticalcineurin monoclonal antibody (Upstate Biotechnology Institute; Catalog No. 05-187; 50 μg/ml) is diluted 50-fold by the Assay buffer on the day of the assay.
7. Alkaline phosphatase-labeled anti-mouse IgG antibody: Alkaline phosphatase-labeled horse anti-mouse IgG from Vector Labs. (Catalog No. AP-2000) was diluted 1000-fold by the Assay buffer on the day of the assay.

IX. CONCLUSIONS

The PFA is a mechanism-based assay for immunosuppressant tacrolimus and CsA. As shown by tacrolimus and CsA metabolites, receptor binding does not always correlate with and predict their immunosuppressive activity. Good consistency between PFA and MLR immunosuppressive activities was found for tacrolimus metabolites isolated in the *in vitro* system. Its uniqueness is to measure the immunosuppressive activity in blood as a total aggregate of an immunosuppressant and its metabolites, and would be significant when the drug produces many metabolites. A typical example would be CsA. It is expected as a scope of PFA that the assay can be applied to another immunosuppressant, rapamycin, that has a similar chemical structure to tacrolimus, but blocks T-cell replication at a different step of its signal transduction pathways of tacrolimus and CsA. Rapamycin's biological activity would be properly assessed in the trimer assay by just replacing Ca^{2+}/calmodulin and calcineurin with FRAP. This is a good example of an assay system to be developed to predict the biological activity in the body, when the functional mechanism is precisely defined.

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Chapter 12

RECEPTOR ASSAYS IN THE CLINICAL LABORATORY*

Steven J. Soldin

CONTENTS

I. INTRODUCTION

The development of immunoassays in the 1950s has greatly improved and enhanced diagnostic ability throughout the entire field of endocrinology. Immunoassays were also applied to drug concentration measurement, and, again, significant improvement was achieved in the optimization of drug therapy as a result of individualizing the patient's drug regimen to achieve drug concentrations within the therapeutic range. The application of immunoassays to therapeutic drug monitoring has resulted in fewer toxic and subtherapeutic events and improved patient care.[1–3]

Yalow and Berson[4] are largely credited with the development of immunoassays and were justifiably rewarded by receiving the Nobel prize in the 1970s. Two and a half decades later, we may pose the questions: Have immunoassays lived up to their potential? Are we measuring what we think we're measuring? The answer to these questions is complex. Certainly, immunoassays have greatly contributed toward improved diagnostic ability and optimization of patient care. However, the specificity and often the sensitivity of some immunoassays leaves much to be desired. In compiling a pediatric reference range book,[5] we cannot but be struck by the widely different ranges found when comparing results obtained using one manufacturers' product with those obtained using the product of a different supplier. Many of these ranges were obtained using the *same* patient samples. Tables 12–1 to 12–3 are examples of these issues.

Clearly, at the present time, immunoassays are still fraught with problems. It seems that many assays cannot reliably quantify the analyte they are purported to measure. Drug receptors assays have a history of approximately three decades. The first drug receptor assay was described in 1966 by Lowenstein and Corrill.[6] Since that time, radioreceptor assays have been described for numerous analytes, including digoxin,[6–9] glucocorticoids,[10–12] neuroleptics,[13,14] cyclosporine,[15–18] tacrolimus,[18,19] sirolimus,[18,20,21] benzodiazepines,[22] vitamin B_{12},[23] folic acid,[26] adrenocorticotropin,[25,26] 1,25-dihydroxyvitamin D,[27] human chorionic gonadotropin,[28] prolactin,[29] and growth hormone.[30] Many of these methods are now in routine use on fully automated analyzers and in many cases are replacing conventional immunoassays (Table 12–4).

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TABLE 12–1
Problems with Immunoassays:
Prolactin-CAP[a] Survey 1995

Method	Mean result (ng/ml [μg/l])
AxSYM	171.3
Technicon Immuno 1	95.1
Opus Magnum	188.8

[a]College of American Pathologists.

TABLE 12–2
97.5th Percentile for Thyroid-Stimulating Hormone Using Kits from Two Different Manufacturers (Results in μU/ml [mU/l])

Kit	0–1 Month	1–5 Years
Abbott® IMx	<16.0	<7.1
Pharmacia ENI Diagnostics, Inc., Delphia®	<6.5	<3.0

Source: Soldin, S. and Hicks, J. M., Eds., *Pediatric Reference Ranges*, AACC Press, Washington, D.C., 1995.

Theoretically, receptor assays possess many advantages over current immunoassays. For example, whenever we have a drug that is metabolized to both active and inactive metabolites, antibodies (monoclonal or polyclonal) will have great difficulty quantifying only the parent drug, or better still the parent drug and the pharmacologically active metabolites relative to their pharmacological potency. One of the potential advantages of receptor assays over immunoassays is that the cross-reactivity of metabolites in receptor assays may well parallel their pharmacological activity. Consider the situation with several drugs that fall into this category: digoxin, cyclosporine, tacrolimus, and sirolimus.

TABLE 12–3
2.5th and 97.5th Percentile from Thyroxine Using Kits for Two Different Manufacturers (Results in mg/dl [nmol/l])

	0–1 Month	
Kit	2.5th Percentile	97.5th Percentile
Abbott IMx®	3.0 (39)	14.4 (185)
Baxter–Travenol Diagnostic, Inc., T_4 Gamma Coat®	5.9 (76)	21.5 (276)

Source: Soldin, S. and Hicks, J. M., Eds., *Pediatric Reference Ranges*, AACC Press, Washington, D.C., 1995.

TABLE 12–4
History of Receptor Assays

Date	Authors	Drug	Refs.
1966	Lowenstein and Corrill	Digoxin	6
1970	Lefkowitz et al.	Adrenocorticotropin	25
1971	Burnett and Conklin	Digoxin	7
1972	Wolfsen et al.	Adrenocorticotropin	26
1975	Ballard et al.	Glucocorticoids	10
1975	Guyda	Growth hormone/prolactin	29
1977	Creese and Snyder	Neuroleptics	13
1977	Garnier and Job	Growth hormone	30
1979	Clemens et al.	1,25-Dihydroxycholecalciferol	27
1983	Van Putten et al.	Neuroleptics	14
1987	Manchester et al.	Digoxin	8
1988	Bednarczyk et al.	Digoxin	9
1989	Donnelly and Soldin	Cyclosporine	15
1991	Russell et al.	Cyclosporine	16
1992	Murthy et al.	Tacrolimus	19
1992	Soldin et al.	Glucocorticoids	11
1994	Bayer, Immuno I	Vitamin B_{12}	23
1994	Nishikawa et al.	Benzodiazepines	22
1994	Soldin et al.	Cyclosporine	17
1995	Bayer, Immuno I	Folic acid	24
1995	Soldin	Cyclosporine, Tacrolimus, Sirolimus	18
1996	Goodyear et al.	Sirolimus	21

II. DIGOXIN

The digoxin receptor assays[6,7] of the late 1960s/early 1970s required large volumes of blood and were very time-consuming. As a result, they were almost immediately replaced by the digoxin immunoassays that were developed in the 1970s. Even though digoxin immunoassays have improved with time, there is still a rather poor correlation between apparent serum concentration and efficacy or toxicity.[31] This is no doubt caused by a number of factors that include lack of specificity of digoxin immunoassays with cross-reactivity occurring to both digoxin-like immunoreactive factors and digoxin metabolites. It is now fairly well accepted that digoxin is significantly metabolized in 20 to 30% of patients receiving the drug and that most often digoxin immunoassays use antibodies that cross-react with many of the metabolites in a manner that does not correlate with their pharmacological potency.[32–34] Dissatisfaction with current immunoassays for digoxin led to a reevaluation of digoxin receptor assays using the human heart ATPase.[9] The specificity of the receptor assay using human heart ATPase was significantly better than current immunoassays, with minimal interference from digoxin-like immunoreactive factors[9] and cross-reactivity of metabolites being proportional to their pharmacological activity relative to digoxin[9] (Tables 12–5 and 12–6). Nevertheless, the receptor assay is tedious, requires human heart ATPase, and is unsuitable for use in the routine clinical laboratory. A soluble or cloned receptor assay may be one direction in which further work could proceed.

III. CYCLOSPORINE

The clinical relevance of cyclosporine metabolites, of which there are around 30, is still controversial. Immunosuppressive activity has been found *in vitro* for AM1, AM9, and AM4N.[16,18,35,36] Only about one third of cyclosporine in the blood is in the form of the parent drug. Approximately

TABLE 12–5
Effect of Digoxin-Like Factors on the Receptor Assay and the FPIA

Source of serum	*n*	Receptor assay (nmol/l digoxin equivalents)	FPIA: nmol/l digoxin	
			TCA	SSA
Cord	14	0.07 (0–0.26)	0.75 (0.62–1.06)	1.29 (1.04–1.78)
Patients				
Kidney failure	8	0.18 (0–0.40)	0.36 (0.11–1.75)	0.36 (0.11–1.87)
Liver failure	8	0.15 (0–0.71)	0.48 (0–1.19)	0.69 (0.11–1.96)
Third trimester of pregnancy	19	0.04 (0–0.06)	0.22 (0–0.48)	0.27 (0–0.61)

TABLE 12–6
Cross-Reactivity of Several Metabolites of Digoxin in the Radioreceptor Assay

Metabolite[a]	Cross-reactivity (%), compared with digoxin		
	FPIA	RRA Human heart receptor	Cardioactivity (%)
Dihydrodigoxin	8	<1	2–6
Bis-digitoxiside	214	51.8	77
Monodigitoxiside	86	47.3	66
Digoxigenin	67	11	4–21
Dihydrodigoxigenin	26	<1	2
Digitoxose	6	0	0

Note: Abbreviations used are—RRA, radioreceptor assay; FPIA, Fluorescence Polarization Immunoassay.

[a] Assayed at 10 nmol/l.

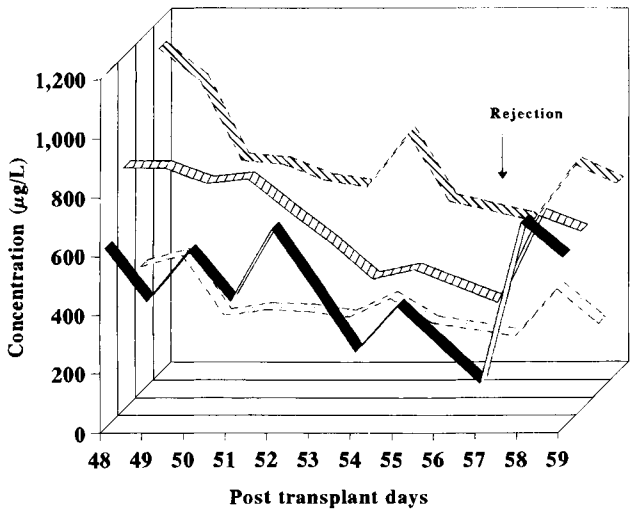

FIGURE 12–1. Comparison of results obtained by two cyclosporine immunoassay procedures (Abbott-specific [▯] and Abbott-nonspecific [▯]) with those of the 52 kDa radioreceptor (▯) assay and the mixed lymphocyte culture (▬) assay in a renal transplant patient.

two thirds are in the form of active and inactive metabolites, although this ratio varies from patient to patient and also within patients. Measurement of only the parent drug by specific immunoassay or high-performance liquid chromatography (HPLC) does not take into account the immunosuppressive activity of the major metabolites that may well be present at concentrations greater than the parent drug. The metabolite immunosuppressive activity when combined could account for about 25 to 30% of the immunosuppressive activity of the parent cyclosporine present. Some immunophilin receptor-based assays may not suffer from this problem, because they tend to cross-react with metabolites proportional to their pharmacological potency.[37] These assays should therefore provide more meaningful clinical data than those provided by conventional immunoassays. Two preliminary studies indicate that this may well be the case for cyclosporine.[16,17] Figure 12–1 gives an example of the excellent correlation achieved between the 52 kDa cyclosporine receptor assay and the mixed lymphocyte culture assay in following a renal transplant patient through a rejection episode.[17] Neither the polyclonal nor the monoclonal Abbott (Abbott Park, IL) fluorescent polarization immunoassays were able to predict the biopsy-proven rejection in this patient. More such data are needed to confirm the clinical superiority of the receptor-based assay for cyclosporine.

IV. TACROLIMUS (FK-506)

Tacrolimus is metabolized into at least nine metabolites.[38] The principal metabolites **M-III** and **M-V** have no pharmacological activity *in vitro*. Both commercially available immunoassays (which use the same antibody) cross-react significantly with these inactive metabolites. One of the metabolites is pharmacologically active (**M-II**) and also cross-reacts significantly in both the Abbott IMx® and the Incstar ELISA® procedures. An immunophilin binding protein still needs to be found that will bind only to the parent drug and the active metabolites relative to their pharmacological potency. Current immunoassays cross-react to a variable extent with cyclosporine metabolites (Table 12–7).

V. SIROLIMUS (RAPAMYCIN)

Sirolimus has been found to have up to ten metabolites.[39–40] The four major metabolites were isolated from the urine of renal transplant patients and their binding to the 14 and 52 kDa immunophilins was compared with their immunosuppressive activity relative to parent sirolimus. The correlation between binding to these immunophilins and pharmacological potency was found to be excellent[41] (Table 12–8), indicating that immunophilin would provide an excellent receptor assay for sirolimus. Commercially available immunoassays are currently unavailable for this drug, so that

TABLE 12–7
Cross-Reactivity of Cyclosporine Metabolites in Some Cyclosporine Assays

Target (100 ng/ml)	No. of labs.	Mean ng/ml	CV
Abbott TDx® monoclonal	219	134.6	7.4
Abbott TDx® polyclonal	40	204.0	6.0
HPLC	40	94.7	16.9
IncStar®–RIA	48	114.7	9.7

Note: Study design (CAP Committee) sample contained approximately 100 ng/ml of AMI and 100 ng/ml of AM9, in addition to 100 ng/ml cyclosporine. Abbreviations used are—CV, coefficient of variation; RIA, radioimmunoassay; CAP, ?.

Source: From the 1994 CAP Survey for Cyclosporine.

TABLE 12–8
Binding of Sirolimus Metabolites to the 14 and 52 kDa Immunophilins and Comparison with Immunosuppressive Activity as Measured by the Mixed Lymphocyte Culture Assay

Sirolimus metabolites[a]	Ratio of metabolite to drug		
	RRA-14 kDa	RRA-52 kDa	MLC[b]
Parent drug	1.00	1.00	1.00
RM-I	0.21	0.25	0.02
RM-II	0.02	0.05	0.09
RM-III	<0.01	<0.01	0.08
RM-IV	<0.01	0.03	0.04

[a] Assayed at 40 μg/l.
[b] MLC, mixed lymphocyte culture.

investigators are limited to HPLC or HPLC/mass spectrometry. The results of the receptor assays for sirolimus compare well with those obtained by HPLC[21] (Figure 12–2), indicating that receptor assays provide a viable alternative to HPLC. The advantages of the former over the latter are many and include smaller sample requirement (200 μl vs. 1 to 2 ml), simplicity, and speed. For example, only 10 to 12 assays/day can be performed using current HPLC procedures for sirolimus, whereas 50 to 70 can be run per day by receptor assay.

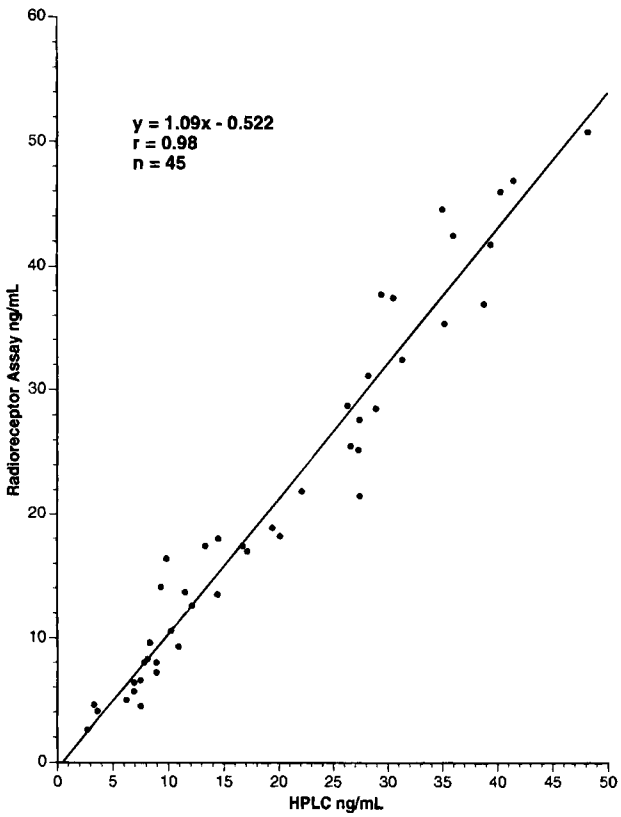

FIGURE 12–2. Correlation of radioreceptor assay for sirolimus with HPLC.

VI. CONCLUSIONS

The field of receptor assays and their clinical utility dates back about three decades. Many of the drug receptors are membrane-bound (e.g., digoxin and neuroleptics), which significantly limits their practical use to develop assays that provide information allowing the therapy of patients receiving these drugs to be optimized. Solubilization and cloning these proteins may enhance their future use. Soluble receptors, such as the immunophilins for immunosuppressive drugs, may well have significant advantages over current immunoassays. The list of receptor assays used routinely in the clinical laboratory is increasing all the time as diagnostic companies realize the many advantages these have for particular assays over current immunoassays. The author predicts a shift away from immunoassays to receptor assays for certain analytes, such as vitamin B_{12}, folic acid, and drugs that undergo extensive metabolism.

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Chapter 13

IMMUNOASSAYS AND IMMUNOASSAY ANALYZERS FOR ANALYTICAL TOXICOLOGY

Edward A. Sasse

CONTENTS

I. INTRODUCTION

The first immunoassay was described by Berson and Yalow in 1959.[1] This assay for the quantitation of insulin was a competitive radioimmunoassay that used radioactively labeled insulin and a limiting amount of antibody to insulin as reagents for measurement of the unlabeled antigen, insulin, in the test sample. The basic ingredients of an immunoassay was defined as: an antibody that binds to a specific antigen or hapten, the antigen or hapten analyte to be measured, and a method of detecting either the free antigen/hapten or bound fraction of the antigen/hapten–antibody complex. Commonly, the system uses a tracer or label, such as a radioisotope for signal detection, that will be proportional to the concentration of the analyte in the test sample. A number of radioimmunoassays were developed and successfully used for many years for the measurement of drugs in forensic and clinical laboratories. However, as newer immunoassay techniques were developed, it soon became apparent that the radioisotopic techniques had certain characteristics that were less advantageous than those of nonisotopic techniques. The newer techniques are different with respect to signal, detection and use enzymes, fluorophores, chemiluminescent molecules, and other properties of the antigen/hapten–antibody complex for measurement.

In 1966, Avrameus and Uriel reported the use of an enzyme-labeled antibody for localization and identification of tissue antigens. This was followed in the next few years by the development of techniques using enzymes as labels for the immunoassay of soluble antigens. The more noteable enzyme-linked-immunosorbent assay (ELISA), developed by Engvall and Perlmann,[3] rapidly became a mainstay for the measurement of proteins, such as immunoglobulins, antibodies, and viral antigens. This technique, more than any other, was the forerunner for the development of the modern enzyme immunoassays and other nonisotopic immunoassays. The disadvantages of radioisotopic assays listed in Table 13–1 have further encouraged the development of nonisotopic immunoassays to the point that today few radioimmunoassays are being used in toxicology and clinical laboratories for drug testing.

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TABLE 13–1
Radioimmunoassay vs. Nonisotopic Immunoassay

	Radioimmunoassay	Nonisotopic Immunoassay
Reagent shelf-life	Limited, due to short half-life of isotopes	Longer
Safety	Isotope handling, disposal	Normal laboratory testing
Regulations	Isotope handling license, disposal	Normal laboratory
Automation	Not good	Yes
High throughput	Limited	Yes
Turnaround time, assay time	Longer	Shorter
Stat testing	No	Yes
Random access, continuous access testing	No	Yes
Precision	Good (duplicate testing, however)	Good
Analytical sensitivity, specificity	Good	Good
Calibration frequency	Each analytical run	Less
Separation step required	Yes	Yes/no
Labor	More, since usually manual testing	Less, since usually automated

All of the immunoassay types are based on the specific reaction of an antibody with the particular analyte (antigen or hapten). The antibody reagent, whether polyclonal antisera or purified monoclonal antibody, must have appropriate quality with regard to binding affinity and specificity or cross-reactivity to be used successfully.

II. IMMUNOASSAY METHODOLOGICAL PRINCIPLES

Immunoassays can be classified in a number of ways based on their respective methodological principles. As previously discussed, an immunoassay can be differentiated based on the type of signal detection or label used:

- Radioisotopic
- Nonisotopic
  - Enzymatic
  - Fluorescence
  - Fluorescence polarization
  - Chemiluminescence
  - Nephelometry and turbidimetry
  - Latex agglutination
  - Colloidal gold aggregation.

In addition, the method may require a separation step, whereby the antibody-bound labeled fraction must be separated from the free labeled material. On the other hand, some methods permit the individual measurement of the bound or free label in the presence of each other, thereby not requiring a separation process. These two types of methods are termed; respectively, heterogeneous or homogeneous. All of the radioimmunoassays are heterogeneous, whereas many of the nonisotopic immunoassays are homogeneous. In this respect, a homogeneous nonisotopic immunoassay has a simpler reaction mechanism, usually making it easier to automate. However, with the development

of more sophisticated analytical instrument systems and methodologies, the heterogeneous assay is commonly automated as well.

Immunoassay reactions may occur entirely in a soluble phase or may involve solid-phase reactants and are correspondingly classified as soluble or solid-phase. Antibody or antigen or hapten reagents may be attached to solid-phase material, such as the walls of glass or plastic vessels or particles of glass, plastic, latex, or colloidal gold. The antigen–antibody reactions can occur on the solid-phase, which facilitates subsequent separation of the bound and free fractions.

Immunoassay reaction processes may be thought of as simultaneous (one-step) or sequential (multistep). All of the reactants and sample analytes may be incorporated into the reaction mixture simultaneously, or sample analyte and reactants may be added in a step-wise fashion followed by sequential incubations and separations. Often, sequential processes use more than a single antibody (first antibody) in the reaction scheme and use additional antibodies (second antibodies) or other binding reagents to effect the labeling process.

Finally, immunoassays are either competitive or noncompetitive. In competitive reactions, the sample analyte typically competes with labeled reagent analyte (tracer) for binding to a limited amount of primary antibody. In competitive radioimmunoassay, the radioactivity signal measured is inversely proportional to the amount of analyte in the test sample. Depending on the type of nonisotopic immunoassay, the signal measured may be inversely or directly proportional to the sample analyte amount.

Noncompetitive immunoassays use nonlimiting amounts of primary antibody in the reaction and capture all of the analyte or an amount proportional to all of the sample analyte. Noncompetitive immunoassays are referred to by type as immunometric, also "sandwich" and "two-site" immunoassays. Hapten analyte assays are usually not noncompetitive, because the hapten molecule may be too small to have at least two antigenic determinants or applicable binding sites required for the "sandwich" process.

It is apparent from the aforementioned classifications and characteristics that there is the potential for a great number of variations in immunoassay methodological principles and reaction processes. In fact, many different combinations and permutations of these assay principles have been used in the development of specific assays.

A. RADIOIMMUNOASSAY

Radioimmunoassay, Roche Abuscreen® (heterogeneous, soluble, sequential, and competitive):

$$\text{Drug (sample)} + \text{Drug} - * + \text{Ab}_1 \leftrightarrows \text{Ab}_1 - \text{Drug (sample)} + \text{Ab}_1 - \text{Drug} - *.$$

$$\xrightarrow{\text{Ab}_2} \text{Ab}_2 - \text{Ab}_1 - \text{Drug} + \text{Ab}_2 - \text{Ab}_1 - \text{Drug} - *.$$

The bound and free fractions are physically separated by second antibody precipitation and centrifugation. The supernate is decanted, and the radioactivity of the precipitate (bound fraction) is counted. Radioactivity is inversely proportional to the amount of Drug (sample), and concentration is determined by interpolation from a calibration curve. Drug – * = ^{125}I- or ^{131}I-labeled drug reagent; Ab_1 = primary antibody; and Ab_2 = heterologous antiimmunoglobulin second antibody (e.g., if the first antibody is a rabbit antidrug antibody, then the second antibody might be goat anti-rabbit IgG).

B. ENZYME IMMUNOASSAY

Enzyme Multiplied Immuno Technique,[4] Syva EMIT®, Beckman Instruments, Diagnostic Reagents, Inc. (soluble, homogeneous, simultaneous, and competitive):

$$\text{Drug (sample)} + \text{Drug} - \text{Enz} + \text{Ab}_1 \xrightleftharpoons{\text{Substrate} - \text{Cofactors}}$$

$$\text{Ab}_1 - \text{Drug (sample)} + \text{Ab}_1 - \text{Drug} - \text{Enz}.$$

The enzyme activity is measured in terms of the rate of spectrophotometric absorbance change at a specified wavelength or fluorescence change if the enzymatic product is fluorescent. This delta absorbance is directly proportional to the amount of drug in the sample, because the enzyme activity is sterically inhibited when antibody binds to the conjugated drug–enzyme moiety. Drug – Enz = enzyme (e.g., glucose-6-phosphate dehydrogenase)-labeled drug reagent, and Ab_1 = primary antibody.

Enzyme-Linked Immunosorbent Assay, Sigma SIA®, Microdiagnostics EIA, Boehringer Mannheim (heterogeneous, solid phase, sequential, and competitive):

$$\text{Drug(sample)} + \text{Drug} - \text{Enz} +]{-}\text{Ab}_1 \leftrightarrows]{-}\text{Ab}_1 - \text{Drug(sample)} +]{-}\text{Ab}_1 - \text{Drug} - \text{Enz}.$$

After washing of the solid-phase material to remove unbound substances, and following substrate addition, the enzymatic activity is measured from the solid phase. The enzymatic activity (rate of change in spectrophotometric absorbance) is inversely proportional to the amount of drug in the sample. Drug – Enz = enzyme (e.g., alkaline phosphatase)-labeled drug reagent, and] – Ab_1 = primary antibody linked to a solid phase (e.g., microtiter well walls, test tube walls, or particles).

Several different approaches to the basic ELISA principle have been developed. For example, the technique of microparticle enzyme immunoassay has been developed by Abbott Laboratories for the measurement of tacrolemus (FK 506) on the Abbott IMx instrument system.[5]

Microparticle Enzyme Immunoassay, Abbott Laboratories[5] (heterogeneous, solid phase, sequential and competitive):

$$\text{Drug(sample)} +] - \text{Ab}_1 \leftrightarrows]{-}\text{Ab}_1 - \text{Drug(sample)}.$$

$$]{-}\text{Ab}_1 - \text{Drug(sample)} +]{-}\text{Ab}_1 + |||\leftrightarrows |||] - \text{Ab}_1 + |||] - \text{Ab}_1 - \text{Drug(sample)}.$$

$$|||] - \text{Ab}_1 + \text{Drug–Enz} \leftrightarrows |||] - \text{Ab}_1 - \text{Drug–Enz}.$$

In the Abbott IMx automated immunoassay system for the measurement of tacrolemus (FK 506), the drug in the sample is incubated with antidrug antibody bound to latex microparticles. After incubation, an aliquot of this solution is transferred (in the second equation above) to a glass fiber matrix that irreversibly binds the latex microparticles. Sequentially (in the third equation above), the enzyme drug conjugate reagent (drug-alkaline phosphatase) is added to the glass fiber matrix and is proportionally captured by remaining unbound antibody sites on the microparticles.

After washing the fiber matrix to remove unbound substances, the substrate 4-methylumbelliferyl phosphate is added. The rate of enzymatic hydrolysis to form the fluorescent 4-methylumbelliferone is measured by a surface reading flurometer. This rate of fluorescence production is inversely proportional to the amount of drug in the sample.] – Ab_1 = primary antidrug antibody bound to the latex microparticle, ||| = glass fiber matrix, and Drug–Enz = analyte drug conjugated to alkaline phosphatase.

Enzyme-Linked Fluorescence Immunoassay, BioMERIEUX Vitek (heterogeneous, solid phase, sequential, and competitive):

$$\text{Drug(sample)} + \text{Drug} - \text{Enz} +]{-}\text{Ab}_1 \leftrightarrows]{-}\text{Ab}_1 - \text{Drug(sample)} +]{-}\text{Ab}_1 - \text{Drug} - \text{Enz}.$$

A pipette tip-like disposable device, the solid-phase receptacle, serves as a solid phase coated with the antidrug mouse monoclonal antibody. The drug in the sample and the reagent drug–enzyme conjugate are cycled from a sample well in and out of the solid-phase receptacle. The sample drug and drug–enzyme conjugate compete for binding to antibody on the solid phase. Wash steps remove unbound conjugate from the solid phase, followed by addition of the substrate.

The solid-phase, antibody-bound drug–enzyme conjugate is inversely proportional to the amount of drug in the sample. The enzyme, alkaline phosphatase, catalyzes the conversion of 4-

methylumbelliferyl phosphate to the fluorescent product, 4-methylumbelliferone, and the resulting fluorescence measured is inversely proportional to the concentration of drug in the sample.] – Ab_1 = antidrug monoclonal antibody bound to the solid phase, and Drug – Enz = analyte drug conjugated to the enzyme, alkaline phosphatase.

Radial, Partition Enzyme Immunoassay, Baxter International, Stratus (heterogeneous, solid phase, simultaneous and competitive):

$$\text{Drug(sample)} + \text{Drug–Enz} +] - Ab_1 \leftrightarrows] - Ab_1 - \text{Drug(sample} +] - Ab_1 - \text{Drug–Enz}.$$

Analyte drug in a sample and drug–enzyme conjugate are added to antidrug antibodies immobilized on a glass fiber pad. After an incubation period allowing competitive binding between the drug in the sample and the drug–enzyme complex, all unbound drug and drug–enzyme conjugate is radially eluted from the field of view by application of a wash solution. A fluorogenic substrate, 4-methylumbelliferyl phosphate, is incorporated in the wash solution and the enzyme reaction is initiated with the wash step. The rate of fluorescence produced by the hydrolysis to 4-methylumbelliferone is measured by front surface fluorometry and is inversely proportional to the drug concentration in the sample. Drug – Enz = analyte drug conjugated to alkaline phosphatase, and] – Ab_1 = antidrug antibody bound to the solid-phase pad.

Cloned Enzyme Donor Immunoassay,[6,7] Microgenics, Boehringer Mannheim Diagnostics (homogeneous, soluble, simultaneous, and competitive): The enzyme β-galactosidase from *Escherichia coli* bacteria has been genetically engineered by recombinant DNA techniques to produce two fragments. Separately, these two fragments are enzymatically inactive. The fragments in solution will spontaneously combine to form complete and fully active tetrameric molecules of β-galactosidase. One of the fragments is termed the enzyme donor (ED) and the other enzyme acceptor (EA). The ED fragment is conjugated to the drug analyte and will compete with the drug in the test sample for binding to a limited amount of specific monoclonal antibody. The analyte drug has been attached to the ED so that binding of antidrug antibody will inhibit the association of the two enzyme fragments.

$$\text{Drug(sample)} + \text{Ed} - \text{Drug} + \text{EA} + Ab_1 \xrightarrow{\text{Substrate}} Ab_1 - \text{Drug(sample)} + Ab_1 - \text{Drug} - \text{ED} + \text{EA} - \text{ED} - \text{Drug} + \text{Product}.$$

Antibody binding to the ED–Drug fragment inhibits binding to the EA fragment, preventing formation of the active enzyme. The more drug in the test sample, the less antibody will be available to bind to the ED–Drug fragment; therefore, more active enzyme will be formed by combination of EA with ED–Drug fragment. Competitive protein binding results in the formation of an amount of active enzyme and product that is directly proportional to the concentration of analyte in the sample.

The rate of hydrolysis of the substrate, chlorophenol red-β-Δ-galactopyranoside, to chlorophenol red is measured spectrophotometrically by the change in absorbance of 560 nm. ED–Drug = inactive enzyme donor fragment of β-galactosidase conjugated to the analyte drug or drug metabolite, EA = inactive acceptor fragment of β-galactosidase, Ab_1 = primary antidrug antibody, and EA–ED–Drug = complete tetrameric enzymatically active β-galactosidase.

Particle-Enhanced Turbidimetric Inhibition Immunoassay,[8] DuPont, Beckman, Roche ONLINE[9] (homogeneous, soluble, simultaneous, and competitive):

$$\text{Drug(sample)} + \text{PR–Drug} + Ab_1 \leftrightarrows \text{PR–Drug} - Ab_1 + Ab_1 - \text{Drug(sample)}.$$

The particle reagent (PR–Drug) is a latex particle or microparticle with the analyte drug linked to the surface. Aggregation of these particles occurs when a specific monoclonal antidrug antibody is present and antigen–antibody complexes are formed. Drug in the sample competes with the parti-

cle–drug reagent for a limiting amount of the antidrug antibody, thereby decreasing the aggregation process.

The rate of aggregation is measured turbidimetrically at 340 nm (or 520 nm, Roche ON-LINE) and is inversely proportional to the concentration of drug in the sample. PR–Drug = latex particle linked to the analyte drug; Ab_1 = antidrug antibody, in some cases the antibody fragment [$F(ab^1)_2$], is used rather than complete antibody to eliminate certain interferences, such as the rheumatoid factor; and PR–Drug–Ab_1 = aggregation species of particles resulting from the formation of particle–drug–antibody complexes.

C. FLUORESCENCE IMMUNOASSAY

Substrate-Labeled Fluorescent Immunoassay, Bayer (Miles–Technicon, Ames Optimate) (homogeneous, soluble, simultaneous, and competitive):

$$\text{Drug (sample)} + \text{Drug} - \text{fluorogenic substrate} + Ab_1 \leftrightarrows Ab_1 - \text{Drug} + Ab_1 - \text{Drug} - \text{fluorogenic substrate}.$$

$$\text{Drug} - \text{fluorogenic substrate} \xrightarrow[\text{hydrolysis}]{\text{Enzyme}} \text{fluorophore}.$$

The enzyme (e.g., β-galactosidase) will hydrolyze the fluorogenic substrate (e.g., β-galactosylumbelliferone) to produce the flurophore, umbelliferone, if the "drug–fluorogenic substrate" complex is not bound to the antibody. Antibody binding to the complex inhibits enzymatic activity. The measured fluorescence produced by the fluorophore is thereby directly proportional to the amount of drug in the sample. A sample with a high drug concentration competitively binds more of the limiting antibody, thus providing more "free" drug–fluorogenic substrate to be available for enzymatic hydrolysis. Ab_1 = primary antibody, Drug–fluorogenic substrate = labeled drug reagent, and Enzyme = reagent that will hydrolyze the Drug–fluorogenic substrate complex.

Fluorescence Immunoassay, Roche, Pharmacia Diagnostics, Baxter International, BioMERIEUX Vitak (heterogeneous, solid phase, simultaneous or sequential, and competitive):

$$\text{Drug(sample)} + Ab_1 - * \leftrightarrows \text{Drug(sample)} - Ab_1 - *.$$

$$Ab_1 - * +] - \text{Drug} \rightarrow] - \text{Drug} - Ab_1 - *.$$

In this method, the primary antibody is labeled with a fluorophore. Drug in the sample and solid-phase reagent Drug compete for a limiting amount of the antibody. After the initial incubation, the solid phase is washed, and then fluorescence is measured from the solid phase. The fluorescence measured is inversely proportional to the amount of drug in the sample. $Ab_1 - *$ = primary antidrug antibody labeled with a fluorescent substance, and] – Drug = analyte drug coupled to a solid phase.

Fluorescence Polarization Immunoassay,[10] Abbott TDx, ADx, TDxFLx, AxSYM; Roche Cobas Bio (homogeneous, soluble, simultaneous, and competitive):

$$\text{Drug (sample)} + \text{Drug} - * + Ab_1 \leftrightarrows Ab_1 - \text{Drug} + Ab_1 - \text{Drug} - *.$$

The drug in the sample competes with the fluorescently labeled drug (tracer) for a limiting amount of antidrug antibody. The more drug in the sample, the less labeled drug can bind to the antibody.

Fluorescence polarization of the resulting solution is measured and reflects the amount of antibody-bound drug–fluorescently labeled complex (Ab_1 – Drug – *). The large molecular complex of Ab_1 – Drug – * has a long rotational relaxation time and a low rotational velocity that permit fluorescence polarization. That is, the large molecules undergo only a few degrees of rotation during the

interval between excitation and fluorescence emission and will emit fluorescence in the same plane, thereby maintaining polarization. The much smaller molecules of free Drug – * rotate rapidly and will be depolarized by the time they emit light.

Fluorescence polarization is inversely proportional to the concentration of drug in the sample. Drug – * = drug labeled with fluorophore, usually fluorescein; and Ab_1 = primary antibody.

Chemiluminescence Immunoassay, Ciba-Corning, Diagnostic Products Corp., MAGIC®Lite (heterogeneous, solid phase, sequential, and competitive): Chemiluminescence is the emission of light associated with the dissipation of energy from an electrically excited substance. The orbital electrons of a luminescent compound are stimulated to a higher energy state by a chemical reaction and give off energy in the form of light as they return to the ground state. Chemiluminescent tracers are conjugated to the analyte of interest in this competitive assay.

$$\text{Drug(sample)} + \text{Drug} - * +] - Ab_1 \leftrightarrows] - Ab_1 - \text{Drug(Sample)} +] - Ab_1 - \text{Drug} - *.$$

Separation of the bound from the free tracer drug may be accomplished by magnets to localize solid-phase paramagnetic particles for washing and removal of the supernatant liquid. The chemiluminescent reaction is initiated by the addition of hydrogen peroxide at the appropriate pH, and the luminescence from the solid–phase particles is measured by a luminometer. Concentration of drug in the sample is inversely proportional to the luminescence.

In the Diagnostics Products Corporation (DPC) automated IMMULITE immunoassay instrument system, the solid-phase, antibody-coated bead is separated from the supernate by high-speed spinning of the reaction tube about its vertical axis expelling the fluid. Drug – * = an acridinum ester (luminescent tracer)-labeled analyte, and]–Ab_1 = a monoclonal specific antibody in limited concentration covalently attached to a micron-sized paramagnetic particle or bead.

Paramagnetic–Particle Chemiluminescent Enzyme Immunoassay, Sanofi Diagnostics Pasteur (heterogeneous, solid phase, simultaneous, and competitive):

$$\text{Drug(sample)} + \text{Drug–Enz} + Ab_1 +] - Ab_2 \leftrightarrows - Ab_2 - Ab_1 - \text{Drug(sample)} +] - Ab_2 - Ab_1 - \text{Drug–Enz}.$$

Drug in the sample competes with the drug–enzyme conjugate for binding sites on a limited amount of specific mouse monoclonal antidrug antibody. Resulting hapten–antibody complexes bind to the paramagnetic particles coated with second antibody, goat anti-mouse immunoglobulin. Separation of the solid phase is achieved in a magnetic field. After washing to remove unbound substances, a chemiluminescent substrate, Lumi-Phos 530, is added and the light generated is measured with a luminometer. The photon production is inversely proportional to the concentration of drug in the sample. Drug–Enz = analyte drug conjugated to alkaline phosphatase, Ab_1 = mouse monoclonal antidrug antibody, and]–Ab_2 = paramagnetic particle coated with goat anti-mouse antibody.

D. PARTICLE IMMUNOAGGREGATION

Latex Particle Agglutination-Inhibition Assay, Roche ABUSCREEN ONTRAK (homogeneous, solid phase, simultaneous, and competitive):

$$\text{Drug(sample)} + \text{Drug} \sim [\ ] \sim \text{Drug} + Ab_1 \rightarrow \text{Agglutinated latex particles by antibody cross-linking.}$$

This test is manually performed and visualized on a slide. The drug in the sample competes with the drug-coated latex particles for a limiting amount of antibody and thereby inhibits agglutination. The presence of agglutination visualized by the formation of white particles is indicative of a negative

drug sample, whereas no agglutination and smooth milky appearance, indicate a positive reaction for the drug under analysis.

Colloidal Gold Aggregation-Inhibition Assay,[11] Biosite Diagnostics TRIAGE™ (heterogeneous, solid phase, sequential, and competitive): This monoclonal-based immunoassay system simultaneously detects multiple drug or drug metabolite analytes in a manual and visual read competitive assay system.

1. In the first step, the urine sample is dispensed volumetrically into a reaction cup on the test device cartridge containing dry reagent beads. The contained reagents (monoclonal antibodies for the targeted drugs, colloidal gold particles conjugated to the representative analyte drugs, and buffer) are reconstituted by the urine specimen.
2. After a 10-min incubation, the reaction mixture is transferred to the detection area on the test cartridge. The detection area is configured as a linear strip that contains a nylon membrane with monoclonal antibodies to the drugs immobilized in seven discrete detection zones.
3. In the first step, free drugs in the specimen compete with the drugs conjugated to the colloidal gold particles for the respective soluble antibody. If no drug is present in the specimen, then a maximum amount of antibody will be bound to the conjugated drug (gold particles). If drug is present in the specimen, then competitively, some or all of the conjugated drug on the gold particles will be unbound to antibody.
4. In the second step, gold particles that have antibody unbound drug sites can be captured by the membrane-immobilized specific antibodies producing a visualized color bar at the zone corresponding to the specific drug (Positive Specimen). Gold particles that have their conjugated drug moieties bound to the antibody from the first reaction will not be captured by the immobilized second set of specific antibodies (Negative Specimen).
5. After a wash solution has been applied to the detection zone, washing through and away the unbound gold particles, the remaining presence of distinct color bars at the specific drug zones are read visually within 5 min. Only specimens containing the drug(s) or metabolite(s) at or above a cutoff level will produce a detectable color response.
6. The specific zones for specific drugs are labeled on the test device, along with system positive and negative control sites to ensure appropriate reagent performance.
7. The following drugs or appropriate metabolites are detectable simultaneously by the currently available TRIAGE™ test device at the following cutoff levels: phencyclidine, 25 ng/ml; benzodiazepines, 300 ng/ml; benzoylecgonine, 300 ng/ml; amphetamines, 1000 ng/ml; tetrahydrocannabinol, 50 ng/ml; opiates, 300 ng/ml; and barbiturates, 300 ng/ml.

Additional versions of the TRIAGE™ menu does now include tricyclic antidepressants and methadone.

III. IMMUNOASSAY ANALYZERS

There are at least 20 automated immunoassay analyzer systems commercially available in the U.S. for toxicology assays produced by at least 12 different manufacturers. These manufacturers provide the methods and reagents, as well as the analytical instruments for testing of drug and drug metabolites. In addition, there are no less than 20 automated clinical chemistry analyzers in the U.S. produced by at least ten different manufacturers that use certain immunoassays for therapeutic drug monitoring and drug of abuse testing. These manufacturers, in many cases, produce their own methods and reagents for this testing. However, a number of the clinical chemistry instruments can also accommodate "user-defined" methods and reagents that are manufactured by other vendors.

All of these automated analytical systems use nonisotopic techniques, such as immunonephelometry, fluorescence polarization, fluorescence immunoassay, enzyme immunoassay, or chemilu-

minescence. Radioimmunoassays have not been automated very successfully, and the respective analyzers have essentially been eliminated from the marketplace through competition with the non-isotopic automated systems. Tables 13–2 and 13–3 list most of the analytical systems and their respective methodologies and/or capabilities.

Generally speaking, the cost of therapeutic drug monitoring and drug of abuse testing assays on automated immunoassay analyzers are more expensive than assays for the respective analytes on automated clinical chemistry analyzers. For this reason, there is a trend developing of movement, when possible, of these assays from the immunoassay analyzers to the clinical chemistry analyzers. Currently, however, the menus on the clinical chemistry analyzers are not as extensive and usually include only the more high-volume analyte assays. The choice of an analytical system for a given laboratory will be influenced by the volume and breadth of testing required and the need for rapid, continuous testing.

Two of the most common therapeutic drug monitoring assays found on most of the analytical systems, immunoassay analyzers, and clinical chemistry analyzers combined, are digoxin and theophylline. The College of American Pathologists Proficiency Testing Survey on Therapeutic Drug Monitoring is useful for reviewing the relative number of laboratories using the respective analyti-

TABLE 13–2
Immunoassay Analyzers for Toxicology/TDM

Abbott Laboratories		
TDx	FPIA	>25 TDMs, 8 DAUs
ADx	FPIA	8 DAUs
IMx	MEIA	Tacrolemus (FK 506)
AxSYM	FPIA, MEIA	14 TDMs, 10 DAUs
Baxter International		
Stratus, Stratus II	FIA, EIA	>15 TDMs
Behring Diagnostics		
OPUS, OPUS Magnum, OPUS Plus	Fluorogenic ELISA	>9 TDMs
BioMERIEUX Vitek		
Mini VIDAS, VIDAS	Enzyme-linked FIA	Theophylline, digoxin
Bayer (Miles/Technicon)		
Immuno 1	Latex agglutination	>11 TDMs
Boehringer Mannheim		
ES 300	Solid-phase EIA	Digoxin
Chiron Diagnostics		
ACS I80 Plus (Ciba Corning)	CLIA	6 TDMs
Diagnostic Products		
Immulite	CLIA	Theophylline, digoxin
F. Merck		
MAGIA 7010, 8000	Magnetic MEIA	Digoxin
Pharmacia Diagnostics		
Fluoro Count 96	FIA	Theophylline
Roche		
Cobas Fara II	FIA	>14 TDMs, 10 DAUs
Sanofi Diagnostics Pasteur		
Access	CLIA	Theophylline, digoxin
Serono		
SR1	Magnetic EIA	Digoxin
Wallac		
Delfia	Time-resolved fluorescence immunoassay	Digoxin

Note: Abbreviations used are—TDM, therapeutic drug monitoring; FPIA, fluorescence polarization immunoassay; DAU, drug of abuse; MEIA, microparticle enzyme immunoassay; FIA, fluorescence immunoassay, EIA, enzyme immunoassay; CLIA, chemiluminescence immunoassay.

TABLE 13–3
Automated General Chemistry Analyzers with Toxicology/ TDM Immunoassay Capabilities

Abbott Laboratories	
ABA, Spectum, EPx	EMIT Enzyme inhibition IA
Bayer (Miles/Technicon)	
Technicon RA 1000, RA 2000, RA-XT, Chemotherapy 1, AXON, OPERA	EMIT
Beckman Instruments	
Synchron CX4, CX5, CX7	EMIT Immunoturbidimetric, PETINIA CEDIA
Behring Diagnostics	DRI
SYVA ETS	EMIT
Boehringer Mannheim	
Hitachi 704, 705, 717, 736, 747, 911, and 917	EMIT CEDIA
Baxter International	DRI
aca, aca STAR, Dimension AR, XL (formerly DuPont)	EMIT PETINIA
Coulter	
Dacos XL, Optichem	EMIT
Instrumentation Laboratory	DRI
IL 900, 1800	EMIT
Monarch Plus	
Olympus	
AU 800/5200	EMIT
REPLY, AU 580	
Roche	
COBAS MIRA Plus, FARA	EMIT, FIA, DRI
COBAS INTEGRO	

The EMIT reagents and methodology are generally used on these chemistry analyzers as "user-defined" tests. The EMIT reagents are sold primarily by Behring Diagnostics (Syva Corp.) however, another vendor, Diagnostic Reagents, Inc., also manufactures EMIT reagents.

Behring Emit Menu:

Urine, DAUs

Amphetamine	Methadone	
Barbiturates	Methaqualone	
Benzodiazepines	Opiates	
Cannabinoids	Phencyclidine	
Cocaine metabolite	Propoxyphene	
Ethyl alcohol		

TDMs

Amikacin	Gentamicin	Phenytoin
Amitriptyline	Imipramine	Primidone
Carbamazepine	Lidocaine	Procainamide
Caffeine	Methotrexate	Quinidine
Chloramphenicol	*N*-Acetylprocainamide	Theophylline
Desipramine	Netilmicin	Tobramycin
Disopyramide	Nortriptyline	Valproic acid
Ethosuximide	Phenobarbital	Vancomycin

Serum toxicology

Acetaminophen	Ethyl alcohol
Barbiturates	Tricyclic antidepressants
Benzodiazepines	

Note: Abbreviations used are—TDM, therapeutic drug monitoring; EMIT, enzyme multiplied immuno technique; IA, immunoassay; PETINIA, particle-enhanced turbidimetric inhibition immunoassay; CEDIA, cloned enzyme donor immunoassay; FIA, fluorescence immunoassay.

cal systems and the relative imprecision of the assays. Table 13–4, from a 1995 survey, demonstrates these characteristics for digoxin and theophylline.

IV. IMMUNOASSAY ATTRIBUTES AND LIMITATIONS

All of these various nonisotopic immunoassays have been used successfully in automated analytical instrument systems. The variety and flexibility of the various mechanisms are quite remarkable, offering a multitude of choices and options for appropriate and unique assay design for respective drugs and optimum performance. Automation of drug assays has reduced labor costs (and, in some cases, material costs) and has improved test turnaround time and precision. Calibration curves are stored up to weeks or months, and calibrations are less frequent because of the stability of these systems. The analytical sensitivity of these methods are generally very adequate for most drugs, with the potential of sensitivities in decreasing order being particle immunoaggregation, enzyme immunoassay, fluorescence immunoassay, and chemiluminescence immunoassay. The combinations of enzyme labeling with fluorescent or chemiluminescent reactions further increase analytical sensitivity. The more sensitive of these approaches are comparable or exceed the sensitivity of radioimmunoassay.

The analytical specificity of immunoassays is a potential problem. Antibodies developed for a given drug may cross-react with another chemically related drug or endogenous substance. Because

TABLE 13–4
College of American Pathologists Proficiency Testing Survey Results, 1995

Drug/method	No. of labs.	Mean	SD	CV
Digoxin (ng/ml)				
Abbott AxSYM	47	1.55	0.10	6.6
Abbott TDx/TDxFLx	1675	1.53	0.11	7.4
Ames Seralyzer	5	—	—	—
BDI Opus/Plus/Magnum	218	1.59	0.14	8.8
Beckman Synchron	359	1.76	0.10	5.6
Becton Dickinson	16	1.67	0.10	6.1
BMC Cedia	83	1.63	0.15	9.4
BMC Cedia XL/SC	12	1.63	0.16	9.9
CIBA Corning ACS:180	135	1.74	0.13	7.2
CIBA Corning Magic	15	1.88	0.15	7.8
Clinical assays	13	1.78	0.23	12.7
Dade Stratus	74	1.78	0.12	6.5
Dade Stratus II	772	1.79	0.11	6.2
DPC Coat-A-Count	31	1.70	0.15	8.9
DuPont aca	669	1.65	0.10	6.1
DuPont Dimension	47	1.70	0.12	7.2
Instrumentation Lab	9	—	—	—
International Bioclinical	7	—	—	
Kodak	58	1.75	0.16	8.9
Microgenics Cedia	108	1.78	0.11	6.4
Microgenics XL, R′	23	1.73	0.12	6.8
Roche Cobas-FP	16	1.84	0.21	11.2
Roche-Micropart AGG IN	41	1.91	0.19	9.8
Sanofi Access	31	1.74	0.13	7.4
Sigma FPIA	11	1.66	0.16	9.4
Syva EMIT	12	1.71	0.20	11.8
Syva EMIT 2000	201	1.69	0.11	6.5
Technicon Immuno-1	28	1.80	0.16	8.9
All methods/all results	4769	1.65	0.16	9.7

TABLE 13–4 *Continued*

Drug/method	No. of labs.	Mean	SD	CV
Theophylline (μg/ml)				
Abbott AxSYM	240	11.17	0.31	2.8
Abbott IMx	8	—	—	—
Abbott TDx/TDxFLx	1634	11.20	0.48	4.3
Ames Seralyzer	8	—	—	—
BDI Opus/Plus/Magnum	112	15.25	1.46	9.6
Beckman Array	26	11.28	0.51	4.5
Beckman Synchron	415	10.98	0.79	7.2
BMC Cedia	157	11.36	0.72	6.3
Dade Paramax System	64	11.19	0.60	5.4
Dade Stratus	7	—	—	—
Dade Stratus II	164	12.07	1.10	9.1
DuPont aca	605	11.83	0.68	5.8
DuPont Dimension	273	11.79	0.69	5.9
HPLC	6	—	—	—
Instrumentation Lab.	6	—	—	—
International Bioclinical	11	11.37	0.49	4.3
Kodak	642	14.71	0.81	5.5
Microgenics Cedia	15	11.39	0.60	5.3
Roche Cobas-FP	90	11.86	0.76	6.4
Sigma FPIA	11	11.81	0.94	8.0
Syva EMIT	60	11.65	0.73	6.3
Syva EMIT 2000	211	11.62	0.80	6.9
Technicon	35	10.75	0.51	4.7
Technicon Immuno-1	23	12.29	0.49	4.0
All methods/all results	4858	11.88	1.38	11.6

Note: Abbreviations used are—SD, standard deviation; CV, coefficient of variation; Cedia, cloned enzyme donor immunoassay; AGG IN, agglutination inhibition; FP, fluorescence polarization; FPIA, fluorescence polarization immunoassay; EMIT, enzyme multiplied immuno technique.

there are so many medications available by prescription or over the counter, individuals may be ingesting substances that will cross-react in a given drug assay. This issue is continually under investigation as newer and more drugs become available. It is essential that a manufacturer provide cross-reactivity data with each assay and continually update these investigations. It is not uncommon to test hundreds of drugs; in one case, for BIOSITE TRIAGE, the company has tested over 1500 drugs for potential interferences. The more and better cross-reactivity data one has, the more confident one can be about the assay. The issue of cross-reactivity or lack of specificity is or has been an item of concern for virtually all of the current immunoassays.

It is also very important that immunoassays be designed to avoid interference by endogenous substances. For example, it is imperative to know of the cross-reactivity of "digoxin-like immunoreactive substances" in a given digoxin assay.

For immunoassays for the detection of drugs within a class, cross-reactivity is an advantage. However, cross-reactivity is not equivalent for all the drugs within a class; consequently, the detection of a "positive" can be variable. Knowledge of the degree of cross-reactivities is important for the proper use and interpretation of these assays. In addition, multiple antibodies may be used in these class-type immunoassays.

With regard to antibody specificity, it is important to know that certain drug or drug class immunoassays may be designed to detect primary metabolites of the drugs rather than the parent compound.

The measurement of fluorescence production in an assay can produce a very sensitive analysis. However, many normal components of biological specimens, as well as xenophobic substances, are

capable of producing fluorescence, causing a background fluorescence that limits the ability to measure separately only the tracer's signal. The measurement of the rate of fluorescence in an enzymatically catalyzed reaction producing a fluorescent product is useful in avoiding the potential background fluorescence and is potentially more sensitive than measuring direct fluorescence. Time-delayed resolved fluorescence immunoassays attempt to minimize this problem as well by delaying readout of the tracer until background fluorescence has decayed. Of course, fluorescence polarization as a measurement is also quite helpful in avoiding certain levels of background fluorescence.

In general, homogeneous assays are more susceptible to endogenous interferences, such as hemoglobin, bilirubin, or lipids. Competitive type assays are generally better in the lower concentrations of the measurable range, in that the signal change per unit of concentration (the slope) is greater in that range. Noncompetitive or sandwich assays are generally restricted to molecules large enough to bind to two antibodies at different sites.

Adulterants are a problem for immunoasays, and there are a variety of substances that if added to the specimen will cause an artifactural result.[12–15] This is particularly important for urine forensic drug testing. Most of these problems can be avoided by appropriate urine collection procedures.

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Chapter 14

HAIR ANALYSIS FOR DETECTION OF DRUGS OF ABUSE

Thomas Cairns, Donald J. Kippenberger, and AnnMarie Gordon

CONTENTS

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I. INTRODUCTION

When Sir Arthur Conan Doyle popularized forensic toxicology through his successful series of fictitious detective stories concerning the exploits of Sherlock Holmes and Dr. Watson, little did he know that in the 1990s hair analysis for drugs of abuse would become a popular method of choice for detection of drug users. This interest in hair as a valuable tissue sample for the identification of drug use has grown rapidly over the last decade. This is evidenced by the sharp increase in papers published in the scientific literature during 1985 to 1995. The work of Baumgartner and others[1–3] has also shown that drugs are exceptionally stable in the hair matrix. For instance, it was Baumgartner who first identified morphine in a historic hair sample (i.e., in a sample from the 19th century romantic English poet, John Keats, who died at age 26 while taking laudanum [tincture of opium] for tuberculosis). During the last 5 years, a number of reviews of the topic have seemed to define the unique properties of hair analysis and the areas where additional research is needed (e.g., a better understanding of the mechanisms of incorporation of drugs and their metabolites, factors influencing analyte stability, and, most of all, the confirmation of drugs by ultrasensitive mass spectrometry (MS).[4–9]

II. MECHANISM OF DRUG INCORPORATION INTO HAIR

The structure of a hair shaft is illustrated in Figure 14–1. Hair growth initiates from a sac-like structure called a follicle that has a diameter of 15 to 120 μm, depending on the body site. This follicle is surrounded by a network of capillaries that nourishes the follicle. One of the main components of hair is a sulfur-containing protein called keratin that forms long, fiber-like structures. The cuticle or outer surface of the hair is composed of a single layer of anucleated cells about 0.5 to 1.0 μm in depth. The main function of the cuticle is to protect the inner structures of the hair. However,

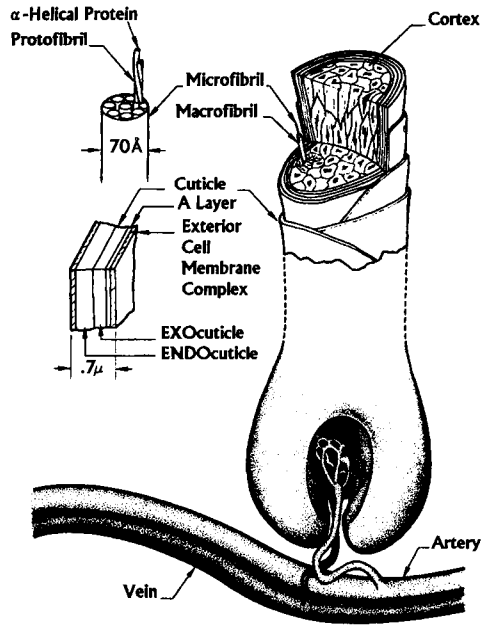

FIGURE 14–1. Transfer of drug from the circulatory system to the hair follicle and its subsequent encapsulation in keratin fibers of the hair shaft.

during normal growth, this cuticle layer tends to break down because of either mechanical and/or chemical damage.

The cortex is the most predominant structural element of hair and primarily consists of tightly packed macrofibrils containing keratin. Pigmentation granules are also located in the cortex. The primary pigment of hair is melanin. The centrally located medulla cells are loosely packed and interspersed with airspaces. The human hair life cycle consists of three major phases—the anagen or growth phase, the catagen or transition phase (wherein the root of the hair becomes keratinized and begins to separate from the bulb), and the telogen or resting phase. The resting phase lasts approximately 2 to 3 months. At any given time, approximately 85% of the head hairs are in the anagen or growth phase. The most uniform growth rate occurs at the posterior vertex (crown) of the scalp. Head hair has been demonstrated to grow at a rate of approximately 1.3 cm/month. It will take approximately 4 to 5 days for the growing hair shaft to emerge from the scalp and be available for chemical analysis. This particular feature of hair growth is very important, because it precludes detection of drug consumption during that time window.

Chemicals in the bloodstream, including both licit and illicit drugs and their metabolites, diffuse from the circulatory system to the rapidly growing hair follicle, with subsequent encapsulation within the keratin fibers.[10] Potsch et al.[11] have recently discussed the various pathways for small molecules, such as drugs, to enter the hair. For instance, drugs may also enter the hair from secretions formed in the eccrine, sebaceous, and apocrine glands before the hair emerges from the skin surface and keratinization has taken place. Henderson[12] has suggested that there may be a more complex mechanism of drug incorporation into growing hair. This author discovered that cocaine, for instance, is the primary analyte found in hair after cocaine ingestion despite its very short half-life in plasma. On the other hand, the major blood and urine metabolite, benzoylecgonine, whose concentrations in plasma far exceed cocaine's, is present in hair at relatively lower concentrations. Similarly, Goldberger[13] found that 6-monoacetylmorphine (6MAM) and heroin have been detected in the hair of heroin users even though both moieties have relatively short plasma half-lives. The factors causing such preferential drug incorporation into hair are incompletely understood; in part, they seem to be caused by selectivity in transport processes across the capillary membranes. However, what is abundantly clear is that, after ingestion, drugs and/or their metabolites are transported into the hair where they remain in a stable environment for long periods of time.

III. SAMPLE DEFINITION

Hair testing involves cutting a small sample from the vertex of the head, because it has the fewest hairs in the telogen (resting) phase. Approximately 60 to 100 hairs (weighing about 25 mg) are cut close to the scalp. The collected sample is placed in a secured envelope with the root ends carefully aligned to allow correct sampling of the first 4 cm. This represents the last 90 days of growth.[14] When scalp hair is not available, body/pubic hair or the clippings or shavings of fingernails may be submitted. Chain-of-custody documentation and Medical Review Officer functions are identical to conventional urine screening. Hair samples, however, can be collected under close supervision without embarrassment, and evasion by substitution or adulteration of the sample is not possible. Hair has no special requirements for storage and/or shipment. Another unique feature of hair analysis over urine or sweat testing is that the initial result can be verified by the collection of a second hair sample, referred to in this laboratory as a "safety net" sample. This action is not possible with urine or sweat testing, because a subject who has abstained from drug use for a few days before submitting the second specimen will easily evade detection.

IV. ADVANTAGES OVER URINE

Hair sampling possesses several unique advantages over traditional urine sampling. The primary advantage is the chronological retrospective identification of drugs over the 90-day window via hair

vs. the short window of 72 hours via urine immediately after ingestion. However, the complementary aspect of both tests should not be overlooked. In the case of monitoring for cause after an accident, urine testing provides effective surveillance, because hair growing at the point of ingestion would take 4 to 5 days to emerge from the scalp surface for subsequent sampling. Comparison of data provided by urine vs. hair testing for drugs of abuse has been extensively reviewed.[15–17] These authors have concluded that such tests often provide complementary features based on their windows of detection.

V. TESTING METHODS

The analysis of hair for drugs of abuse involves a tripartite approach: (1) The hair is extensively washed to avoid false-positives caused by external contamination with drugs; (2) the remaining drug analytes, released from the hair matrix by dissolution or various solvent extraction procedures[18–21] of the hair fiber, are analyzed by an initial immunoassay screen; and (3) presumptive positives are confirmed by gas chromatography (GC/MS).

Because it cannot be assumed that normal hygienic practices are of sufficient frequency or effectiveness for removal of externally deposited drugs, there is general agreement that hair specimens should be subjected to additional decontamination procedures in the laboratory before extraction and analysis of the drugs. A variety of different procedures have been used. For example, Kintz and Mangin[22] wash hair for 15 min at 37°C with dichloromethane. Although this is not a hair-swelling solvent, such as water or methanol, there is the possibility that any externally deposited drugs that may have been transported into interior regions of the hair by water (e.g., by perspiration) will not be removed by this solvent. Most analysts prefer to use solvents that access deeper lying hair structures because of their hair-swelling action. Thus, Cone et al.[23] use a 15-min methanol wash at 37°C, and Nakahara et al.[24] do three washes with 0.1% sodium dodecyl sulfate detergent followed by three washes with water for an unspecified period of time. Selavka and Rieders[25] wash hair extensively with water until no significant quantities of drugs are removed from the hair specimen. Baumgartner and Hill[26] use a similar approach, which involves an initial wash with a nonhair-swelling solvent, isopropanol, for removal of drugs or oily residues deposited on the hair surface, followed by a minimum of three 30-min phosphate buffer washes at 37°C for the removal of drugs from deeper lying hair structures, designated as the semiaccessible domain of hair. The efficacy of the wash procedures is monitored by three kinetic parameters involving measurement of the drugs in the washes and those present in the final hair digest. If the kinetic analysis indicates that washing was suboptimal, a new specimen is obtained that is subjected to the same treatment as before plus two additional 60-min phosphate buffer washes.

Residual analytes are liberated by a variety of methods that have been summarized by Selavka and Rieders.[25] A most convenient method, because of its universal applicability to essentially all drugs, is the enzymatic digestion procedure of Baumgartner and Hill.[26] The method has the additional advantage of not extracting drugs from the melanin fraction, a fraction that is removed from the hair digest by centrifugation before analysis as a preventive measure against possible bias caused by hair color effects.

Another additional advantage of the digestion procedure is that all analytes are removed from the dissolved protein matrix with 100% effectiveness. In contrast to this, Rothe and Pragst[27] have shown that optimum extraction efficiencies for a particular drug by solvent-based methods can only be established with great difficulty. The method developed by Baumgartner and Hill[26] has been evaluated in over 150 blind field studies and has been used in the analysis of over 800,000 hair specimens for workplace testing. These studies have included the analyses of the heroin metabolite, MAM, which is effectively trapped by hair. In contrast to these findings, morphine is mostly found in the urine, thereby contributing to the overturn of over 95% of morphine-positive urine samples,[28] because of the probability that the morphine in urine is caused by prescribed codeine or poppy seed ingestion.

For analyses under mass production conditions and with populations containing a preponderance of negative samples, it is advantageous to identify presumptive positive samples by a preliminary immunoassay screen. Such samples are subsequently confirmed by ultrasensitive MS.

A. FORENSIC CONFIRMATION OF PRESUMPTIVE POSITIVES BY GAS CHROMATOGRAPHY MASS SPECTROMETRY (GC/MS)

1. Structural Confirmation via MS

The burden of confirmation for various drugs of abuse detected in hair by RIA (such as amphetamine, methamphetamine, cocaine and benzoylecgonine, cocaethylene, codeine, morphine, 6-acetylmorphine, and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol[cTHC]) has been placed on MS because of its strengths in reproducibility, repeatability, specificity, and limit of detection.[29] Current trends have indicated that this increased reliance is based on the ability to provide detection, confirmation, and quantitation in a single analysis. Scientists, however, are not exempt from the mundane rigors of supporting their findings using predescribed criteria that are generally recognized as scientifically sound.

With the various mass spectrometric methods available for the identification and confirmation of various drugs in human hair, the criteria for confirmation of presence have been constructed on logic as applied to the application on a case-by-case basis. At the low drug levels found in hair samples, various data manipulations or alternate choices of approaching the analytical problem of confirmation have been used to give an acceptable result. The problems experienced when dealing with nanogram- and picogram-level analysis are much more complex than when recording a mass spectrum of an ample supply of a reference standard. The increased reliance on MS to provide the proof of presence has resulted in a widely differing comprehension in the scientific literature of what constitutes a confirmation. To clarify this situation, a general set of guidelines has been formulated by the authors from practical experience in the field to articulate the logic behind what actually constitutes a confirmation, whatever the ionization technique used.

a. Basic Elements of Structural Confirmation

As early as 1989, it was concluded, on purely statistical grounds, that under electron ionization (EI) conditions, a minimum of three structurally significant ions are necessary to provide proof of presence.[29] A full mass spectral scan would, however, continue to provide the maximum and highest level of evidence. The intensity variation for ion abundance ratios was recommended to be within 5% when compared with a reference standard analyzed under similar conditions. However, because many of the softer ionization techniques (e.g., chemical ionization [CI]) do not cause extensive fragmentation, a severe limitation might exist in meeting the suggested general criteria. Moreover, the additional experimental parameters governing the application of these techniques may not permit the same reproducibility observed under EI conditions.

The general principles governing the evolving criteria for confirmation of trace levels by MS has been discussed in detail[29] in terms of the various component factors leading to data acceptance, namely, quality assurance controls, Good Laboratory Practices, Good Measurement Practices, Standard Operating Procedures (SOPs), Protocols for Specific Purposes, recognized official methods status, tuning and calibration, mass spectral quality indices, the reality of variability, and limit of detection.

b. GC Retention Time and MS Scan Rate

The synergistic support received in a mass spectral identification by the retention time of the eluting drug or metabolite is of obvious importance. For drug confirmation, the retention time correlation should normally be within the 2% error factor, compared with repeated injections of the sample or a reference standard. The improved reliance on retention time correlation can be attrib-

uted to the fact that sample and suspected standard are recorded within a relatively short period of time (i.e., contemporaneously).

In the case of capillary column GC/MS, the scan rate plays a important role. Because the elution time frame of the average compound is only 2 to 4 sec, a scan rate capable of acquiring several full scans is often necessary. Under such cases, however, it should be recognized that the sample concentration in the source during a scan varies considerably. At scan rates of 1 sec, ion statistics are usually not severely compromised.[29] Representative spectra, however, will be best acquired by combining all scans over the profile and taking the average. Capillary column conditions offer the main advantage of allowing a full mass spectral scan to be obtained on smaller quantities of material because the compound is concentrated in a very narrow band.

At slow scan speeds relative to overall capillary elution times, the chromatographic reproduction of the retention time can only suffer from a slight perturbation of retention time correlation with a reference standard (at most, 1 to 2 sec). Such deviations are usually well within the 2% error factor expected from injections by the operator. Quantification using capillary GC/MS can only be satisfactorily achieved by employing single-ion monitoring descriptors, whereby dwell times are selected to give multiple data points over the elution profile.

c. Electron Ionization

Historically, the fingerprinting capability of EI spectra, which benefits from the extensive number of fragments formed after ionization, has been the cornerstone of identification either by comparison with published data bases or structural identification via fragmentation patterns. In general, the reproducibility of the EI spectrum of a particular compound by magnetic instruments from one laboratory to another has been acceptable to the extent that direct comparisons were commonplace and reliable. However, with the advent of quadrupole mass spectrometer and more recently ion traps, this ability has been reduced in importance. Because quadrupole instruments and ion traps had the ability to mass discriminate during the tune-up procedures, direct comparisons with EI-generated spectra on magnetic sector instruments caused the correlation of relative abundance ratios from the previously accepted 5% to be extended to as much as 50%. It is recognized that there often exists a variance between a previously recorded spectrum of a reference standard and an actual reported sample finding. For proof of presence, the recording of both sample and reference standard should be conducted on the same instrument on the same day. For confirmation purposes, it has been clearly demonstrated[29] that the desired statistical need for two or more ions of structural significance observed in the correct relative abundance ratios is desirable but not mandatory.

d. Chemical Ionization

The use of CI techniques to favor production of a molecular ion species for characterization purposes has been used for a number of reasons to resolve identification problems. First, the ability to determine the molecular weight of the compound under investigation has often been considered of paramount importance as the initial step in the identification process. Second, the use of CI may avoid the need to perform intensive sample clean-up by suppressing the interfering fragment ions. Third, the use of both methane and ammonia as reagent gases in separate experiments can often reveal the necessary structurally significant ions for confirmation, whatever the polarity of the analyte. However, the CI approach can suffer from a potential deficiency in that the availability to produce more than three ions for confirmation may not always be attainable.

e. Negative-Ion CI (NICI)

The advantages of NICI lies in the possibility of greater sensitivity than positive-ion CI, especially where electron-capturing derivatives of the target compound have been prepared usually with fluorine incorporation. The distinct advantage provided by such compounds is that their detection levels by NICI are in the low picogram range, wherein quantitative support can reinforce GC measurements.

f. Mass Spectrometry/Mass Spectrometry

The production of a protonated molecular ion for a particular drug via soft ionization methods, such as methane chloride, is generally preferred for primary identification purposes. Observance of a molecular ion can be considered the most important criterion for identification, but the burden of proof of presence placed on a single ion species cannot be regarded as sufficient for confirmation. Although a single ion representing the molecule at the correct retention time on a high-resolution capillary column might seem to have furnished sufficient evidence for confirmation, the need to provide additional proof of structural dimensions still remains. With the introduction into commerce of MS/MS instruments, the possibility of improving the degree of specificity by soft ionization techniques has strongly emerged for practical drug confirmation. Reliance on product ions (often referred to as daughter ions) from collision experiments of a parent ion has effectively reinforced the former chromatographic retention data as a prime criterion for identification.

g. Quantitative Aspects of Daughter Ions

The exploration of daughter ions for quantitative purposes was first investigated in the case of ethyl carbamate in wines and spirits at the parts per billion level.[30] With the availability of a stable isotopically labeled ethyl carbamate reference standard, both confirmation and quantification were accomplished using methane CI/MS/MS. These authors demonstrated that reliable quantitative data at low levels could be generated by isotope dilution techniques involving ratio measurements between daughter ions belonging to the sample and reference standard.

2. Specific Criteria Adopted for Hair Analysis

The use of CI/MS and negative CI/MS/MS in detecting the five drug of abuse groups has resulted in the following generic criteria being applied in this laboratory to all analyses (detection, quantification, and confirmation).

a. Instrument Performance Check and Contemporary Criteria Establishment

Before proceeding with sample analysis each mass spectrometer is subjected to a sequence of injections involving solvent blanks, standards, controls, and negatives to establish the ability to detect the target drug below the cut-off required by the SOP. During this stage of instrument performance, the limit of detection and limit of quantification are calculated based on signal-to-noise ratio (S/N) being greater than 3:1 and 10:1, respectively. From the data generated during the initial analysis of the standards, several criteria are established for sample analysis (e.g., GC retention time, acceptable ion abundance ratios, and the precision and accuracy of the assay).

b. GC Retention Time

The GC retention time of the target drug is established from the established mean from the contemporary standard assays so that subsequent samples must not deviate more than 2% from that mean. Furthermore, the elution profile (and hence ion profiles via MS) should adhere to a gaussian-shaped distribution.

c. Ion Abundance Ratios

Ion abundance ratios used in the analytical protocol are calculated over the concentration range of the standards analyzed. The acceptable range for this data set is a deviation of 25% from that mean. This level of deviation was determined based on two scientific facts. One, the generally acceptable level for such ion abundance ratios under CI in the scientific literature for regulatory purposes is about 20%.[29] Two, the determination of experimental ion abundance ratios at ultra trace levels from repeated injection of a series of standards and controls has revealed over a long time period (several years) that the acceptable deviation about the mean should be set at 25%. The cause for such deviations is based on the source pressure fluctuations during the ion-molecule collision necessary to produce ions under CI. This situation is more critical in MS/MS experiments, whereby an addi-

tional collision experiment is conducted in producing daughter ions. Therefore, the establishment of the accepted 25% deviation in ion abundance ratios has been deemed appropriate, based on evidence from ion abundance ratios gathered by this laboratory over a large number of analyses.

d. Accuracy and Precision

The establishment of calibration curves with an acceptable standard deviation (SD) = 2 (before sample analysis acceptance) is in concert with the ion abundance ratio measurements being within 25% of the mean. In the case where multiple standards at the cut-off level are injected, the mean and SD are calculated based on a 2-point calibration curve and must be within the predescribed SD = 2.

e. Number of Ions

Although there exists a variation in application of MS methods, the underlying theory that at least three structurally significant ions are necessary for confirmation has been experimentally established. With the increasing practice of using soft ionization methods, the universal problem of meeting these established criteria has been overcome by MS/MS using a combination of parent and daughter ions. In some instances, the measure of two ions have been used as definitive proof of presence and then the supporting evidence of GC retention time and analytical extraction have been considered important. Also, the ability of GC/MS to detect metabolites concurrently, as well as the parent drug, is an added benefit not available from RIA methods.

In most quantification assays, the employment of one ion representing the compound of interest has been acceptable,[29] provided confirmation has been conducted *a priori*. The linearity of detection of a mass spectrometer has been demonstrated under most ionization modes by calibration plots covering at least two orders of magnitude in concentration.

VI. CASE HISTORIES

A. ROUTINE PRODUCTION ANALYSIS OF CARBOXY-TETRAHYDROCANNABINOL (cTHC) VIA NICI-GC/MS/MS

By far, the most difficult analytical issue in testing for the various groups of drugs of abuse is the detection of marijuana. For sound scientific reasons to avoid passive contamination issues, the moiety of choice for detection in hair is the primary metabolite of THC, cTHC. Although samples are screened by RIA to detect positives, the structural confirmation step involves derivatization of cTHC to a volatile derivative containing sufficient fluorines to permit low-level detection via NICI.[31,32] The analytical approach in this laboratory[23] permits detection to the femtogram level, with concurrent reduction in the sample matrix background contribution. The appearance of base peak corresponding to the molecular anion after loss of HF at *m/z* 670 and a major fragment ion at *m/z* 492 are insufficient evidence to provide unambiguous proof of presence. Therefore, the power of tandem MS/MS has been used to provide characteristic daughter or product ions to satisfy the criteria for structural identification. In addition, the use of a deuterated (d3) internal standard of cTHC provides quantification of trace levels via accepted isotope dilution analysis.

The experimental protocol chosen to perform routine detection of cTHC in hair digest via GC/MS/MS involved selected reaction monitoring (SRM), using the two parent ions (*m/z* 670 and 673) belonging to cTHC and its d3 analog. The corresponding daughter ions produced in Q2 by collision processes, *m/z* 492 and 344 for cTHC and *m/z* 495 and 347 for the d3 analog, were recorded for structural confirmation concurrent with quantification via isotope dilution.

The sensitivity of this technology as applied to derivatized cTHC is illustrated in Figures 14–2 and 14–3, where 1 pg injected on column has provided sufficient sensitivity to indicate such trace levels are detectable. Confirmation of structure in samples at trace levels is positive, provided the ions are present in the ratio observed for the standard (within 20%). Quantification using the *m/z* 492 ion is conducted by direct ratio measurements of d0 vs. d3 for the internal analog standard.

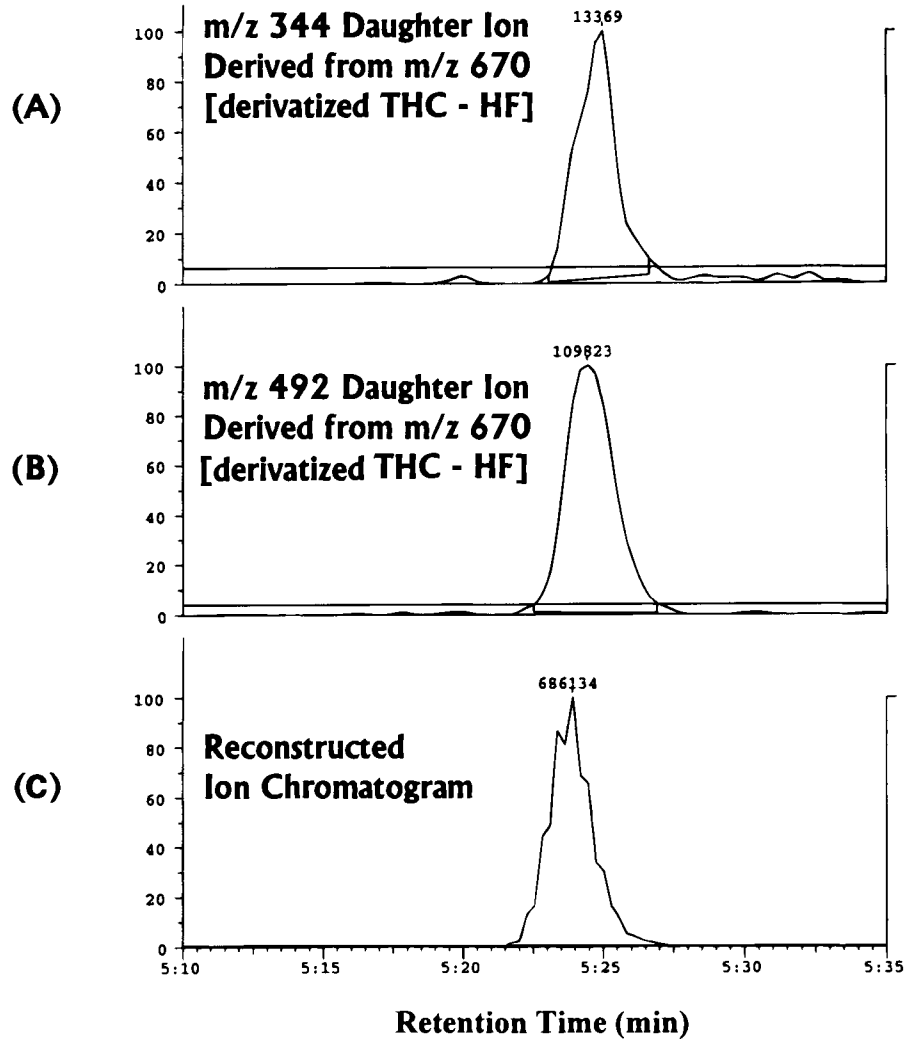

FIGURE 14–2. Daughter ion chromatograms for a derivatized 1-pg cTHC/10-mg hair-spiked control using the parent ion at *m/z* 670: (A) *m/z* 344, (B) *m/z* 492, and (C) reconstructed total ion chromatogram. [HF indicated loss of hydrogen fluoride.]

This case history demonstrates the utility of TSQ technology in the SRM mode to provide reliable structural confirmation data, in addition to acceptable quantitative data for the metabolite of THC at low femtogram levels in hair digests. The unique combination of derivatization to add fluorine to the molecule to enhance its electron-capturing capability and the use of NICI to drastically reduce sample background have proved to be highly successful in an MS/MS production mode operation.[33]

B. METHAMPHETAMINE AND AMPHETAMINE

In 1984, Suzuki et al.[34] published a technical note outlining a GC/MS confirmation procedure based on hair extracted samples, derivatized with trifluoroacetic anhydride (TFA) and using *N*-methyl-benzylamine as an internal standard. These authors selected to use packed-column GC in conjunction with methane CI to enhance the production of the protonated molecule ions for all three compounds: *m/z* 246 for methamphetamine, *m/z* 232 for amphetamine, and *m/z* 218 for the internal standard. Under methane CI conditions, there were two or more structurally significant fragment ions available for confirmation of structure. Quantification, however, was conducted using the pro-

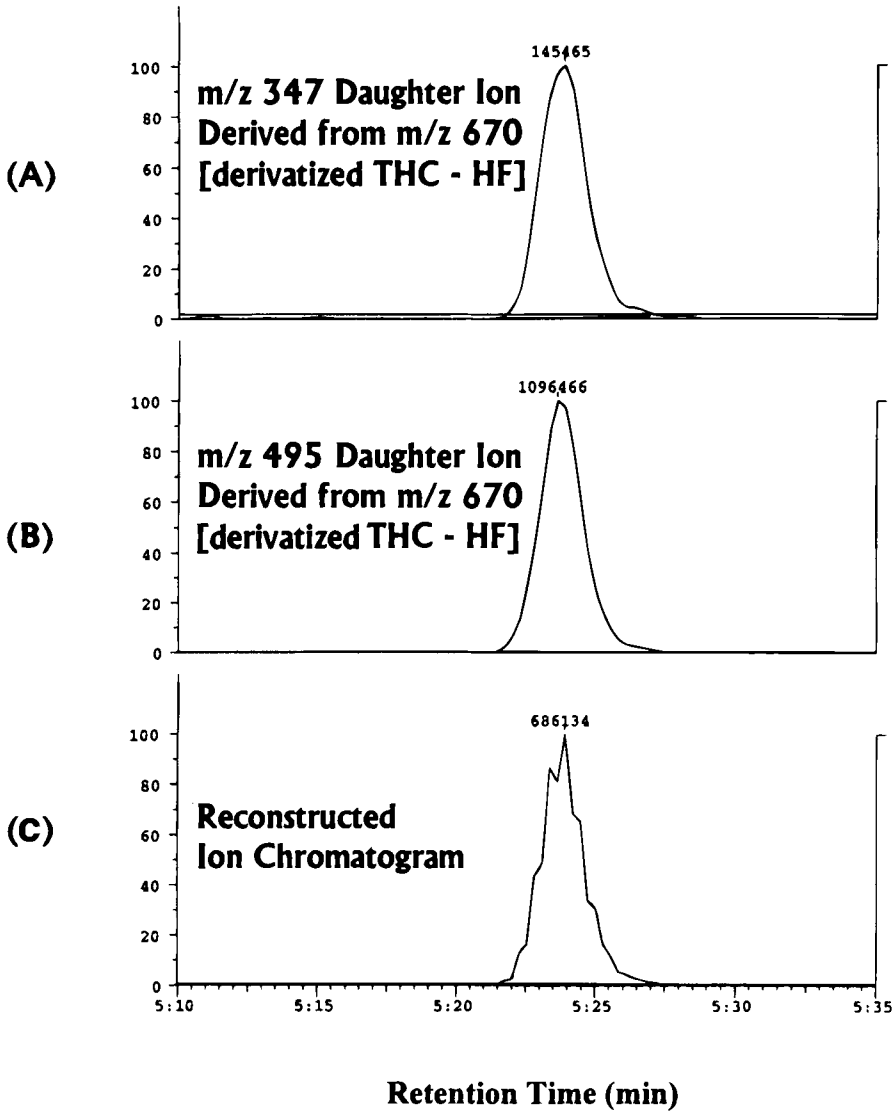

FIGURE 14–3. Daughter ion chromatograms for a derivatized 1-pg cTHC-d3/10-mg hair-spiked control using the parent ion at *m/z* 673: (A) *m/z* 347, (B) *m/z* 495, and (C) reconstructed total ion chromatogram. [HF indicated loss of hydrogen fluoride.]

tonated molecule ions under a multiple-ion detection mode. The detection limit claimed, 100 pg injected on column, allowed a single strand of hair to be used for a typical analysis. Calibration curves established indicated linearity over several orders of magnitude for both drugs. On the same theme, Nakahara et al.[35,36] advanced this derivatization approach using TFA by employing stable isotopes, the d4 analogs of methamphetamine and amphetamine, to improve the quantification step. Chromatographic separation was done by using a megabore column. These authors chose the fragment ions under EI, namely the base peaks, for the necessary isotope ratio measurements, *m/z* 154/158 for methamphetamine and *m/z* 140/144 for amphetamine. Under such conditions, the detection limit was found to be 0.5 ng/mg hair.

C. MORPHINE

Pelli and Traldi[37] applied MS/MS techniques via heated probe introduction to hair samples from herion addicts. Using EI, these authors demonstrated the detection and confirmation of 10 pg/mg

morphine at a S/N level of 5:1. The selected parent ion, the molecule ion of morphine at *m/z* 285, when in collision with argon, produced four structural significant daughter ions at *m/z* 256, 242, 228, and 215. Without extraction and subsequent chromatographic separation, these authors had provided a protocol for morphine in hair that satisfied the basic tests of confirmation of presence. Improvements in analytical approaches[38–40] have recently been discussed in some detail with regard to detection of both heroin and its metabolite 6-acetylmorphine.

D. COCAINE, BENZOYLECGONINE, AND COCAETHYLENE

Using a capillary column for chromatographic separation, Balabanova and Homoki[41] developed an assay for cocaine in hair samples based on GC/MS/EI. For confirmation purposes, these authors used a full mass spectral scan for direct comparison with a reference spectrum. Under EI, the molecule ion for cocaine was present at *m/z* 303, together with five other prominent fragment ions: *m/z* 272, 198, 182, 105, and 82. The detection limit quoted for this approach was 100 fg. In this application, the highest level of confirmation had been used, that of full mass spectral scans. A year later, in 1988, Martz[42] published a technical paper using a GC/MS/MS approach, with deuterated analogs for cocaine (d3) and benzoylecgonine (d5) for stable isotope quantification. This author elected to use methane CI to enhance the protonated molecule ions for these drugs for subsequent collision with argon yielding daughter ions for confirmation.

E. PHENCYCLIDINE

In 1988, Kidwell[43] presented a paper at the annual conference of the American Society for Mass Spectrometry, wherein he described a pyrolysis analysis procedure for phencyclidine in hair samples. His approach avoided the extraction process by placing the sample into the direct insertion probe, thermally increased to 250°C, of the mass spectrometer for analysis. By using isobutane CI, the protonated molecule ion, (M + H)+ at *m/z* 244, of any phencyclidine present would be favored. By selecting this ion for subsequent collision with argon, the daughter ion at *m/z* 159 and 86 would be formed. Although this procedure eliminated the extraction step and the chromatographic separation common to most analytical methods, the use of MS/MS still maintained the basic minimum elements of confirmation, namely the presence of three structurally related ions. The sensitivity of the method was determined to be 50 pg/mg.

VII. EVOLVING ISSUES IN HAIR TESTING

Despite the widespread acceptance of hair analysis, there exist a number of scientific areas receiving intense investigation and evaluation.

A. PASSIVE CONTAMINATION LEADING TO FALSE-POSITIVES

It has been suggested by several researchers[44] that hair may become contaminated by drugs present in the environment, making it impossible to distinguish users from nonusers. These claims have been refuted by numerous blind field studies[26] and most recently by Mieczkowski.[45] In the latter study, undercover narcotics officers were monitored by hair analysis for evidence of passive contamination. These officers who were extensively exposed on a daily basis to cocaine did test positive by RIA for cocaine in the wash solutions. However, no measurable quantities were found in the hair digest. These results attest to the resistance of hair to contamination and the effectiveness of the isopropanol and phosphate buffer wash procedures for the removal of external contamination. Measurement of metabolites in the inaccessible domain of hair (i.e., the regions in hair to which environmental contaminants have no access) provides an additional measure of safety against false-positives caused by external contamination. This conclusion does not apply to methods that do not use aggressive wash procedures for removal of drugs from the accessible and semiaccessible domains and extraction procedures, preferably dissolution methods, which remove drugs from the

inaccessible domain. More recent studies have addressed the adaptation and evaluation of decontamination procedures.[46–48]

B. RACIAL BIAS IN HAIR TESTING PROCEDURES

The potential for racial or hair color bias has been raised.[49,50] Although potential racial bias issues can be raised against urine testing, they have not been investigated to date. However, in the case of hair analysis, this question has been addressed by a definitive study by Mieczkowski and Newel,[51] at least for methods in which the melanin fraction has been removed by centrifugation before analysis. These authors evaluated the hypothesis that hair assays for cocaine were subject to racial bias. As their population, they selected 315 African-American and 846 Caucasian arrestees in Pinellas County, FL, whose cocaine use had been evaluated by urine, hair, and self-reports. Each measure of drug use showed that the African-American group had twice the drug use as that of the Caucasian group. On the assumption that self-reports are not racially biased, these results point to the same conclusion for urinalysis and hair analysis.

C. COSMETIC TREATMENTS PREVENT DETECTION

As with any drug test, drug users have attempted to apply a variety of maneuvers to evade detection. In the case of hair, this involved efforts to leach out of the hair the drugs of interest by such measures as extensive washing with special shampoos, as well as perming, dyeing, or bleaching[52] of hair. To date, these treatments have proven unsuccessful because such evasive measures cannot completely remove the blood-derived drugs that have become trapped in the inaccessible domain. It should be noted that this conclusion may not be valid for methods that use less efficient solvent-based extraction methods instead of hair dissolution procedures.

VIII. PROFICIENCY TESTING

There is currently a global concern that hair analysis adopts a proficiency testing system to evaluate both the diverse analytical methods used by practitioners, as well as the development of appropriate reference materials for quality assurance purposes.[53] To address these issues, a new professional society to be called "The Society for Hair Testing" has recently been formed in Europe. High on their agenda are the topics of evaluation, validation, and a well-constructed quality assurance program complete with blind proficiency samples for participating laboratories.

IX. CUT-OFF LEVELS

The question regarding the most appropriate GC/MS cut-off level to determine when a sample is to be declared positive has recently been addressed by Kintz et al.[54] Based on numerous field studies,[26] the analytical cut-off values used by this laboratory in reporting confirmed positive results (i.e., avoidance of environmental and/or passive exposure) for the various drug groups are as follows, based on analysis of a 10-mg hair sample:

Marijuana metabolite	1.0 pg/10 mg hair
Cocaine	5 ng/10 mg hair
Opiates	5 ng/10 mg hair
Methamphemtamine	5 ng/10 mg hair
PCP	3 ng/10 mg hair.

X. CLINICAL STUDIES

The expansion of hair analysis to the field of clinical studies has been demonstrated by Goulle et al.[55] whereby the levels of phenobarbital in hair have been used for drug monitoring purposes.

Another interesting use of hair analysis for clinical purposes has been the detection of tricyclic antidepressants in post-mortem human scalp hair.[56] In addition, Wilkins et al.[57] have examined hair in a clinical quantitative determination of codeine and its major metabolites. A major clinical effort has been directed toward nicotine and its metabolite in hair by Nilsen et al.[58] to answer the question of environmental or passive smoke transfer to the hair of nonsmokers. Most recently, the elegant clinical experiment to determine the exact pharmacokinetic incorporation of cocaine and its metabolites into hair from the blood has been conducted using stable isotopes administered intravenously and intranasally.[59] In this study, the authors concluded that a simple compartment model between blood and hair was clearly not the mechanism for incorporation. More complex pharmacokinetics were obviously involved that required additional study to reach an accepted solution.

XI. CONCLUSIONS

The analysis of trace levels of drugs of abuse in hair has rapidly become the preferred method by many corporations to provide data on drug use of preemployment candidates stretching over the past 90 days rather than the 3 to 4 day time window provided by urine analysis. Clearly, the emphasis placed on forensic toxicology and trace analysis has stimulated technological advancements to the present-day accelerated pace. History has clearly demonstrated that it has been the development of innovative technologies for drug analysis by analytical chemists that has stimulated the development of new forensic approaches for the detection of drug users. This relationship between the new and traditional approaches should not be viewed as one of competition between blood, urine, and hair analysis, but rather as one wherein the different techniques are in synergistic interaction in solving the unique forensic challenges associated with the identification of drug use. In this respect, the particular value of hair analysis lies in the ability to determine long-term usage of drugs of abuse rather than the short-term elimination provided by blood and urine.

The greatest challenge in the area of hair analysis has been the development of ultrasensitive MS technologies to provide reliable confirmation of structure. The case histories described in this review stand as testimony to the evolution of criteria for confirmation of presence that are generally recognized as scientifically sound. Although the variation in application of MS methods is large, the underlying theory that at least three structurally significant ions are necessary for confirmation has been experimentally established. With the increasing practice of using soft ionization methods, the universal problem of meeting these established criteria has been overcome by MS/MS using a combination of parent and daughter ions. In some instances, two ions have been used as definitive proof of presence, and then the supporting evidence of GC retention time and analytical extraction have been considered important. Also, the ability of GC/MS to detect metabolites concurrently, as well as the parent drug, is an added benefit not available from RIA methods. In most quantification assays, the employment of one ion representing the compound of interest has been acceptable, provided confirmation has been conducted *a priori*. The linearity of detection of a mass spectrometer has been demonstrated under most ionization modes by calibration plots covering at least two orders of magnitude. Although the use of isotope dilution techniques has been used extensively using CI methods to avoid additional sample clean-up, as well as to avoid potential interferences, its recent extrapolation to daughter ion chemistry has now extended the linearity concept to collisionally induced dissociation experiments. Moreover, the introduction into commerce of the low-cost ion trap mass spectrometers with sensitivity in the low picogram range might well bring this type of MS into more general applications to drugs of abuse testing in hair. Despite the 15% coefficient of variation predicted[29] for quantification of the nanogram and low picogram level between laboratories, the precision and accuracy demonstrated by a single laboratory can be as low as 10%. The scientific excellence portrayed by members of this discipline in publication of results of reliable methods for analysis of drugs of abuse in hair stands as evidence that no mandated carved-in-stone criteria are necessary for confirmation. The good judgment of the MS expert, coupled with established quality

assurance procedures, results in analytical practices that are considered scientifically sound by forensic science standards.

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Chapter 15

SWEAT IT OUT

Irving Sunshine and Jacqueline P. Sutliff

CONTENTS

I. INTRODUCTION

One of the most important responsibilities of an analytical toxicologist is the determination of whether or not an exogenous chemical substance contributed to a given individual's indisposition. Traditionally, this would involve the analysis of a biological specimen. In nonfatal situations, blood and/or urine have been the specimens of choice. Blood is preferred when one or two analytes are in question. If several analytes may be involved, urinalysis may be a better choice because this would require a large volume of blood. Usually, a large volume of urine can be obtained easily, and urinalysis methods are simpler and less demanding technically than are those required to analyze blood. Because blood and urine specimens have been analyzed extensively, their limitations, chemical interpretation, and forensic defensibility have been well established. One limitation of these

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analyses is their limited ability to determine a history of exposure to drugs unless frequent analyses are performed. This frequency would be determined by the half-life of the substances in question and imposes fiscal restraints that may preclude obtaining the desired history. In recent years, interest in alternative biological specimens has grown. The presentation that follows focuses on sweat. This is not meant to disparage the use of other alternative biological specimens, such as hair, meconium, nails, saliva, and vitreous fluid that merit the separate presentations that are presented elsewhere in this volume.

II. STRUCTURE OF THE SKIN

Skin has two major layers: the epidermis and the dermis. The outer layer is a stratified epithelium, the epidermis, that varies between 75 and 150 μm in thickness over most of the body, except on the palms and soles. The outer surface of the epidermis is called the stratum corneum. It acts as a barrier to restrain passage of water and solutes in either direction across the skin.[1] Under the epidermis is the second major layer, the dermis, that is a dense fibroelastic connective tissue. The dermis supports extensive vascular and nerve networks, and specialized excretory and secretory glands.[2]

III. SWEAT AS A BIOLOGICAL MATRIX

Moisture can be lost from skin by two distinct pathways. The first pathway, called insensible sweat, seems to be caused by diffusion through dermal and epithelial layers.[3] Insensible sweat results from the passive diffusion of volatiles, including water, through the skin.[4] The rate of fluid lost through insensible sweat depends on body location, ambient temperature, body temperature, and the relative humidity of the environment.[5] For example, rates from 6 to 10 g/m^2/hr have been produced by the arm, leg, and trunk, whereas 100 g/m^2/hr for palmar, planar, and facial skin have been reported.[3] Total body insensible fluid loss rates per day also vary considerably (between 381 and 695 ml), depending on temperature.[6]

The second pathway for moisture loss, called sensible sweat, is commonly referred to as sweat and is secreted from the eccrine glands[5] and the apocrine glands.[20] The innermost cells of the eccrine gland serve as secretory elements that empty into the lumen or duct of the glands. The duct terminates at the surface of the skin.[8] The number of actively secreting eccrine glands depends on the body location of the type of sweat response created.[9] Specific triggers of eccrine sweat include exercise, stress, thermal stress, mental stress, and emotional stress.[10] Eccrine gland density varies between 200/cm^2 on the forearm to over 400/cm^2 on the thenar eminence,[9] and their aggregate weight in the average human is about 100 g.[2] Maximum rates of sensible sweat produced by the body can be as high as 2 l/hr in average subjects and 4 l/hr in trained athletes.[5] The amount secreted is affected by emotional, physical, and thermal stress.[11] The variability of these factors and the uneven distribution of the sweat glands make it difficult to obtain specimens of sweat systematically.

Apocrine glands are located in the axillae, pubic, and mammary areas. They are not thought to have significant thermoregulatory effect, and their role and mechanism in humans are not clear.[2]

Sebaceous glands are lipid-secreting structures in the skin, especially in the scalp, forehead, and face. The sebaceous glands secrete a substance called sebum that is primarily lipid. These glands appear over most of the body, with average concentrations of 100/cm^2.[21] They are not thought to affect the regulation of body temperature,[2] and their mechanism in the body does not seem to be related directly to the production of sweat.

IV. COLLECTION OF SWEAT

Numerous methods have been developed to induce sweat and to collect samples from human skin. Smith[22,23] extracted sweat from clothing worn by suspected drug users and analyzed the

extract. Investigators interested in measuring the concentration of specific components of sweat have collected run-off perspiration induced by exercise and/or thermal stress.

V. PREVIOUS TRANSDERMAL DIAGNOSTIC DEVICES

Earlier studies by Darling et al.[24] led to the development of a device to collect sweat. Darling's research, which was done on cystic fibrosis patients, showed that sweat rates could be accelerated with pilocarpine and that the sweat could be collected on skin. Medtronic, Inc. (Saint Paul, MN) subsequently marketed a collection device called the CF Indicator that accelerated iontophoreses on the skin. This device incorporated a miniature sweat stimulator and disposable chloride sensor. Wescor (Logan, UT) also developed a sweat conductivity analyzer that was approved for cystic fibrosis screening.

Other researchers made attempts to detect drugs with sweat collection devices. Phillips et al.[11] developed a patch that consisted of absorbent cotton sandwiched between waterproof polyurethane on one side and layers of porous, nonwoven rayon on the other. These patches were intended to measure levels of ethanol, digoxin, and lithium in patients who used these drugs. To increase the amount of sweat during the 8-day wear period, these researchers applied dry absorbent pads impregnated with sodium chloride crystals under the waterproof dressing. The assumption was that any sweat excreted would dissolve salt in the pad and bathe the skin with a saturated salt solution. This early patch was time-consuming to apply because each patch required construction at the time of use. Furthermore, the patch was large, leading to discomfort and detachment, and much of the sweat specimen could not be extracted from the collection pad.

Phillips[13] developed a second patch with a collection pad of polyester fiber with a hydrophilic finish. Again, the pads were soaked in saturated sodium chloride to bathe the skin with a saturated salt solution. Phillips et al.[14] used these devices to conduct field tests for the detection of ethanol. This research concluded that, in an individual subject's behavior, temperature and backdiffusion of ethanol from the patch onto the skin or possible leakage from the patch made it impossible to determine the rate of ethanol entry onto the patch. This study was unable to replicate Phillips' earlier studies.[13] It could not provide a sensitive measure of ethanol in transepidermal fluid, nor could it determine the amount of ethanol consumed. These researchers concluded that further design modifications and pilot testing were essential.

Peck et al.[15] developed a mathematical model for the pharmacokinetics of transdermal drug detection. They determined that backtransfer of the drug into the skin could be a substantial limitation to the technology. Peck[16] then developed a slightly different transdermal patch design from the original Phillips' design. This design retained the occlusive patch approach of Phillips, but included chemical binding in the patch to discourage backdiffusion of the diagnostic signal. In 1985, Peck applied for a patent for this saline-activated, carbon aquagel, dermal substance collection device. This device consisted of substance-binding reservoirs and was wetted by a liquid transfer medium to transfer the soluble substances from the skin surface. Like the earlier patches, Peck's patch had an occlusive pad designed to capture all of the sweat the body produced during the wear.

In 1985, Peck filed at least two more U.S. patents on improvement to this design. Conner et al.,[7] Murphy et al.,[18] and Conner et al.[19] used Peck's design to monitor theophylline and caffeine in preterm infants.

VI. DETECTION OF DRUGS IN SWEAT

Over the years, there have been attempts to analyze sweat for various drugs. Johnson and Malbach[26] thermally induced sweat and administered drugs with various pK_a's. They concluded that there was a strong correlation between the pK_a of a drug and the amount found in sweat and in the sweat-to-plasma ratio. Vree et al.[27] gave controlled oral doses of amphetamines and then measured

the amphetamine content of sweat. He concluded that analysis of sweat would be useful in doping control and that excretion of amphetamines in sweat was largely independent of sweat pH. Ishiyama et al.[28] found that methamphetamine was excreted by sweat glands and concluded that analysis of sweat would be a valuable in forensic practice. Henderson and Wilson[29] measured methadone and its metabolites in both sweat and urine of patients in methadone maintenance programs, primarily to determine optimal doses of methadone. They were not able to determine optimal doses from sweat, but concluded that sweat may be a significant route of elimination of methadone and, thus, may significantly affect the amount of methadone required. Shah[30] studied the excretion of the antimicrobial agent griseofulvin. His research indicated that this drug passively diffused into sweat for delivery to the skin's surface.

Overall, very few of these determinations used insensible sweat. The transdermal patch-type devices described were occlusive. They were primarily designed to collect sensible sweat produced by thermal stimulation or vigorous exercise. By harvesting all of the sweat thus produced, investigators hoped to correlate the drug concentration in the collection device with the dose used. The studies on drugs in sweat were, thus, preliminary at best, and the utility of the results was often unclear. The need for systematic collection of sensible sweat became apparent, and efforts were initiated to develop such a device.

The need for an improved device led to the development of a nonocclusive unit that permitted the evaporation of the entrapped water content of sweat. If the device did not retain the sweat's water, the transport characteristics of skin would not be altered and skin irritation caused by this water trapped against the skin would be minimized. Thus, it would be possible to collect sweat constituents other than water over time and to investigate better dose/patch constituent correlations. To this end, such a patch test device was developed by Sudormed, Inc. in 1990.

VII. HISTORY OF SWEAT PATCH DEVELOPMENT

The patch test device was adapted from 3M's Tegaderm™ 1625 transparent dressing that has been used since 1980 for wound dressings. The sweat patch was developed as a general sweat collection device (Food and Drug Administration [FDA] Document Control No. K902442). In October 1990, Sudormed was given market approval for the *Sudormed Sweat Patch Specimen Container* by the FDA. Upon receipt of FDA clearance, Sudormed decided to apply the technology to the detection of drugs of abuse in sweat. The worldwide marketing and distribution rights for the sweat patch for use in the field of drug and alcohol detection was purchased by PharmChem Laboratories, Inc. of Menlo Park, CA in 1992. The name "PharmChek™" was chosen for this application of the *Sudormed Sweat Patch Specimen Container*.

In 1992, a premarket notification 510(k) was submitted to the FDA for the use of the patch for the detection of cocaine. The FDA subsequently notified Sudormed that its cocaine submission could not be approved without an approved method for screening for cocaine in sweat. The FDA requested that a separate 510(k) submission for a screening assay for detection of cocaine in sweat be submitted along with data on the patch as a collection device for cocaine. Furthermore, the FDA requested that Sudormed provide data in separate 510(k) submissions for each of the drugs (cocaine, opiates, phencyclidine [PCP], amphetamines, and marijuana), for which it intended to have the sweat patch approved as a collection device along with co-dependent 510(k) submissions for screening assays for each of these drugs in sweat. To comply with the FDA's request, Sudormed met with manufacturers of screening assays to assess their suitability for detection of drugs in sweat. As a result of these discussions, SolarCare Technologies Corporation (STC) (Bethlehem, PA) agreed to modify its enzyme immunoassay (EIA) microplate screening assays for detection of drugs of abuse in sweat and to prepare 510(k) submissions to the FDA for these modified screening assays as co-dependent submissions with the PharmChek™ sweat patch. The STC EIA microplate screening

assays had originally been approved by the FDA for the detection of drugs of abuse in urine. By the end of December 1993, Sudormed and STC had completed co-dependent 510(k) submissions for cocaine, amphetamines, opiates, PCP and marijuana.

VIII. THE PharmChek™ DRUGS OF ABUSE PATCH

There are three components (Figure 15–1) to the PharmChek™ sweat patch: (1) the polyurethane/adhesive layer, (2) the release liner, and (3) the collection pad. The polyurethane/adhesive layer is approximately 6 cm wide, 7 cm long, and 0.025 mm thick. The adhesive is described by 3M as a hypoallergenic, water-resistant adhesive. The polyurethane/adhesive layer is identical to that used in 3M's Tegaderm™ 1625 transparent dressing. The overall dimensions of the polyurethane/adhesive layer are also identical to that used in 3M's Tegaderm™ 1625 transparent dressing. More than 1 billion Tegaderm™ transparent dressings have been sold throughout the world as wound dressings since 3M received FDA 510(k) market approval in 1981.

One characteristic of Tegaderm™ makes it especially valuable for the sweat patch. 3M developed the adhesive so that it could not be reapplied to the skin once it was removed. Exfoliated stratum corneum cells stick to the adhesive when it is removed from the skin and prevent the adhesive from resticking. This characteristic of the adhesive greatly diminishes the possibility of tampering with the patch.

A unique 9-digit serial number is printed underneath the polyurethane between the adhesive and the polyurethane layer. It can be read through the polyurethane layer and can serve as an aid in chain-of-custody control of the patch.

The second component of the patch is the release liner. It is a very thin medical-grade cellulosic tissue (1-ply-17# drape) supplied by James River Corporation (New York). The release liner is approximately 3 cm wide, 5 cm long, and 0.003 mm thick. The purpose of the release liner is to allow removal of the collection pad from the adhesive after patch use. Without it, the collection pad sticks to the adhesive and cannot be readily removed. After wear, the release liner is discarded along with the polyurethane/adhesive layer.

The third component of the patch is the sweat collection pad. It is composed of a medical-grade cellulosic paper (#939-039) supplied by Ahlstrom Filtration (Mt. Holly Springs, PA). The collection pad is approximately 3 cm wide and 5 cm long. It is approximately 0.7 mm thick and functions as a reservoir for the nonvolatile components of sweat. It collects a minimum of 300 µl of insensible perspiration in a 22°C environment. This is the volume of sweat required for testing if exercise or other factors that increase sweating are prevalent. To avoid contamination, the collection pad should be separated from the patch with gloved hands or disposable tweezers.

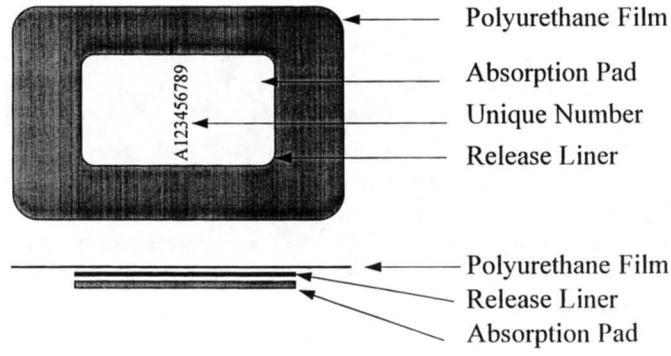

FIGURE 15–1. PharmChek Sweat Patch. Hypoallergenic, non-occlusive retains solids from sweat.

IX. THEORY OF SWEAT PATCH OPERATION

After wear, dry concentrates of the nonvolatile components of sweat, including drugs of abuse, remain on the collection pad of the sweat patch. The impermeability of the patch's polyurethane layer prevents loss of the collected sweat components into the environment. The polyurethane used in the Tegaderm™ permits water molecules to pass through the Tegaderm™. Because the smallest drug molecule for which the patch is intended, amphetamine (with a molecular weight of 135 Da), is ten times heavier and ten times larger in size than a singlet water molecule, neither amphetamine nor the drugs of abuse with even larger molecules can fit between the intramolecular spaces of the polyurethane. Therefore, applying the drug to the outside of the patch or being exposed to the drug in the environment will not cause the drug to enter the patch. Furthermore, the drugs of abuse, once collected on the patch, cannot diffuse to the outside environment. Finally, according to 3M, solvents (such as water, alcohols, ethers, and alkanes) do not increase the permeability between the polyurethane molecules. This means that solvents applied to the patch in an attempt to tamper with or dissolve the drug content of the patch will be ineffectual.

The sweat patch seems to operate like an ion trap. The hydrophilic drugs of abuse are weak bases with pK_a's around 8.0. Thus, as the pH of the solution is lowered, the drugs become more ionized. Skin has a normal pH of about 5.0, and this normal pH is maintained under Tegaderm™ dressings. Interstitial fluid and plasma have a normal pH of about 7.0. Weak base drugs with high pK_a's will be more highly ionized on the skin than in the body by a factor of about 100. This means that unionized drug molecules leave the blood and interstitial space at a pH of 7.0 and arrive on the surface of the skin, wherein they can be absorbed by the patch.

X. DESCRIPTION OF THE STC METABOLITE ASSAYS

The STC metabolite assays are competitive enzyme immunoassays (Elisa, enzyme-linked immunosorbent assay) in which the antibodies are bound to microplate surfaces (i.e., wells). When a sweat specimen or standard containing drug is applied to the microplate, along with an enzyme labeled with hapten derivative, there is competition between the drug in the specimen and the enzyme conjugate to bind to the antibody that is fixed on the microplate. The wells are washed six times with distilled water to remove excess enzyme conjugate and drug once the competition for the antibody coated on the surface of the microplate well is complete. A substrate is added (3,3″,5,5″-tetramethylbenzidine). After 30 min incubation, the reaction is stopped with 100 µl of 2 *N* sulfuric acid. The color that is produced is measured at two absorbances, 450 and 630 nm, using a microplate reader (BioTec Instruments, Inc. Winoski, VT). Interference caused by fingerprints or scratches is determined by the reading at 630 nm. The instrument automatically subtracts the absorbance at 630 nm from the reading at 450 nm, thus compensating for this potential interference.

These assays are generally sensitive to particular analytes, such as benzoylecgonine, in the cocaine assay and morphine in the opiate assay. However, they have a significant cross-reactivity with parent drugs or other metabolites. (Table 15–1)

For example, the antibodies used in the STC cocaine metabolite assay are raised against benzoylecgonine, but there is also significant cross-reactivity with parent drug cocaine. This cross-reactivity is critical, because the primary compound produced in sweat when cocaine is used is the parent drug cocaine. In addition, the assay exhibits significant cross-reactivity with ecgonine methyl ester and cocaethylene. This is important because finding metabolites of cocaine in sweat demonstrates that the cocaine was actually ingested and serves to negate any inference of contamination.

The immunoassays only provide a preliminary analytical test result. A more specific, chemically independent method must be used to confirm the initial result. Gas chromatography/mass spec-

TABLE 15–1
Cross-Reactivity, Limit of Detection, and Screening Cutoff

Drug	% Cross-reactivity	LOD patch (ng/ml)	Screening cutoff (ng/ml)
Cocaine	102	1	10
Benzoylecgonine	20		
Ecgonine methyl ester	18		
Cocaethylene	143		
Heroin	28	1	10
6-MAM	30		
Codeine	588		
Hydromorphone	16		
d-Amphetamine	30	5	10
l-amphetamine	23		
MDMA	144		
MDA	21		
Pseudoephedrine	31		
PCP	100	0.8	7.5
4-OH-PCP	31		
9-THC (by RIA)	100	5	5
9-THC-9-COOH	180		
11-hydroxy-THC	300		

Note: Volume of eluate per patch is 2.5 ml. Abbreviation—LOD, limit of detection.

trometry (GC/MS) is the preferred confirmatory method. If GC/MS fails to support the positive results obtained through EIA, the result should be reported as negative, despite any initial positive test result.

XI. USE OF THE PATCH

Several published studies reveal the potential of the PharmChek™ sweat patch test. In one study, 18 male volunteers took 50 or 126 mg of cocaine intranasally.[31] Before drug use, patches were applied to the subjects' back, torso, and biceps. One day after drug use and at varying times thereafter, patches were removed and analyzed. These analyses accurately reflected drug use, but the mean cocaine concentrations in the patches were significantly different for the two doses used. However, the variability of these results was such that any one result could not be used to reflect the dose involved or the time of use. There is no doubt though that a positive PharmChek™ patch result indicated cocaine use in the previous 7 days. Data also indicated that the patch could not differentiate between multiple small doses or one large dose of drug.

Parallel urine specimens obtained at the time of patch removal gave positive results for 48 hr after drug use. Thereafter, the urine concentrations were below the National Institute on Drug Abuse guideline criterion for a positive result. The sweat patches (70%) contained more than 15 ng/ml of cocaine or cocaine metabolites after 7 days of wear. (A consensus has not yet been reached for the screening cutoff values of drugs of abuse in sweat.) Figure 15–2 shows the comparison of percentage positive urine results with the percentage positive patch results from a single dose of cocaine over 7 days of drug testing.

Another study involved humans who used either cocaine hydrochloride (intravenously or intranasally), cocaine base (by smoking), or heroin (intravenously).[7] After drug use, up to 14

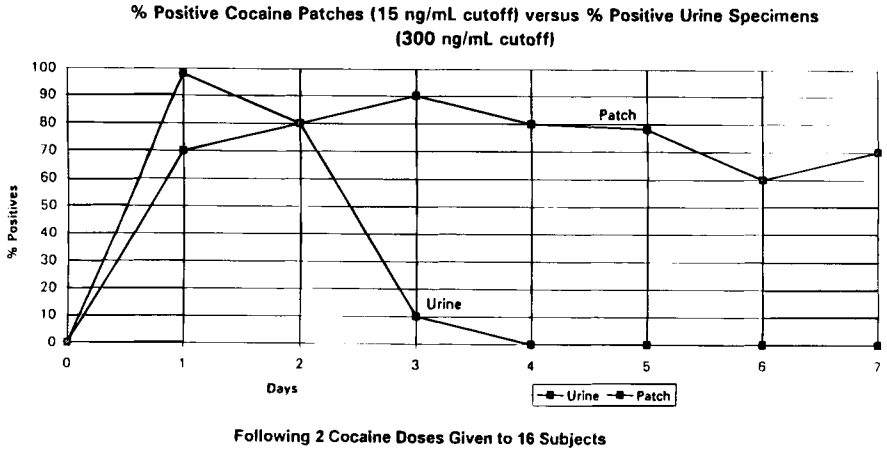

FIGURE 15–2. Positive urine and patch results after two cocaine doses.

patches previously applied to the back and abdomen were removed before and periodically after drug use. The patches were removed at different time intervals, stored at –30°C and subsequently were analyzed by GC/MS. Figure 15–3 shows the results of the patch analysis taken from four subjects 24 hr after the intravenous injections of several concentrations of cocaine hydrochloride. The standard error of the mean shown in this figure indicates that there was substantial intersubject variability. Analysis of patches taken at the same time and those taken at later times gave comparable concentrations of cocaine, but there were substantial differences in the amounts of cocaine on the patches of each subject tested. After heroin use, the concentrations of heroin varied with time. In one subject, the *l*-acetylmorphine (6-MAM) increased as the heroin concentration decreased. This suggested that heroin may have been hydrolyzed on the patch's pad. When patches were applied to 17 heroin users from a drug treatment center, heroin, 6-MAM, or morphine was detected by the patch in all but one subject.

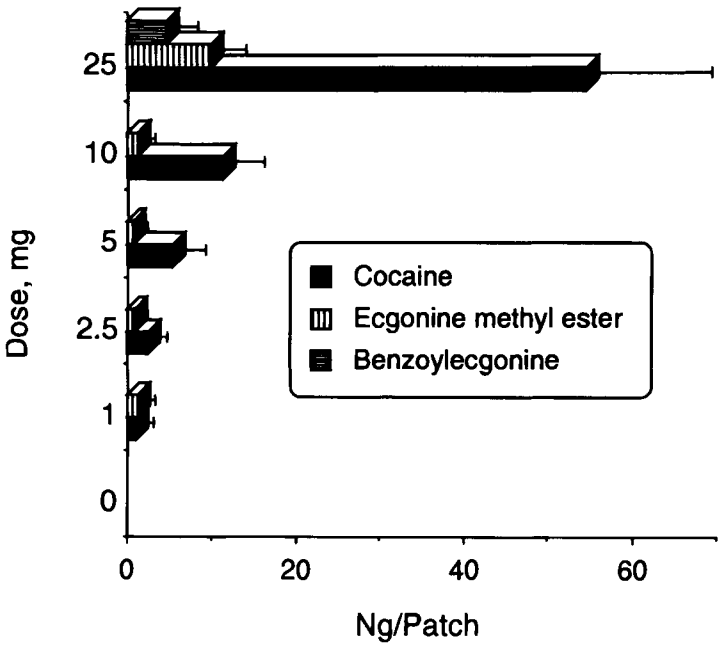

FIGURE 15–3. Patch results after intravenous injections of cocaine hydrochloride. *Source:* Cone, E.J. et al. 1994. *J. Anal. Toxicol.*, 18:298–305. With Permission.

In addition to the published studies previously cited, there are many field studies on the use of the patch test in progress. Preliminary data from one of these studies (see A below) are briefly presented to indicate the potential use and value of the patch test.

A. MICHIGAN STATE DEPARTMENT OF CORRECTION FACILITIES

Subjects from five units in these facilities participated in this study. Three of the facilities were residential and electronic monitoring (REP) sites, and two were prisons.

Subjects within the REP sites sleep in and have their evening meal at the site where they are under close supervision. During the day, they engage in appointed tasks off the REP site. Thus, they may have some access to street drugs. Study subjects wore patches for 7 or 14 days and were required to give urine specimens every 3 days. The resulting data could be used to estimate the effectiveness and the relative economy of the two testing procedures. This study ran for 3 months.

Inmates in the two prison sites were tested each month according to a computer-generated random list. Urine specimens were obtained when the patches were applied and when the patches were removed. This study ran for 6 months.

Patches were tested using the STC immunoassay and GC/MS. Urines were tested using EMIT® and GC/MS. The urine screening cutoff levels included: cocaine, 300 ng/ml; opiates, 300 ng/ml; amphetamines, 1,000 ng/ml; marijuana, 100 ng/ml; and PCP, 25 ng/ml. The screening cutoffs for the patch specimens were as follows: cocaine, 10 ng/ml of eluate; opiates, 10 ng/ml; amphetamines, 10 ng/ml marijuana, 5 ng/ml; and PCP, 7.5 ng/ml (Table 15–1).

Positive screening tests initiated a confirmation by GC/MS. Because the volume of patch eluate was small, confirmation of more than 1 drug per sample was not possible. If more than one drug was detected in the patch eluate, GC/MS was performed in the following order: cocaine, opiates, amphetamines, PCP, and tetrahydrocannabinol.

The number of subjects tested was 1,054 (Table 15–2). About 2,885 patches and 10,080 urine specimens were tested. A study of this table shows that 26 more subjects, 95 versus 69, (25%) were detected positive for drug use by patch testing than by urine testing.

Data collected in the Michigan Pilot Program demonstrated that, during this study, the patch was able to be worn safely for up to 14 days. Patch wear times alternated between 7 and 14 days to test the effectiveness of both wear periods. In 85% of the cases in which wear data were reported on the chain-of-custody form (2,547 patches), it was found that the intended wear period was completed without the patch being taken off, falling off, or the skin becoming irritated.

The ability of the subjects to be able to wear the patches successfully varied between sites and between types of sites. Corrections staff in Michigan were not given patch test results for specific prisoners. Prisoners were not be punished for having a positive patch test; however, subjects who refused to wear patches received sanctions. The effect of these sanctions on the testing subjects was very different between the prison sites and the REP sites. A sanction on a prisoner who may be serving a 20-year sentence seemed to have little effect on his decision not to wear a patch. A sanction on

TABLE 15–2
Michigan Study (1,054 Subjects—2885 Patches, and 10,080 Urines)

Drug	Subjects (%)	Patches (%)	Urines (%)	Subjects (%)
Cocaine	60 (5.8)	98 (3.4)	25 (0.22)	15 (1.4)
Opiates	21 (2.0)	28 (1.0)	29 (0.3)	20 (1.9)
Amphetamine	2	2	0	0
THC[a]	11 (1.0)	12 (0.4)	50 (0.5)	34 (3.2)
Total	95 (9.0)	140 (4.9)	104 (1.0)	69 (6.5)

[a]THC, tetrahydrocannabinol

someone in a REP situation, who may be free in a few months and could have his release date delayed if he did not wear a patch, had quite a large effect.

In the prisons, often one third to one half of the prisoners would refuse to wear patches, and they had a high rate of patches that "fell off" (70.4% of the subjects completed their intended patch wear period, but 12.3% of their patches "fell off" between 0 and 7 days). In the REP sites, almost no one refused to wear the patch and the "fall-off" rate was lower (86% of the subjects completed their intended wear period, and only 5% of the patches "fell off" between 0 and 7 days).

Complaints about irritation from the patch were few at the REP sites. Some subjects (0.9%) experienced itching during the 0- to 7-day wear period, and 0.4% experienced itching between 8 to 14 days of wear.

Some prisoners (9.2%) experienced itching or redness for the 0- to 7-day wear period and 0.3% for the 7- to 14-day wear period. There are two potential explanations for this. One of the prisons is associated with a medical facility and has a large proportion of subjects who are on many medications or have preexisting skin conditions. Also, if a prisoner did not want to wear a patch, stating that the patch caused itching was a legitimate reason to have the patch removed without sanctions.

XII. EASE OF PATCH APPLICATION AND REMOVAL

The average time to apply a patch and complete the chain of custody was 4 min. The average time for removal of the patch and completion of the chain of custody was 4 min.

The average collection time for one urine specimen that covered drug use of 12 to 72 hr was 6 min. This does not take into account the time expended on subjects who cannot urinate when they come into the testing area and had to wait the allotted 2 hr to complete the test or the time for subjects who could not urinate in an observed situation and had to strip, urinate, and be dressed again.

In the REP situations, there were both male and female officers and male and female prisoners. There was no effort to provide same-sex personnel to apply or remove the patches. Patches were worn on the upper, outer arm. There seemed to be no objection to having a person of the opposite sex apply or remove the patch.

XIII. ACCEPTANCE OF THE PATCH

A questionnaire written by the project coordinators at the Michigan Department of Corrections and PharmChem Laboratories was given to the Corrections Department Officers who applied and removed the patches. They were asked questions about how they viewed the sweat patch and how they felt it would be accepted by the prisoners. They were specifically asked to decide whether they would prefer urine testing or patch testing for their prison group.

Overall, 78% of the individuals who participated in the program felt that a sweat patch testing system, either alone or in combination with urine testing, would work in their facility. Seven percent thought the patch would not work in their facility.

XIV. CONCLUSIONS

Sweat is a component of the body's excretory system. Thus, the analysis of sweat could reveal a person's drug use. A novel, nonocclusive sweat collection device, PharmChek™, was developed. It contains an absorbent pad that continuously collects and concentrates the nonvolatile components of sweat when applied to a person's upper arm or midriff. The patch is meant to be worn for a minimum of 24 hr to 7 days or more. Any drug the patch wearer uses would accumulate on the absorbent pad. Analysis of the patch would reveal whether or not the person being tested used a drug.

Available experimental reports indicate that patches will contain different drug concentrations that are a function of the amount of drug used. Intersubject and intrasubject variability exists.

Consequently, analysis of a patch can distinguish between a drug user and an abstainer. A positive result provides no clue to the amount of drug used or when it was taken.

In the Michigan Department of Corrections Pilot Program, the patch was worn for up to 14 days with minimal discomfort. Some of the REP subjects (0.9%) and 9.2% of the prison subjects reported skin irritation as a result of using the patch. On-site application and removal are simple, feasible, and cost-effective. The patch provides for continuous monitoring during the time of wear. In contrast, 4 or 5 urine specimens might be required to be comparable.

The initial published reports are encouraging. However, they suggest that the pharmacokinetics and pharmacodynamics of sweat need additional research.

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Chapter 16

DRUG TESTING OF MECONIUM: DETERMINATION OF PRENATAL DRUG EXPOSURE

Leo Kadehjian

CONTENTS

I. INTRODUCTION

The problems of maternal prenatal drug use and its effects on fetuses and newborn infants have received widespread attention. These problems have been felt in the clinical, public health, public policy, regulatory, legislative, and even legal communities. The tragedies of crack babies, fetal alcohol syndrome infants, and low birth weight infants from maternal smoking, and their corresponding costs to society have been well-publicized. The responses to these problems have been varied, from efforts at improved early diagnosis and treatment in the clinical arena to dramatic legal reactions, even including criminal prosecution of mothers for drug delivery to their newborn infants via the umbilical cord in the 60 to 90 seconds after birth.[1] As of 1995, 26 states have passed legislation addressing the identification, reporting, and/or treatment referral of drug-using pregnant women and their exposed infants.[2] But, central to all of these efforts is the ability to identify drug-using pregnant women and their drug-exposed fetuses and infants. It is toward this end of identifying prenatal drug exposure that meconium analysis at birth is being examined.

However, some authors have indicated that drug testing at birth may be too late, in that the fetal drug exposure and any concomitant fetal damage have already occurred.[3] Rather, good clinical practice and public health care policy should strive to identify drug-using women early in pregnancy, if not before pregnancy, and provide counseling and treatment. In this prenatal period, improved and diligently implemented clinical screening and risk assessment tools, coupled with routine testing,

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may be very valuable. Nonetheless, meconium testing at birth has shown itself to be a valuable tool in addressing the problems of prenatal drug use.

A. SCOPE OF THE PROBLEM

The prevalence of maternal drug use has been determined in many ways (from self-reports, physician diagnostic assessments using various screening instruments and questionnaires, to maternal and neonatal drug testing). The accuracy and reliability of each of these tools have been assessed, with drug testing generally proving to be the most sensitive and specific. Despite methodological differences and acknowledged limitations, these studies all show that maternal drug use is extensive. The National Institute on Drug Abuse recently completed its 1994 National Pregnancy and Health Survey.[4] The survey was based on self-report data collected from 2,613 women delivering live-born infants in 52 metropolitan and nonmetropolitan hospitals throughout the U.S. The survey showed that, in 1992, more than 220,000 women used illicit drugs during pregnancy. This represents about 5.5% of the 4 million annual live births in the U.S. However, the statistics for illicit drug use are dwarfed by those for the use of cigarettes (820,000 women, 20% of all births) and alcohol (757,000 women, 19% of all births).

There have been many other studies on the prevalence of substance abuse in pregnant women with the conclusion that this is not an isolated problem, but is pervasive. The number of clinical publications on perinatal substance abuse rose dramatically in the late 1980s as the fallout from the crack cocaine epidemic began showing up in delivery rooms. Without question, cocaine is the drug receiving the greatest attention in the perinatal substance abuse literature. Some authors have criticized the media (both clinical and popular) for allowing the hyperbole surrounding crack babies to overshadow the equally tragic consequences of maternal alcohol and cigarette use, which are much more widespread. Clearly, our society needs to be concerned about prenatal use of all drugs.

B. COSTS TO SOCIETY

Maternal drug use has profound consequences for society. The costs associated with maternal drug use in terms of specialized care for their drug-exposed neonates are many times greater than for nondrug-exposed neonates. Several studies on the costs of cocaine-exposed infants have been done, with extra costs for stays in the neonatal intensive care unit amounting to $10,000 or more.[5–7] In one study of 82 infants using meconium analysis, 41 were positive for either cocaine or opiate exposure, with an average length of hospital stay of 26 days, which at a neonatal intensive care unit daily rate of $1,250 (excluding physicians' fees) amounted to more than $1 million in drug-related costs.[8] The U.S. Government's General Accounting Office estimated that infants exposed to illicit drugs cost society from almost $400 million to up to $3 billion annually.[9] Another consideration is the association of human immunodeficiency virus (HIV) with intravenous drug use and the maternal-to-fetal transmission of HIV.[10,11] The lifetime costs of treating an HIV-infected infant whose mother is an intravenous drug user has been estimated at $102,000.[12] Of course, the costs from the more widely used licit drugs, alcohol and nicotine, must also be considered. The annual costs of the more than 8,000 diagnosed fetal alcohol syndrome infants have been estimated at $321 million.[13–15] The annual costs of low birth weight infants as a result of maternal cigarette use have been estimated at $725 million.[16–18] Thus, prenatal use of all of the aforementioned drugs combined costs society at least several billion dollars annually.

The sequelae of maternal drug use have been felt not only in the medical and public health communities, but also in the legal community. The courts have been drawn into the debate with many civil cases and more than 200 criminal prosecutions against women for using drugs during their pregnancy. These cases have been brought under child abuse and neglect statutes and even criminal drug delivery statutes. Not only have drug-using women had their parental rights curtailed or terminated, some have also been arrested and incarcerated. However, some courts have actually indicated in their published opinions that such prosecutions do not make for sound public policy. Judges have

acknowledged that drug-using pregnant women, fearing such legal threats to themselves and their children, are likely to be driven away from getting the help they and their infants need. There have been more than 100 law review articles comprising over 2,600 pages addressing the legal issues surrounding public policies on maternal drug use. Interestingly, the dramatic rise of law review publications in the early 1990s parallels that in the clinical literature.

II. USE OF MECONIUM TO DETECT PRENATAL DRUG EXPOSURE

Meconium is a dark green mucilaginous material in the intestine of the full-term fetus. It is comprised of accumulated bile, mucous, intestinal secretions, epithelial cells, and swallowed amniotic components. It is up to 70% lipid. It begins accumulation at about 16 weeks' gestation and is not normally excreted by the fetus *in utero*, and thus theoretically represents a window of prenatal drug exposure of about 20 weeks prepartum. In support of meconium formation at 16 weeks' pregnancy is a case of spontaneous abortion at 16 weeks' pregnancy, wherein cocaine was identified by meconium radioimmunoassay (RIA) analysis.[19,20] However, some authors have questioned the ability of meconium analysis to detect drug usage more than 3 weeks before birth.[21] At birth, meconium is excreted by the neonate several times a day for the first 1 to 3 days postpartum. However, premature infants may not pass any meconium within the first 2 days after birth, so any testing using meconium must be accordingly delayed. Up to 10 g of meconium has been collected in pooled specimens, although 0.5 to 1 g usually suffices for drug analysis. Meconium is not to be confused with the infant's first milk stool.

Based on a review of the literature on meconium drug testing using the MEDLINE database and cross-referenced articles, at least 40 full papers and 33 scientific meeting abstracts on human neonatal meconium studies have been published since the first report in 1980.[22] (Not all 73 meconium references are cited in this text, but the uncited articles are included as "Additional References.") These studies have described the analysis of more than 11,000 meconium specimens and more than 2,500 positive results. More than 50% of all the meconium specimens analyzed and reported on in these studies were from just two reports involving very large studies; one involving 4,409 specimens,[23] portions of which had been included in an earlier report,[24] and the other involving 2,270 specimens.[25] This latter study ultimately involved the analysis of more than 10,000 specimens.[26] Furthermore, more than 85% of all meconium specimens analyzed were from only eight large studies, each of which analyzed more than 350 meconium specimens.[23–31] The remainder of the studies each analyzed an average of about 40 meconium specimens. These studies involved meconium analysis for a variety of drugs, using several procedures and analytical methods. Most of these studies have also compared results for meconium testing with those for other specimens, mainly neonatal urine. A few studies compared meconium with self-report.

The first report on drug analysis using meconium was from 1980 and involved a study of the fetuses of six morphine-addicted rhesus monkeys. The fetal tissue which showed the highest concentration of morphine was from the gastrointestinal tract. Two human fetuses from heroin-addicted mothers were also studied. Although their intestinal tracts showed no detectable drug, their meconium specimens did show the presence of morphine and in much greater amounts than found in neonatal urine. Furthermore, much higher concentrations of morphine were found in the meconium specimens collected on the second postpartum day than on the first (62.9 vs. 10.3 μg/g and 6.2 vs. 1.9 μg/g for the two fetuses).[22] Since then, the value of meconium as a specimen for the detection of gestational drug exposure has been repeatedly demonstrated.

Testing of meconium has received interest primarily because it theoretically provides information about drug exposure in the last 20 weeks of pregnancy, whereas a single postpartum urine specimen would provide a window of only a few days. One study, however, challenged the view that meconium provided such an extended window, in that meconium testing detected maternal cocaine use only in those cases wherein the mother reported use within the previous 3 weeks. Although this

study used a sensitive gas chromatography/mass spectrometry (GC/MS) method with a 5 ng/g cut-off for benzoylecgonine, the extraction procedures had a very low recovery (21%) of this analyte.[21] The placenta has drug binding and metabolizing capabilities and thus acts as a protective barrier against fetal exposure to drugs and other xenobiotics.[32] Thus, not every drug-using mother will have an exposed infant who tests positive, whether using meconium, neonatal urine, or neonatal hair.[33]

Meconium has also been shown to be useful in detection of intrapartum drug use (i.e., drug use just before delivery). In a study of the use of meperidine and codeine during labor, both codeine and meperidine were readily detected in meconium. In one third of the meperidine containing specimens, normeperidine was also detected, in concentrations about one half those of meperidine.[34] Thus, unlike hair testing, which may not show evidence of drug exposure until about 1 week after exposure (because of the time it takes for the growing hair shaft to exit from the skin surface), meconium analysis can indicate very recent drug exposure.

A. COLLECTION AND STORAGE

Meconium can be collected by simple scraping with a spatula from the infant's diapers. The specimen can be put into a test tube and tested straightaway or stored at reduced temperatures for later testing. Only about 0.5 to 1 g of meconium is needed for analysis. Opinions seem divided as to whether meconium is easier to collect than urine. There have been concerns that the adhesive tape used to attach a urine collection bag may irritate the newborn's skin and that the collection bag may become dislodged. Such specially designed urine collection bags are commercially available.

Meconium can be collected for 1 to 3 days postpartum, and there seems to be some uncertainty about the value of collecting only the first meconium, as opposed to collecting meconium for subsequent days as well. In addition, a concern has been raised that drugs and metabolites may not be evenly distributed throughout the meconium specimens.

Only a few studies have examined serially collected meconium specimens. The very first study on drug analysis in meconium (1980) indicated that simply sampling the first meconium excreted may not give as great an assay sensitivity as extended sampling for another day or two until the infant's first stool.[22] In one infant, on day 1, the morphine concentration in meconium was 10.3 μg/g, whereas on day 2 the concentration was 62.9 μg/g. For a second infant, the concentration on day 1 was 1.9 μg/g and on day 2 was 6.2 μg/g. In a more extensive study by the same group, 20 infants were tested serially for 3 days for cocaine metabolite, morphine, and cannabinoids. For cocaine metabolite, most sequential specimens remained about the same or declined, but some declined dramatically. However, a few specimens showed large increases in concentration from day 1 to day 2. Similar results were obtained for morphine and tetrahydrocannabinol (THC).[35,36] In another study of serially collected meconium specimens, relative benzoylecgonine levels were higher in specimens collected 25 to 36 hr after birth than in specimens collected within the first 24 hr. The authors attributed this increase to possible contamination of the meconium specimens with extracorporeal urine.[21] An earlier study demonstrated the contamination of meconium specimens with urinary metabolites.[37] For 14 infants with specimens serially collected over two 12-hr periods, results for cocaine, benzoylecgonine, and ecgonine methyl ester generally declined (for 50% of specimens) or remained about the same (for 38% of specimens). Only 12% of the results showed an increase. Others have indicated that the concentration of drugs in meconium drops off after the first 2 days.[36,38] In a study of intrapartum use of meperidine, meconium specimens collected on the first day postpartum averaged 283 ± 821 ng/ml, whereas specimens collected on the second day averaged only 145 ± 178 ng/ml, but the ranges were comparable (0 to 3,500 ng/ml).[34] In a study of cocaine metabolites and opiates in eight infants' meconium specimens collected serially over 3 days, there were no consistent changes in concentrations, with some increasing, some decreasing, and some remaining about the same.[39] It has been proposed that meconium collected over 0 to 10 hr postpartum reflects gestational exposure at <20 weeks, meconium collected at 11 to 20 hr represents 21 to 30 weeks gestation, and meconium collected at 21 to 36 hr represents >30 weeks gestation.[40] Thus, serial collection may reflect the chronology and degree of exposure.

Thus there may exist a trade-off between potentially increased sensitivity vs. early discharge. Also, collecting meconium from diapers beyond the first available specimen runs the risk that the specimens may become contaminated with urine, perhaps affecting interpretation.[21,37]

Specimens may be stored at reduced temperatures (e.g. –20°C) before analysis. Storage at room temperature for 24 hr led to a 25% decrease in measured cocaine metabolite levels, a 62% increase in morphine levels (caused by hydrolysis of morphine glucuronide), and a 30% decrease in THC levels.[41]

III. ANALYTICAL ISSUES

In performing meconium analyses, the specimen is generally first extracted in either an aqueous or organic solvent (most often methanol) and then tested by an immunoassay and/or chromatographic procedures, such as high-performance liquid chromatography (HPLC) or GC/MS. Unlike immunoassays for urine testing, there is as yet no U.S. Food and Drug Administration (FDA)-approved commercial method for meconium testing, although a collection kit including solvent (Mectest®, Meco Industries, Walnut, CA)[23] has been submitted for FDA approval, and an extraction procedure has been patented by a commercial drug testing laboratory (U.S. Drug Testing Laboratories, Inc., Chicago, IL).[42] Each laboratory performing meconium testing must develop its own procedures. Reported extraction efficiencies for various procedures, and for various drugs and metabolites have also been highly variable and must be taken into consideration.

A. EXTRACTION

Because of the mucilaginous semisolid nature of meconium, an extraction step is required before further analysis. A variety of extraction media have been used depending on the analyte and method of subsequent analysis. The extraction media used in the various studies include aqueous HCl, aqueous ammonia (for amphetamines), aqueous NaOH (for THC-CO_2H), aqueous $NaHCO_3$:Na_2CO_3 (5:1), normal saline, aqueous phosphate buffer, pure methanol, buffered methanol, and acetonitrile. Pure or buffered methanol has been the most widely used extraction medium. After suspension in the extraction medium, the mixture is usually vortexed for a few minutes and then centrifuged. The supernatant is then either analyzed directly or subjected to further processing steps, such as further back extractions, evaporation and reconstitution, and derivatization for GC/MS analysis. In addition, solid-phase columns (reversed phase or cation exchange) have also been used to further clean up the specimen, especially for subsequent GC/MS analysis. It is important to account for both extraction efficiency, as well as any dilution or concentration of the specimen as a result of these processing steps when reporting final concentrations.

In the first study of drugs in meconium from 1980, 0.1 *N* aqueous HCl extraction was used for the analysis of morphine. Recovery was 97%, but the sensitivity of the spectrophotometric method was low, with a limit of detection of only 5.6 μg/g.[22] Current methods now have limits of detection in the nanogram per gram range. The use of acidified aqueous extraction, followed by RIA, as used in the early studies, was considered impractical for mass screening because it required separate extraction procedures for the various drugs. The authors of these early studies switched to a one-step buffered methanol extraction followed by Emit® (known as enzyme multiplied immunoassay technique), and found that it gave very high sensitivity and specificity and was practical for mass screening.[43] Many other subsequent studies used pure methanolic extraction, but reported recoveries for the different drugs vary widely, and from study to study. For benzoylecgonine, most studies using pure methanolic extraction report recoveries of 80 to 100%, but several studies using pure methanol extraction have obtained much lower recoveries, some as low as 20 to 30%.[37,44,45] Some of the studies reporting these very low recoveries also used solid-phase columns to further clean up the specimen after the initial extraction, and it has been postulated that the conditions of initial extract application to the columns were not optimal.[46] Using methanol extraction for the analysis of five

drugs, recoveries of 95 to 100% were reported in contrast to 48 to 75% for normal saline extraction.[47] The use of acidified methanol (0.2 *N* HCl:MeOH, 1:1) extraction over pure methanol was later introduced and was reported to increase extraction efficiency to 100% for benzoylecgonine, giving a 5-fold increase in selected ion monitoring ion abundance in GC/MS analysis.[48] However, studies using the original acidified aqueous extraction method have also reported acceptable extraction efficiencies. Acetonitrile extraction was used by one group for the analysis of benzoylecgonine, but recoveries were not reported.[30,49] A single methanol extraction was found to give turbid specimens unsuitable for subsequent Emit® analysis; thus, a chloroform:isopropanol (3:1) extraction from an aqueous carbonate:bicarbonate meconium suspension was used. Extracts were dried and reconstituted in aqueous phosphate buffer, and ethyl acetate was used to remove lipids before Emit® analysis. This gave clearer extracts with lower background absorbance.[39,50]

A recent report addressed the rate of false-negative results caused by inefficient extraction procedures. This study compared four extraction procedures: aqueous HCl, methanol, phosphate-buffered methanol, and glacial acetic acid/diphenylamine/acetone, followed by evaporation and reconstitution leaving a concentrated extract. In comparing the four methods in retesting 100 previously confirmed positive specimens for amphetamines, THC metabolite, cocaine metabolite, and opiates, the first two procedures identified only 20% of the positive specimens, the third procedure 54%, and the last procedure 100%. This study also addressed the rate of unconfirmed positives caused by use of immunoassay screening tests without further confirmation. Of 535 meconium specimens that screened positive, only 285 (53%) were confirmed by GC/MS.[51]

B. USE OF SOLID-PHASE COLUMNS

Solid-phase columns have also been used after solvent extraction in over a dozen studies, especially where GC/MS analysis is performed. The original extracts were applied to the solid-phase column, and the column is washed with water, aqueous acid, and methanol. Drugs and metabolites are then eluted from the columns using, for example, methylene chloride:isopropanol:ammonium hydroxide (78:20:2) for cocaine analysis.[46] The pH of the extract applied to the column may have an important effect on overall recoveries, because application to the columns from a pH 6 phosphate buffer may have led to low recoveries (30%) of benzoylecgonine,[44,45] whereas application from a pH 2 phosphate buffer led to much higher recoveries (89 to 93%).[46] Application to the columns from drug-free urine also had low reported recoveries (21%) of benzoylecgonine.[37] Use of a strong cation exchange column after initial pure methanol extraction also led to very high recoveries (98 to 100%) for benzoylecgonine.[52]

C. ANALYSIS

After extraction, the specimen has been most often analyzed by an immunoassay. The immunoassays used have included RIA (Diagnostics Products Corporation Coat-a-Count®, Roche Abuscreen®), Syva Emit®, Abbott FPIA (known as fluorescence polarization immunoassay), Roche ONTRAK™, and Roche Online™. (Although in one study, this last technique, which uses latex beads, demonstrated many unconfirmed positive results apparently caused by interaction of the beads with the comparably sized [1 to 5 μm] lipid droplets in the extracted and methanolic buffer reconstituted meconium specimens).[53] The chromatographic techniques used have been primarily GC/MS and occasionally HPLC. Drugs and metabolites that have been detected in meconium include opiates (morphine and morphine glucuronide, codeine, and hydrocodone), methadone, meperidine and normeperidine, cocaine and metabolites (norcocaine, benzoylecgonine, benzoylnorecgonine, ecgonin methyl ester, *m*-hydroxybenzoylecgonine, and cocaethylene), THC (parent and major acid metabolite, and other immunoreactive metabolites), amphetamine and methamphetamine, phencyclidine (PCP), benzodiazepines, and cotinine (nicotine metabolite). In addition, fetal exposure to ethanol has been determined by meconium analysis for various fatty acid ethyl esters: ethyl laurate, ethyl palmitate, and ethyl stearate.[54]

D. CUT-OFFS AND LIMITS OF DETECTION

The cut-offs chosen for determining positivity are critical when comparing various assay methods and specimens for their respective sensitivity, specificity, positive predictive value, and negative predictive value. Thus, a noninstrumented immunoassay (Roche ONTRAK™) with a fixed cut-off of 300 ng/ml for opiates and cocaine metabolite in meconium proved to have lower sensitivity relative to a RIA, with much lower cut-offs of 15 to 25 ng/g, as would be expected.[55] RIAs have demonstrated limits of detection for various analytes in meconium on the order of 15 to 25 ng/g; although, in a few studies, much higher cut-offs were used. In one study of meconium specimens using RIA to detect cocaine exposure, the detection rate was determined for several cut-offs. At a 1,000 ng/ml cut-off, 10 specimens were positive; at 300 ng/ml cut-off, 18 were positive; at 50 ng/ml cut-off, 31 were positive; and at 25 ng/ml cut-off, 38 were positive.[56] Homogeneous immunoassays, Emit®, and FPIA, have been used in many studies on meconium, but often using much higher cut-offs, up to several hundred nanograms per gram. However, several studies have used these immunoassays not at conventional urine testing cut-offs of several hundred nanograms per gram, but rather used cut-offs determined from 2 or 3 standard deviations above the rate for negative specimens. This allowed cut-offs on the order of 50 to 60 ng/g.[23,29,30,49] HPLC has demonstrated limits of detection of 30 to 100 ng/g. As expected, GC/MS has demonstrated the lowest limits of detection, on the order of 5 to 10 ng/g; but, in some studies, much higher cut-offs have been used (50 to 300 ng/g).

E. METABOLIC CONSIDERATIONS

Generally, the metabolites found in meconium are the same as those found in urine, and in comparable proportions, although some differences have been noted. For cocaine, several papers have indicated that the parent cocaine may be present in greater amounts than its metabolite benzoylecgonine, which is the major urinary metabolite. But, this is not a consistent finding. In examining the ratio of cocaine to benzoylecgonine in 37 positive meconium specimens from 20 infants, the average cocaine concentration was 388 ng/g (16 to 2,917), whereas the average benzoylecgonine concentration was 1,130 ng/g (75 to 4,978). However, three specimens had a greater amount of cocaine than benzoylecgonine, and four had only cocaine.[21] The authors also cautioned that extracorporeal contamination of meconium with urine may have accounted for higher amounts of benzoylecgonine in meconium specimens collected at 24 to 36 hr postpartum, as opposed to meconium collected within the first 12 hr.

A few reports have indicated the presence in meconium of *m*-hydroxybenzoylecgonine, a minor metabolite in adult urine.[57,58] The presence of this metabolite in meconium has been proposed to be responsible for inconsistent results between screening and confirmation assays, where its immunoreactivity was shown to be comparable with that of benzoylecgonine in FPIA and Emit® assays, and comprised up to 6.3 times the amount of benzoylecgonine in meconium specimens.[58] In another study, 197 (95%) of 208 cocaine-metabolite FPIA-positive meconium specimens had this metabolite present; and, for 23% of the specimens, this was the only cocaine metabolite detected.[57]

Another study has demonstrated the presence of the previously unreported benzoylnorecgonine in 7 of 11 meconium specimens from cocaine-exposed infants in amounts up to 5,000 ng/g.[46]

Cocaethylene, which is formed *in vivo* when both cocaine and ethanol are consumed together, has also been detected in meconium.[38,59–61] In one study of 36 cocaine and/or metabolite-positive meconium specimens, 17 of them had cocaethylene, indicating that their mothers used both cocaine and ethanol.[38] Another study also identified cocaethylene in 1 of 26 cocaine/metabolite-positive meconium specimens.[59] There are concerns that cocaethylene may be more toxic than cocaine itself. Another study addressed the potential for toxicity of other cocaine metabolites, especially benzoylecgonine.[62]

One study involving fetal autopsies from two spontaneous abortions and one stillborn infant proposed that the cocaine concentration in meconium is proportional to the amount of maternal use and that the presence of cocaine in various segments of the fetal intestine is related to the time of

maternal use. Experiments on rats supported these conclusions.[19,20] A cocaine dose–response relationship was also demonstrated in a study of benzoylecgonine levels in meconium and birth weight, length, and head circumference. The dose–response relationship was maintained even after controlling for gestational age, and maternal use of cigarettes, alcohol, and marijuana.[63] There have been many other studies demonstrating a correlation between meconium positivity for drug exposure and adverse neonatal outcomes.

In a study of detection of opiate exposure, where both free and conjugated morphine are eliminated, the hydrolysis of glucuronide conjugates after acidic aqueous extraction did not significantly increase the detection rate. The total morphine detected after hydrolysis of 12 meconium specimens was on average 35% greater than for the nonhydrolyzed specimens, but this was not statistically significant.[64]

IV. COMPARISON WITH OTHER METHODS

In an effort to identify drug-exposed newborns, a variety of both maternal and neonatal specimens have been examined. These include maternal and neonatal urine, maternal and neonatal hair, amniotic fluid, and meconium. Each of these specimens offers its own advantages. The chief advantage of maternal hair, neonatal hair (which grows during the last 4 months of pregnancy and is available for 3 to 6 months after birth), and meconium is that they potentially offer the ability to determine drug exposure over an extended period of time of several weeks to months before birth. However, these specimens and techniques are not widely used; the specimens may not always be readily available (neonatal hair), do not allow for easy laboratory automation (meconium and hair specimens requiring extraction), and can be expensive. Furthermore, there are still unresolved technical and interpretive issues in hair testing, although much active research is ongoing.[33,65–75] There have been reports of false-positive maternal hair test results from passive exposure to cocaine and THC.[40,76] In contrast, urine testing is very widely used with FDA-approved commercial procedures and kits, is easily automated, and is relatively inexpensive. Although urine has the limitation of offering a window of only a few days for most drugs, its use in maternal testing throughout pregnancy is a major advantage, allowing early detection of drug-using mothers hopefully before serious fetal damage is done. In 19 studies, wherein meconium was compared with urine, 6 showed comparable results, 10 showed meconium more sensitive than urine, and 3 showed urine more sensitive than meconium. Overall, in these 19 studies, meconium demonstrated about one third more positive test results than urine.

In addressing sensitivity of various specimens and assay technologies, it is important to examine the assay cut-off used for determining whether a specimen is called positive or negative. In comparisons of the diagnostic sensitivities of meconium relative to other specimens, the cut-offs used for the different specimens are often different, precluding an accurate assessment of each specimen's sensitivity. In some studies, neonatal or maternal urine specimens were tested at conventional immunoassay cut-offs of 300 ng/ml for benzoylecgonine, whereas meconium was tested at a much lower GC/MS cut-off of only 5 ng/g. Several researchers using commercial immunoassay kits have chosen to use cut-offs that are 2 or 3 standard deviations above the negative rates, rather than the higher kit manufacturer recommended cut-offs. It is important to note that the concentrations of drugs in meconium are generally reported as nanograms per gram, whereas for urine specimens the concentrations are reported as nanograms per milliliter. Sometimes, meconium results are reported as nanograms per milliliter in the final assay liquid after extraction. In those cases, it is important to be aware of the efficiency of extraction, any concentration or dilution effects caused by the extraction and reconstitution procedures, and whether the studies have taken these into account when reporting assay sensitivity and cut-offs.

The importance of assay cut-off and sensitivity in these specimen comparisons was emphasized in a study of 18 paired meconium and neonatal urine specimens tested at different cut-offs. Meconium specimens were tested at a 100 ng/g FPIA cut-off and generated nine positive results.

When the paired urine specimens were tested at a 150 ng/ml Emit® cut-off, only seven positive results were obtained; but, when the paired urine specimens were tested at a 10 ng/ml GC/MS cut-off, 11 positive results were obtained. The authors concluded that, "The preliminary data indicate that the key factor in determining the effectiveness of neonatal screening for cocaine exposure is the sensitivity of the assay utilized rather than the specimen type."[77]

A. REVIEW OF THE LARGEST STUDIES

In the largest meconium study from a single hospital, a prospective study of 3,010 newborns from a high-risk Detroit population, meconium testing (by RIA) was shown to be far superior to self-report data in the detection of cocaine, morphine, and cannabinoids. The RIA cut-offs were 15, 25, and 50 ng/g for cocaine, morphine, and marijuana metabolites, respectively. Meconium analysis identified 44% of the subjects as positive, whereas self-report identified only 11%. When specimens from neonates of admitted maternal drug users were tested, meconium showed much improved detection rates vs. neonatal urine (88% vs. 52%).[24] Partial data (1,000 specimens) from this study was reported in an earlier report.[78]

A large government-sponsored study reported on the analysis of 2,270 specimens from four sites using Emit® (at slightly reduced cut-offs) after methanol extraction, followed by GC/MS confirmation. The drugs analyzed were cocaine metabolite (at 100 ng/g), opiates (at 200 ng/g), cannabinoids (at 20 ng/g), amphetamines (at 200 ng/g), and PCP (at 10 ng/g). Relatively low confirmation rates were observed that were attributed to specificity differences between the immunoassay and GC/MS.[25] Since the initial report, more than 10,000 specimens have been analyzed in this study.[26]

A large study from Rochester, NY involved anonymous testing of all mothers (urine) and infants (meconium and urine when specifically ordered). The testing of 1,030 meconium specimens for benzoylecgonine by FPIA (at 60 ng/g) yielded 52 positives (5%). Testing of 903 maternal urine specimens (at 50 ng/ml) yielded 39 positives (4.3%). Both meconium and maternal urine were available for 737 mother–infant pairs, with both specimens positive in 26 pairs. Maternal urine was positive for 30 of the paired specimens, whereas meconium was positive for 35. Thus, in four instances, the maternal urine was positive when meconium was negative; in nine instances meconium was positive when urine was negative. Neonatal urine specimens were collected only for 119 infants when there was a physician-ordered drug screen. Of these 119 neonatal urine specimens, 24 (20%) were positive for benzoylecgonine. For 100 of these infants, both urine and meconium specimens were collected. Neonatal urine gave positive results in 21 of these paired specimens, whereas meconium gave positive results in 28. There were no instances wherein the neonatal urine was positive when meconium was negative. Thus, meconium was slightly more sensitive than either maternal or neonatal urine. This study also reported on the sensitivity of screening assessment criteria for physician-ordered testing vs. urine and meconium test results. The screening criteria showed good sensitivity of 89%, but a relatively poor specificity of 69%. The positive predictive value was a poor 15%, and the negative predictive value was 99%.[30]

In a study of meconium, neonatal urine and maternal urine for the detection of cocaine use in 423 consecutive deliveries at a Bronx, NY hospital, all three specimens gave virtually identical positive rates of about 12% by Emit® (37/303 maternal urine specimens, 42/351 neonatal urine specimens, and 41/345 meconium specimens). Specimens giving results above the negative calibrator were considered positive. A few of the specimens gave positive results by one specimen, but not with the others. The authors felt that meconium had an advantage over neonatal urine because of greater reliability of collection, although this view is not shared by all authors on the subject. For the THC metabolites, there were 18 maternal urine specimens with positive results, whereas all neonatal urine and meconium specimens were negative. The authors concluded that meconium did not seem to offer an advantage over maternal or neonatal urine in the detection of cannabinoids, codeine, morphine, or methadone.[29] A similar conclusion was reached by other authors.[21,39,79,80] Some authors have noted that the additional processing steps involved with meconium analysis make it unsuitable for routine screening purposes.[39]

In a study comparing maternal urine and meconium results for detection of cocaine use, maternal urine was collected in 258/426 deliveries, with a positive rate of 3.9%; whereas, meconium was collected in 367/426 deliveries, with a positive rate of only 1.6%.[27]

In a study of meconium from 621 consecutive newborns from two university-affiliated urban hospitals in Connecticut, 21 specimens were positive for cocaine metabolite (3.4%) using FPIA (at 100 ng/ml after extraction). Another 10 specimens had results above the negative rate, but these were excluded because previous experiments had shown poor recoveries when meconium was spiked with such small amounts of benzoylecgonine. In contrast, the nurses' opinions about cocaine exposure were correct only 22% of the time.[28]

In a study of 500 meconium specimens from a suburban medical center outside of Philadelphia, 59 tested positive for cocaine metabolite, whereas only five mothers admitted using cocaine. This study also noted a dramatic difference in the incidence of infants testing positive, depending on whether the mother had private insurance, was covered by Medicaid, or had no insurance. Almost 27% of women who had either no insurance or were covered by Medicaid had infants with positive meconium tests, whereas only 6.3% of the women covered by private insurance had positive infants.[31]

B. OTHER REPRESENTATIVE STUDIES

In a study of the detection of prenatal cocaine usage in 40 inner city women using four different specimens (maternal hair, maternal urine, neonatal hair, and meconium), hair and meconium specimens tested by GC/MS showed greater positive rates (24/40 neonatal hair at 600 ng/g cut-off, 28/40 for maternal hair at 600 ng/g cut-off, and 28/40 for meconium at 40 ng/g cut-off) than maternal urine specimens tested by Emit® or GC/MS (17/40 at 20 ng/ml cut-off).[74]

In another study involving 59 mother–infant pairs, test results were compared for meconium, infant hair, maternal hair, infant urine, and self-report. Maternal hair test results showed excellent sensitivity relative to self-report, identifying 40/41 (97.5%) of mothers with a history of cocaine use. In contrast, infant hair was positive for 25/32 whose mothers had positive hair test results for a sensitivity of 78%. Meconium showed a corresponding sensitivity of 74 to 52%, depending on the analyte and cut-off (cocaine by GC/MS at a 300 ng/g cut-off or benzoylecgonine by FPIA at a 150 ng/g cut-off, respectively). Finally, neonatal urine (using a 300 ng/ml cut-off for benzoylecgonine) had a sensitivity of only 38%.[73]

In a study of cocaine exposure in 232 very low birth weight infants and their mothers, 86 were cocaine-exposed, based on either testing or self-report. Self-report showed a sensitivity of 65%, maternal urine of 64%, meconium of 51%, and neonatal urine of 25%. Five of the 86 exposed infants were identified by either their urine tests or their mothers' urine tests alone; 13% were identified by maternal history alone, and 30% were identified by meconium alone. Of 33 positive meconium tests, cocaine was identified in 21, norcocaine in 15, and cocaethylene in 1. No benzoylecgonine was identified in any meconium specimen. For 62 exposed infants with both urine and meconium tests, only 3 positives and 17 negatives were in concordance for the two methods.[60]

There have been a few reports on the analysis of amniotic fluid.[21,79,81–84] A comparison with amniotic fluid was made in one study, wherein amniotic fluid collected by amniocentesis for assessment of fetal lung maturity was also available for drug testing, but only from six subjects. In this study, 20/30 meconium specimens were positive for cocaine or its metabolites, whereas 3/6 amniotic fluid specimens were positive. The authors noted that amniotic fluid did not offer any substantial benefit.[21,79] In another study, comparing amniotic fluid to maternal and neonatal urine for detection of cocaine exposure in 23 known cocaine users, amniotic fluid was positive in 74%, whereas maternal urine was positive in 61% and neonatal urine was positive in 35%.[84]

In addition to comparisons between various specimens, comparisons between specimen testing and patient history or self-report have also been reported. The limitations of self-reported drug use by pregnant women are well-known.[24,85–93] Repeatedly, studies have demonstrated that testing (whether through maternal or neonatal urine, maternal or neonatal hair, or meconium) is superior to

self-report. In five studies wherein patient history was compared with meconium testing, history identified fewer than one third the number of positives identified by testing (history 412 vs. meconium 1452).[24,28,31,45,75] One reason postulated for low self-report rates is the fear of sanctions, even up to criminal prosecutions for drug use. It has been noted that well-designed screening questionnaires with appropriate health care provider training can improve self-report rates.

Even health care provider assessments have shown poor sensitivity.[94] For example, in one study, nurses' opinions about neonatal cocaine exposure were correct only 22% of the time, relative to meconium test results. Only 12 of the mothers of 21 infants with positive meconium results were identified by the nurses, for a sensitivity of only 57%. The positive predictive value of the nurses' assessments was a poor 22%.[28] In another study, neonatal urinalysis ordered after physician assessment using established screening criteria yielded a sensitivity of 89%, but also yielded a poor positive predictive value of 15% when compared with results from universal meconium and maternal urine testing.[30]

V. CONCLUSIONS

Over the last few years, there has been growing interest in the use of meconium to detect gestational exposure to drugs. Meconium analysis theoretically offers the advantage of detecting drug exposure over the last trimester of pregnancy, although use within the few weeks before birth may be required for ready detection. Accurate and reliable laboratory procedures have been developed, although there are those who question the ease of collection of meconium and the necessary extraction procedures. Nonetheless, meconium has shown itself to be a sensitive and accurate gauge of gestational drug exposure. Whether meconium testing will become as widely used as urinalysis remains to be seen. Urine does have the advantage of being usable throughout pregnancy (as does maternal hair). One writer summed up the situation by indicating, "It is unfortunate but there is no gold standard for drug detection in the prenatal or neonatal period."[95]

Even though the value of meconium analysis for identification of drug-exposed neonates has been repeatedly demonstrated, solutions to the problems of maternal drug use and its sequelae will come from identifying drug-using mothers early in their pregnancy and providing them with counseling and treatment before fetal damage is done.

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Chapter 17

ANALYTICAL TOXICOLOGY OF VITREOUS HUMOR

Robert O. Bost

CONTENTS

I. INTRODUCTION

The forensic pathologist must consider many questions about each fatality investigated. Among these questions are the following five that are usually referred to the toxicologist:

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1. Did the decedent use a therapeutic or toxic substance?
2. If so, which?
3. What was the concentration?
4. What does the result mean?
5. Was the fatality a direct consequence?

To answer the first three questions, the toxicologist must be provided with one or more specimens for analysis. The toxicologist is also guided in selecting the optimal analytical protocol if a history is provided along with the specimen(s). The following items can provide useful information: age, race, sex, medical history (diseases, medications prescribed or used before death), social history (drinking and/or use of illicit drugs), circumstances of death, condition of the body, whether embalmed before collection of specimen(s), findings at the scene of death (medication bottles, alcoholic beverage containers, drug use paraphernalia, unexpected containers of chemicals [such as roach bait or other pesticide at the bedside], aerosol containers near a young decedent, household products/cleaners, bottle of antifreeze or drinking glass containing residue of antifreeze, charcoal grill or hibachi inside the living area, etc.), and the pathologist's initial findings.

The analytical scheme will depend on the (1) capabilities of the laboratory's staff and equipment; (2) circumstances of the individual case; and (3) specimen(s) provided to the laboratory. Only this third factor is within the scope of this chapter. Some analytes can be determined only in specific specimens (carbon monoxide in blood or other hemoglobin-containing specimens), but the majority of drugs or other toxic substances can be qualitatively identified, with greater or lesser success, in any of the specimens usually obtained during autopsy. In the event that multiple specimens are provided to and analyzed by the toxicology laboratory, comparison of the results for the various specimens will aid in interpretation of the findings for the case (i.e., in trying to answer questions 4 and 5).

The usual biological specimens submitted for toxicological analyses are blood, bile, urine, and aliquots of tissues. Occasionally nails, hair, and vitreous humor may be considered. Vitreous humor is a useful, although frequently ignored, specimen. Comparison of results in vitreous humor with those in blood can assist in distinguishing between absorption and elimination states after ethanol use and in distinguishing between exogenous and endogenous sources of ethanol. The presence of a high vitreous humor concentration of glucose is useful information in the diagnosis of a diabetic patient. In the absence of a blood specimen, results obtained from vitreous humor may provide some insight into the death of the patient. Limited information is available regarding the pharmacokinetics of drug disposition into and out of vitreous humor. As discussed herein, it is believed that ethanol (and probably other small, water-soluble substances) can pass quickly into and out of the vitreous humor from blood, whereas morphine seems to distribute into and out of vitreous humor slowly. Because vitreous humor is not appropriate in most clinical/pharmacokinetic studies and because the time interval in fatalities between ingestion and death is most often not known, pharmacokinetic information will only be available by inference from accumulations of large numbers of cases, from extrapolations from animal studies, or from those rare cases in which all of the information is available. For these reasons, forensic toxicologists need to be knowledgeable about and capable of performing analyses of vitreous humor specimens. They also need to be willing to perform analyses with greater frequency and to share their results with their colleagues.

II. OBTAINING VITREOUS HUMOR SPECIMENS

A. STRUCTURE OF THE EYE

The anatomy of the eye is diagrammatically represented in Figure 17-1. The term "vitreous humor" refers to the gel that fills the vitreous body of the eye. Although technically a gel, the vitreous humor is fluid enough to be manipulated like other fluid specimens (pipetted, diluted with other solutions, and passed through flow systems). The vitreous humor should be obtained during the autopsy. If an autopsy is not required, vitreous humor may be extracted at the convenience of the

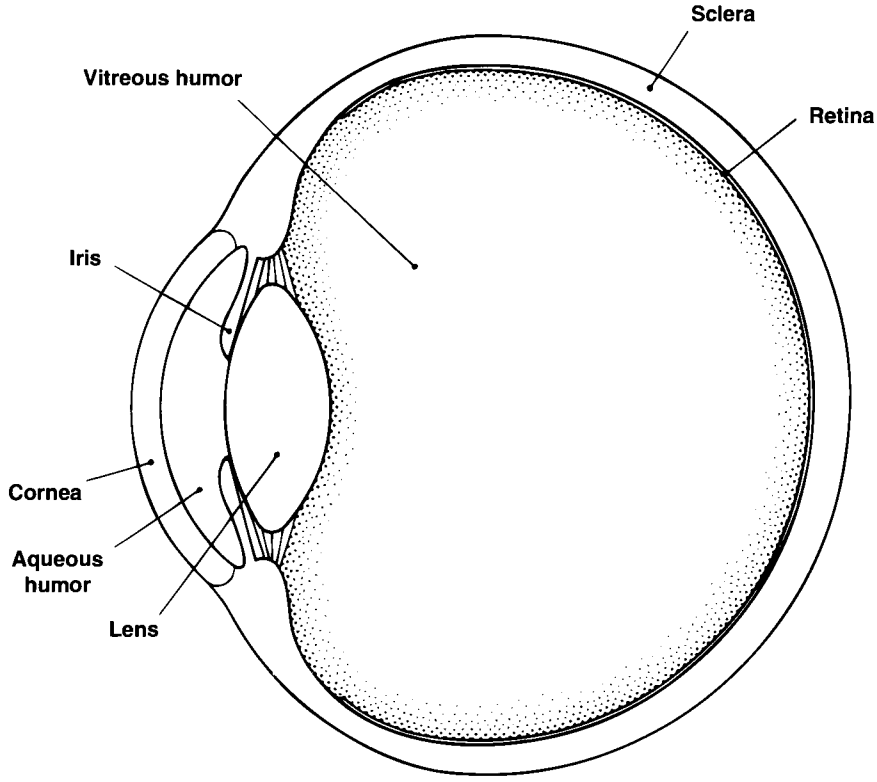

FIGURE 17–1. Structure of the human eye.

medical legal agency's staff. To avoid confusion in nomenclature regarding other portions of the eye, the term "vitreous humor" will be used in this chapter to refer to the gel in the vitreous body and to the specimen that is provided to the laboratory.

B. COLLECTION OF THE SAMPLE

Vitreous humor specimens are obtained using a 5-ml or a 10-ml syringe. The needle is placed against the eye at the lateral canthus (i.e., the lateral aspect just above the junction between the upper and lower eyelids). The needle is pressed through the membrane and into the eye to a depth of approximately 2 cm (3/4 to 1 in.). Using gentle suction, the vitreous humor is withdrawn into the syringe until a volume of 2 to 3 ml is obtained. Use of excessive suction is to be avoided, because this could draw cellular components into the specimen and result in artifactually altered results for some analytes. If a larger volume of specimen is desired, then collection from the second eye can be done. Once the specimen has been withdrawn from the eye(s), it can be placed into a suitable container for transport to the laboratory. Some authors add a preservative to vitreous humor specimens, but most do not use any preservative. Suitable containers can include vacutainer tubes or screw-capped test tubes, or the syringe itself can be sent to the laboratory. Submission of the syringe is undesirable unless the needle has been removed and replaced with a leak-proof cap before transport. The container should be properly labeled to identify the case number, pathologist responsible for the case, person taking the sample, and any other information routinely used within the system for proper identification of evidence being submitted to the laboratory. It is proper to replace the vitreous humor with a similar volume of saline to ensure the cosmetic appearance of the eye(s). The family can be spared the unpleasant experience of observing the vitreous-depleted eyes.

After the specimen has been received and properly accessioned into the laboratory, storage at refrigerator temperature (4°C) is satisfactory for most analytes. In a study of cases of digoxin overdose, DiMaio et al.[1] either performed the analyses within 24 hours or else froze the specimens until

analysis could be performed. Several years ago, in the author's laboratory, specimens for insulin analysis were frozen until they could be delivered to a laboratory performing the analysis; however, freezing is no longer required. Coe[2] has recommended that vitreous humor specimens be centrifuged and the supernatant portion be used for analysis, especially in those analyses in which the specimen is sampled directly into the instrument and must flow through small diameter tubing.

C. CAUTIONS

Infection control must be a concern when working with vitreous humor samples, just as it is with other biological specimens. Acquired immune deficiency syndrome (AIDS), hepatitis, and tuberculosis are among the diseases that can be spread by improper exposure to a contaminated sample. Klatt and Noguchi[3] have properly summarized the need for *routine* use of universal precautions.

> The CDC recommends that universal precautions be employed for routine infection control.[4] This means that workers must assume that all persons, specimens, or bodies may be harboring infectious agents, including HIV. This is because—as we stated earlier—you cannot always tell from appearances who is infected and who is not. Moreover, many infectious diseases—such as hepatitis or tuberculosis—are more easily transmissible than HIV. In fact, attention should not be focused only on HIV while ignoring other potentially harmful diseases.

Numerous publications have shown that both the human immunodeficiency virus (HIV) antigen and antibodies can be detected in vitreous humor samples.[5,6] Klatt et al.[5] reported that vitreous humor specimens were positive for HIV antibody up to 34 hours post-mortem; some vitreous humor specimens gave "false-negative" results when tested longer than 34 hours post-mortem.

Karhunen et al.[6] reported, ". . . that HIV antibodies can be verified in samples of sera, whole blood, vitreous fluid, or bile that were stored for months at room temperature." Nyberg et al.[7] reported finding evidence for infectivity in specimens obtained at autopsy from patients with clinical AIDS, even when the autopsy was delayed for up to 6 days after death. Cowan et al.[8] collected vitreous humor specimens from 11 patients with AIDS; for five of the patients, antigen was detectable, and antibody was detectable in seven patients. Although published or official guidelines often do not include vitreous humor among the specimens covered, it must be remembered that most of these guidelines refer to clinical situations that would not include vitreous humor specimens. Furthermore, the absence of a mention of vitreous humor in these guidelines should not be assumed to imply that vitreous humor is any less dangerous or infectious than any other body specimen.

III. ANALYTICAL METHODS

In general, the same analytical procedures can be used with vitreous humor specimens as with other specimens. The limited specimen volume available may place some restrictions on the procedures used and/or the type of analytes investigated. The following analytical procedures have been reported by various investigators: Conway microdiffusion/dichromate, alcohol dehydrogenase, Widmark distillation method, glucose oxidase-peroxidase, hexokinase, ultraviolet spectrophotometry, gas chromatography (GC) (or gas-liquid chromatography [GLC]), liquid chromatography (LC) (or high-performance liquid chromatography [HPLC]), immunoassays (such as radioimmunoassay [RIA], enzyme multiplied immunoassay technique [EMIT], and fluorescence polarization immunoassay), and atomic absorption.

Not surprisingly, earlier investigators used procedures for vitreous humor specimens that they used for other specimens for the same analytes, procedures that are not used very often at the present time. Felby and Olsen[9] used alcohol dehydrogenase and the Widmark method for ethanol. In a separate study, Felby and Olsen[10] used ultraviolet spectroscopy to determine barbiturates and meprobamate in vitreous humor, blood, and liver. Coe and Sherman[11] analyzed for ethanol with a diffusion/dichromate procedure. Sturner and Garriott[12] described using ultraviolet spectrophotometry

and GLC for detection and quantitation of numerous different drugs in both vitreous humor and blood. They also used RIA for determination of digoxin and insulin. Blumenfeld et al.[13] used a variety of methods to measure various analytes in sudden infant death syndrome cases: enzymatic (urease) method for urea nitrogen; atomic absorption for calcium and magnesium; coulometric–amperometric method for chloride; flame photometry for potassium and sodium; a Jaffe method for creatinine; and a trichloroacetic acid–Ponceau S dye method for total protein. Several reports describe using automated analyzers for determination of various electrolytes and similar analytes. The automated analyzers at that time incorporated colorimetric procedures for chloride and glucose and flame photometers for sodium and potassium. In addition to using a Technicon AutoAnalyzer for sodium, chloride, potassium, urea nitrogen, glucose, creatinine, SGOT and SGPT, Leahy and Farber[14] also used electrophoresis on a cellulose acetate medium for separation of lactate dehydrogenase isoenzymes. One of the problems with analyzing vitreous humor specimens in automated analyzer systems occurs because of the viscosity of the specimens. At the Southwestern Institute of Forensic Sciences/Dallas County Medical Examiner's Office,[15] vitreous humor specimens were submitted to the adjacent hospital clinical chemistry laboratory for electrolyte analyses. The clinical laboratory had significant difficulty with plugged flow lines associated with analyzing our specimens. After our laboratory purchased an automated analyzer system, we had similar problems caused by formation of fibrous precipitates in some of the lines. Ultimately, we discovered that the acid solution needed for the CO_2 analysis was the responsible reagent; because we did not use the CO_2 results, we replaced the acid solution with water, and the problem was resolved. (This automated analyzer system used ion-selective electrodes for the detection of the various analytes.) As described previously, Coe[2] recommends that vitreous humor specimens be centrifuged and that the supernatant portion be used to prevent clogging of the small diameter tubes often used in modern analytical instruments.

Little has been discussed regarding sample preparation or extraction before instrumental analysis. Most work has been done just as blood would be (i.e., direct introduction into autoanalyzers, dilution with aqueous internal standard and headspace analysis for alcohol, or liquid–liquid extraction for drugs). Evenson and Engstrand[16] described their procedure for using SepPak C_{18} cartridges for solid-phase specimen cleanup before HPLC of tricyclic antidepressants.

As newer technology became available and put to general use in the analysis of drugs, this technology was used for analysis of vitreous humor specimens. Gas chromatographic (GC or GLC) analysis for ethanol was used by Winek and Esposito[17] in comparing ethanol concentrations in blood, bone marrow, vitreous humor, bile, and urine. Likewise, Clark and Jones[18] used GC to determine ethanol in a study of possible putrefactive ethanol production. Wu Chen et al.[19] used GC in their investigation of a case of methanol intoxication. Of course, many other publications have also included the use of GC for the determination of ethanol or other similar volatile substances. In some laboratories, the fluid specimen is injected directly into the GC without any prior treatment; other laboratories dilute the specimen with water before injection. Often, the aqueous diluent also includes an internal standard for use in quantitation. Liquid samples (diluted or not) can be injected manually or by autosampler injector systems. Headspace analysis has also been used in many laboratories and, again, may be done either manually or by autosampler.

The problems associated with analysis of vitreous humor specimens in autoanalyzers designed for handling serum/plasma specimens have been described previously. Watts and McDonald[20] have examined several different specimen types (blood, plasma, serum, urine, vitreous humor, and water) for possible matrix effects on the headspace gas chromatographic analysis for ethanol or other volatile compounds. Water spiked with the various volatiles was used as the basis for comparison. For all specimen types, both headspace injection and direct injection were used. For most analyses, sodium chloride was added to "salt out" the volatiles; use of sodium sulfate gave greater variability. For each compound, the difference in peak heights for the test specimen and for water was calculated. Furthermore, the differences were tabulated for both headspace injection and direct injection. The results obtained with vitreous humor specimens were virtually indistinguishable from water specimens with direct injection and also with headspace injection.

GC has been the analytical technique applied to the determination of the majority of drugs of concern to the forensic toxicologist. As mentioned previously, Sturner and Garriott[12] used GC to analyze blood and vitreous humor for the presence of several drugs, including propoxyphene, barbiturates, tricyclic antidepressants, stimulants, pentazocine, and methadone. In a study of 484 fatally injured drivers and pedestrians, Cimbura et al.[21] used several techniques (ultraviolet spectroscopy, thin-layer chromatography, colorimetric tests, RIA, and GC) for the determination of a variety of drugs. GC was used by Poklis et al.[22] for analysis of cocaine.

RIA has been used by Sturner and Garriott[12] for digoxin and insulin; by Vorpahl and Coe[23] for analyses for digoxin; and by Ziminski et al.[24] for barbiturates, methadone, and morphine. Bermejo et al.[25] reported on RIA analyses for morphine in vitreous humor both with and without enzymatic hydrolysis. Sutheimer et al.[26] evaluated the Syva ETS-Plus ethanol assay with both ante-mortem blood and routine post-mortem specimens; they found that the correlation between ETS results and GC results was quite good, although they note that the 14-sample study group was small.

More recently, HPLC has been applied to the analysis of numerous drugs. It is particularly applicable to those drugs that are not easily volatilized and analyzed by GC. Stajic et al.[27] used HPLC for the analysis of metoprolol in an overdose case. Micellar electrokinetic capillary chromatography is a form of capillary zone electrophoresis; Ferslew et al.[28] have used micellar electrokinetic capillary chromatography for the analysis of barbiturates in several biological fluids, including vitreous humor.

In keeping with current desires for more rapid and inexpensive analyses, Penttila et al.[29] investigated the use of the Alcoscan test strip for rapid screening of samples for the presence of ethanol. This study involved saliva from living subjects and urine and vitreous humor from deceased individuals. The test is based on the reaction between ethanol and alcohol oxidase in the presence of an indicator dye system to form an indamine-type dye with a stable blue color. Visual comparison with a color chart or, as done in this study, use of reflectance photometry (Glucoscan), would provide quantitation.

Two cautions must be remembered with regard to analysis of vitreous humor specimens. First, some variations have been observed for results obtained with different analytical methodologies. Daae et al.[30] studied five different methods for glucose analysis and found significant differences between the methods. Likewise, Coe and Apple[31] found variations for glucose, urea nitrogen, sodium, potassium, and chloride using a variety of procedures for each of these constituents. Thus, a laboratory performing routine analyses of vitreous humor for endogenous analytes should establish a normal range of values within their own laboratory, instrumentation, and procedures before comparing results with those published by others and offering interpretations. Second, an early assumption was that there should be no difference between eyes for the concentration of a given analyte. Recently, however, Balasooriya[32] reported that the concentrations of potassium, sodium, and/or urate were varied between the two eyes, even though the specimens were drawn at the same time. Madea et al.[33] have also reported deviations of up to 10% from the mean of the two eyes in analyses for potassium. Thus, any interpretation of post-mortem change based on differences between specimens drawn at different times must be made cautiously.

IV. INTERPRETATION OF RESULTS

Coe has been one of the most prolific workers and writers in the field of post-mortem chemistry involving endogenous substances (carbohydrates, nitrogen compounds, electrolytes, lipids, hormones, etc.). In addition to numerous publications of individual studies, Coe has also prepared extensive reviews of post-mortem chemistry. The earlier review[34] covers the literature before 1974; the later review[2] covers papers appearing in the intervening 20 years.

A. ETHANOL AND METHANOL

Although vitreous humor was used for investigations of other analytes earlier, the first publication of analysis of vitreous humor for toxicological purposes involved the analysis for ethanol and appeared in 1966.[35] In this paper, the authors compare the concentrations of ethanol in blood and vit-

reous humor, as determined by GC. They concluded that, "There is a close correlation between levels of ethyl alcohol in the blood and the vitreous humor; . . . ." This conclusion is in agreement with earlier research work that has reported that ethanol could pass rapidly into the eye.[36–39]

Subsequently, the analysis for the presence of ethanol is the most extensively published area of investigation involving toxicology in vitreous humor. A MedLine search (for the period 1966 through September 1995) produced the following numbers of references listed: vitreous—7440; vitreous humor—419; vitreous humor and ethanol—16; vitreous humor and alcohol—23; vitreous and ethanol—29; and vitreous and alcohol—49. Both early papers[9,11,16,35] and recent papers[40–42] have given attention to the relationship of the concentration of ethanol in vitreous humor to the concentration in blood. Felby and Olsen[9] reported a blood alcohol concentration/vitreous humor alcohol concentration of 0.74 ± 0.11 (Widmark method) and 0.72 ± 0.09 (alcohol dehydrogenase method). Using a diffusion and dichromate reduction method, Coe and Sherman[11] obtained a ratio of 0.89 ± 0.023. Winek and Esposito[17] used a gas chromatograph, and both direct injection and headspace sample injection; they report a blood/vitreous humor ratio of 0.93 ± 0.17 (range = 0.73–1.51) for direct injection and 0.94 ± 0.17 (range = 0.68–1.52) for headspace sample injection. Backer et al.[40] reviewed the data for several fluids and brain to determine the post-mortem distribution ratios and to determine whether any one specimen could be used to estimate a blood ethanol concentration if blood was not available. Their results showed a blood/vitreous humor ratio of 0.95, with a range of 0.58–2.08. When they subdivided their cases according to absorptive or postabsorptive phase, they found a ratio of 0.84 (range = 0.58–1.16) for cases in the postabsorptive phase and 1.12 (range = 0.50–2.08) for cases in the absorptive phase. Briglia et al.[41] reported on the data obtained for 60 cases, including ethanol concentrations in blood from several sites (femoral vein, right atrium, root of the aorta, brain, cerebrospinal fluid, urine, pericardial fluid, and vitreous humor). The paper does include data for vitreous humor, although the objective was to examine possible site differences in blood ethanol concentrations. Caplan and Levine[42] studied the results of cases for which specimens were collected as part of the routine protocol. For 101 cases with a blood ethanol concentration <0.10 g/dl, 67% had a vitreous humor ethanol concentration within 0.02 g/dl of the blood concentration. For the 205 cases with a blood ethanol concentration ≥0.10 g/dl, 64% were within the blood/vitreous humor ratio range of 0.71 to 1.00; the mean ratio was 0.85, and the median ratio was 0.84. Bost[15] has accumulated data for 2411 cases, in which ethanol concentrations in both blood and vitreous humor (as well as bile and urine) were obtained as part of the routine processing of case specimens. Statistical evaluation of this data gave a blood/vitreous humor ratio of 0.838, with a standard error of 0.008.

Several authors have discussed the theoretical relative distribution of ethanol between blood and vitreous humor. Each notes that, at equilibrium, the distribution should be reflective of the relative water content of each specimen. Felby and Olsen[9] calculate that the ratio of the water content of blood to the water content of vitreous humor should be 0.79. Of course, this depends on the hematocrit of the blood being within normal range; variations caused by thickening of the blood specimen before collection or by coagulation, resulting in a low hematocrit specimen, will cause the relative water content to be lower or higher, respectively. Coe and Sherman[11] reviewed the data in three other papers, using the same statistical treatment as for their own data, and reported that the ratio ranged from 0.75 to 1.01. They noted that the following factors may influence the results: (1) variations in hematocrit readings; (2) variations in laboratory methodology and specificity; (3) laboratory error; and (4) inadvertent bias in case selection, including a larger proportion of cases in absorptive phase in one study and cases in the elimination phase in another study, particularly if a given study involves a small number of cases.

Several authors have stated that the concentration of ethanol in vitreous humor cannot be presumed to provide an accurate and precise prediction of the corresponding ethanol concentration in blood. However, the correlation can be of some benefit. In the absence of a specimen of blood (severe traumatic injury or incineration of the body), analysis of the vitreous humor and multiplication by an appropriate conversion factor can provide an estimate of the blood alcohol concentration (BAC) that can give guidance to investigators. In cases in which possible contamination is suspected

of causing a post-mortem or postcollection elevation of the blood ethanol concentration, analysis of vitreous humor can provide evidence to either substantiate the blood result or support the theory of contamination. Since the early days, it has been recognized that the eye is an isolated and protected organ; the vitreous humor is likely to be available when other specimens are not, and it is less likely to have been contaminated by bacteria that might produce ethanol during the putrefactive processes.[43] In one of the early studies, Felby and Olsen[9] withdrew specimens from each eye at different times from four cases; the specimens were analyzed separately. The interval from time of death to time of obtaining the second specimen varied up to 99 hours; the interval between obtaining the first and second specimens varied from 22 to 74 hours. In none of the four cases was a significant difference observed between the alcohol concentration in the first and second specimens. Thus, under these controlled conditions, the vitreous humor was stable with respect to possible post-mortem generation of ethanol. But, in cases in which post-mortem decomposition suggests concern about the validity of blood alcohol results, ethanol concentration in the vitreous humor may help distinguish between exogenous and endogenous sources of the ethanol found in the blood specimen. Zumwalt et al.[43] examined 130 decomposed bodies for endogenous ethanol (post-mortem formation); of these, vitreous humor was obtained for analysis in 91 cases. The presence of ethanol in the "bloody fluid" and absence in vitreous humor was one of the criteria used to infer that the detected ethanol was produced after death. Nineteen cases fit into this designation of "presumed endogenous production."

In an analogous study, Gilliland and Bost[44] retrospectively reviewed the results for 286 decomposed cases. Using the criteria of (1) blood positive and other specimens negative, (2) a history indicating no ingestion before death, or (3) the presence of three-carbon alcohols, they determined that endogenous production had occurred in 55 cases (19.2%) of the 286 cases examined. This proportion compares with 18.7% reported by Zumwalt et al.[43] Levine et al.[45] reviewed cases for which BAC was within the range of 0.01 to 0.04 mg/dl (n = 381). After subdividing the cases according to the BAC, they reported that 54% of the cases with a BAC = 0.01 mg/dl were associated with a positive vitreous humor and/or urine result. For samples with a BAC = 0.02 mg/dl, the association rose to 63%; at a BAC of 0.03 mg/dl, the association was 73%; and at a BAC = 0.04 mg/dl, the association was 92%. Based on this information, they offered the interpretation that, in the absence of vitreous humor or urine, one could reasonably predict that a BAC = 0.04 or greater resulted from consumption of ethanol. It must be remembered, however, that a 92% association still allows for 8% that could involve post-mortem formation of ethanol. Zumwalt et al.[43] and Canfield et al.[46] have reported cases of post-mortem formation in which the ethanol concentration exceeded 0.15 mg/dl. Canfield et al.[46] reported on an interesting group of cases (i.e., victims of fatal aircraft accidents). They used urine and/or vitreous humor, when available, in conjunction with blood, to distinguish between ante-mortem consumption and post-mortem formation of ethanol. Of the 975 cases studied, 79 were positive for ethanol. In 45% of the ethanol-positive cases, neither alternative specimen was available, and no determination of origin could be reached. In 21 ethanol-positive cases (27%), the ethanol was determined to have been produced post-mortem and, in 22 cases (28%), the ethanol had been ingested. In two of the cases, post-mortem ethanol production exceeded 150 mg/dl. Note was also made of one additional case in which the post-mortem blood ethanol concentration was in excess of 300 mg/dl, but no ethanol was detected in vitreous humor or urine.

Cases in which the body has been embalmed before collection of samples present another situation, wherein the results of analysis of vitreous humor can be useful. Scott et al.[47] conducted tests on a series of bodies, collecting blood and vitreous humor before embalming and then also vitreous after embalming. In each of their eight cases, the relationship between blood and vitreous humor ethanol concentrations was within the expected range. For 6 of the 8 cases, the postembalming vitreous humor ethanol concentration was lower than the preembalming specimen; for two cases, the postembalming result was slightly higher. They also report the concentration of methanol in the postembalming vitreous humor samples for each case to show that significant diffusion of embalm-

ing fluid does occur into the vitreous humor, but that this does not cause significant alteration of the ethanol concentration. They include one case in which the blood and preembalming vitreous humor specimens were negative for ethanol, but the postembalming specimen showed 0.34% ethanol. The explanation was that the embalmer had cleaned the globus of the eye with ethanol on a cotton swab before placing an eye cap in position. In the author's laboratory, similar unexpected results in unembalmed bodies were being produced; investigation revealed that technicians from the tissue transplant center were using ethanol solutions to clean the eye before harvesting corneas for transplantation. Changing to alternate cleaning solutions eliminated this problem. Coe[48] also studied the effect of embalming by collecting the vitreous humor from one eye at the time of examining the body at the morgue and then from the other eye after embalming had been done. Of 35 cases studied, ten were positive for ethanol. A dilutional factor was observed as a result of embalming. In 8 of the 10 cases, the difference in concentration between the two eyes was considered to be insignificant; but, in two cases, the ethanol concentration in the second specimen was at least 0.10% w/v less than the first.

In some situations, the toxicologist may be requested to offer an opinion regarding whether the deceased was in the absorptive phase or elimination phase of ethanol distribution. If the toxicologist is provided a blood specimen with a normal hematocrit, along with a vitreous humor specimen, and if the results show a blood/vitreous humor ethanol ratio greater than one, then the toxicologist may suggest that the deceased was within the absorptive phase. Without these conditions being met, without the hematocrit being known, and/or with a lower ratio, then no conclusion can be offered about the absorption or elimination. The question of whether the individual was a relative naive drinker or a chronic abuser of alcohol is often raised. Serum concentrations of γ-glutamyltransferase have been considered to be a marker of chronic ethanol abuse. Devgun and Dunbar[49] attempted a small survey to determine whether vitreous humor data could be used in a similar interpretation. Of their series of 14 cases, γ-glutamyltransferase was detected in only three specimens. Although these results do not suggest significant usefulness, the authors do recommend that larger scale studies should be performed.

Vitreous humor methanol concentrations in fatalities have been reported by two groups. Wu Chen et al.[19] described the hospital course and post-mortem results of a hospitalized man who survived 40 hours. The post-mortem blood and vitreous humor methanol concentrations were 142 and 173 mg/dl, respectively. The blood/vitreous humor ratio of 0.82 compares closely with the ratios discussed herein for ethanol. The case reported by Pla et al.[50] involves a man found dead at home. The blood and vitreous humor methanol concentrations were 0.284 and 0.396 mg/dl, respectively; the blood/vitreous humor methanol ratio was 0.72, again reasonably close to the ratios reported for ethanol.

B. ELECTROLYTES, GLUCOSE, AND NITROGEN COMPOUNDS

Coe,[34] in a review of the literature on post-mortem chemistry, states that there are no normal concentration values for vitreous humor available from clinical studies. The only way to obtain apparent normal values is to extrapolate from post-mortem results or to use animal data, which is questionable because of species differences. As described herein, analysis of vitreous humor for electrolytes has been performed by a variety of techniques. The results of these analyses have been used both for routine case handling and for investigations of special circumstance cases. The concentrations of electrolytes in blood or serum from living patients are expected to be within specified ranges, and any deviation from normal is indicative of disease or other malady within the body. After death, the concentrations of these substances do change in erratic ways; thus, analysis of blood or serum after death cannot provide the same diagnostic information as can ante-mortem samples. Believing that the relative isolation of the vitreous humor would reduce any significant change in electrolyte concentrations, Leahy and Farber[14] obtained specimens from patients dying in hospitals and from patients arriving dead on arrival at hospital. Using ante-mortem blood urea nitrogen data or the absence of any evidence of chronic illness to establish the normal health condition of the patient, they were able to offer the post-mortem normal vitreous humor urea nitrogen concentration

range as being 5 to 29 mg/dl. Similarly, the normal post-mortem concentration range for creatinine in vitreous humor was given as 0.31 to 1.05 mg/dl. In patients who had normal ante-mortem blood glucose values or who died from sudden traumatic injury, the vitreous glucose concentrations ranged from 28 to 89 mg/dl. The normal post-mortem sodium concentration in vitreous humor was reported to be in the range of 128 to 158 meq/l; the chloride concentration range was 108 to 142 meq/l. However, the potassium concentration in vitreous humor rose after death, with no discernible pattern. Thus, no "normal" post-mortem range could be offered. Coe's[34] extensive review of post-mortem chemistry in blood, cerebrospinal fluid, and vitreous humor quotes some of his own previous work[51] and work by Naumann[52] on the normal concentrations of electrolytes and other analytes in vitreous humor, as well as the work of Leahy and Farber discussed herein. The results of these three studies are summarized in Table 17–1.

Leahy and Farber[14] and Coe[51] also examined results from patients who were hypernatremic before death and found a correlation with an elevated sodium concentration in post-mortem vitreous humor. Review of the data from their cases indicated that concentrations <130 meq/l or >155 meq/l matched with hypo- or hypernatremia before death. Similarly, concentrations of chloride ion <105 meq/l or >135 meq/l reflected ante-mortem hypochloremia and hyperchloremia, respectively.

Leahy and Farber[14] and Naumann[52] stated that the concentration of glucose was found to be approximately half the concentration in serum and that the concentration seemed to be stable after death. However, Coe[51] demonstrated that the concentration initially was about 85% of the serum concentration and that it decreased in the interval after death, sometimes precipitously. Sturner and Gantner[53] felt that low vitreous humor concentrations were indicative of hypoglycemia before death; however, Coe's data discounted this hypothesis. All of these authors did concur that a high glucose concentration, especially when coupled with a positive test for ketone bodies, in the vitreous humor was useful for determining diabetic acidosis.

The concentration of potassium ion in the vitreous humor increases after death. This was first reported in 1962 by Jaffe[54]; subsequently, Coe,[51] Adelson et al.,[55] Hanson et al.,[56] Hughes,[57] Lie,[58] Sturner,[59] and Sturner and Gantner[60] all concurred with the finding. Sturner, Gantner,[60] and Lie[58] found the correlation between rate of increase and post-mortem interval to be close enough to permit a time of death estimate within ±5 hours. Adelson et al.,[55] Hughes,[57] Hanson et al.,[56] and Coe[51] found enough variation that the estimate could not be made within ±10 hours within the first day after death. Furthermore, the error margin increased as the interval increased. Adjutantis and Coutselinis[61] collected vitreous humor from each eye at different times, obtained the rate of change for this specific body, and extrapolated the concentration back to an initial concentration of 3.4 meq/l. Using this method allowed them to make estimates within ±1.1 hours, but only during the first 12 hours after death. This subject has continued to draw attention. A recent exchange via an Internet e-mail listserv discussed the utility of vitreous humor potassium concentrations; the prevalent opinion was that any correlation was so poor that no interpretation should be made on this basis. Specifically, Forrest[62] stated,

TABLE 17–1
Normal Concentrations of Electrolytes and Other Analytes in Vitreous Humor

Na^+ (meq/l)	Cl^- (meq/l)	K^+ (meq/l)	VUN (mg/l)	Glucose (mg/l)	Creatinine (mg/l)	Ref.
128–158	108–142	*	5–29	28–89	0.31–1.05	14
131–151	104–132	*		**	1.2 (avg.)	51
118–154	114 (avg.)	*				52

Note: VUN, vitreous urea nitrogen. *Increased after death in an unpredictable manner.
**Decreased after death in an unpredictable manner.

> The measurement and interpretation of vitreous K^+ is not straightforward. These days K^+ will usually be measured in clinical laboratories by ion selective electrodes, rather than by flame emission or atomic absorption. ISE methods may be subject to interference from the products of putrefaction, and may show some bias dependent on sample viscosity. Vigorous centrifuging and measuring the supernatant does seem to reduce the variability of the analyses. Once you have measured K^+ in vitreous, one then has to interpret it. I haven't been impressed by its utility in case work. Which is a polite way of saying that IMHO [in my humble opinion] it is practically useless and potentially misleading.

In response, Lauridson[63] stated, "This has been my experience also. I have just finished looking at a series of cases with known time since death. Vitreous K^+ is not reliable."

The utility of vitreous humor electrolytes in drowning cases has been considered by Rammer and Gerdin[64] and by Sturner et al.[65] Rammer and Gerdin[64] studied 38 cases of drowning and used 35 cases with other causes of death as controls; they considered both measured electrolyte concentrations and the ratios of concentrations in various specimens. They found that no single result could distinguish drowning death from other causes. The difference in osmolality between left-heart blood and cerebrospinal fluid and between left-heart blood and vitreous humor were significant. In all but one control case, the osmolality in left-heart blood was higher than in cerebrospinal fluid; in 18 of 38 drowning cases, the blood result was lower. Likewise, only one control case had blood osmolality lower than that in vitreous humor; 6 of 26 drowning cases has lower osmolality in blood. Analogous results were observed when considering the sum of sodium and potassium concentrations. In 7 of 21 drowning cases, the difference of (Na + K) total concentration between left-heart blood and vitreous humor was >20 meq/l (blood was lower); in only 1 of 26 control cases was the difference this great. Acknowledging that cerebrospinal fluid may be more useful than vitreous humor, the authors conclude that comparison of osmolarity or of (Na + K) between left-heart blood and one of these fluids may provide evidence to substantiate a diagnosis of drowning. Sturner et al.[65] studied the concentration of magnesium and other electrolytes by immersing bovine eyeballs in sea water for varying times. Although they detected significant increases in magnesium concentration, they concluded that vitreous humor magnesium "appears to be an imperfect marker in estimating the length of immersion. . . . Establishing or excluding the diagnosis of drowning solely by this technique seems unlikely because of ion diffusion into the eye fluids."

Coe[48] examined the effect of embalming on the concentrations of several analytes in vitreous humor by collecting the vitreous humor from one eye when the body was examined at the morgue and then from the second eye after the embalming had been completed. Sodium and chloride concentrations were observed to be lower in the postembalming specimen than in the preembalming specimen in all but one case. No correlation between amount of decrease and time interval could be ascertained. The concentration of potassium was always higher in the second specimen; again no correlation was detected. Urea nitrogen and glucose did seem to provide useful information. Although a decreased urea nitrogen concentration was observed in the second specimen, Coe believed that this was caused by a dilutional effect. For four cases with mildly elevated values preembalming, the results were still high postembalming for three. In the one case with a markedly elevated value in the first specimen, the second specimen was also quite high. Similarly, for glucose, the specimens from three diabetics gave elevated values in both the first and second specimens. The summary interpretation was offered that individuals suffering from nitrogen retention or diabetes could be recognized, even if specimens were not obtained until after the body had been embalmed.

Bray et al.[66] had the opportunity to examine 13 adult victims of a plane crash that fell into near-freezing water and to conduct electrolyte analyses in the vitreous humor specimens of these victims after they were recovered 7 to 8 days after the crash. They reported that the glucose concentration had not decreased as much as would be expected in nonimmersed bodies after a similar post-mortem interval. Rapid chilling of the bodies apparently inhibited the glycolysis and concomitant post-mortem decrease in glucose concentration. They also note the apparent dilution of vitreous humor electrolytes.

Psychogenic polydipsia (compulsive water drinking), although generally thought to be relatively benign, has resulted in several fatalities. DiMaio and DiMaio[67] note that, in three patients hospitalized before death, blood electrolyte analyses were available to substantiate the history and diagnosis. In their reported fatality, the patient was found dead at home. After a 6-day hospitalization for psychogenic water intoxication, she was discharged. The following day, she was found dead at home. Because serum electrolyte results were not considered reliable, vitreous humor was submitted for electrolyte analysis. The results were: sodium, 115 meq/l; chloride, 105 meq/l; potassium, 7.6 meq/l; glucose, <25 mg/dl; and creatinine, 0.4 mg/dl. Using the prior history, lack of evidence of pathological or other toxicological injury, and the hyponatremia revealed by the vitreous humor results, the pathologist ruled the death to be caused by water intoxication.

In both of his reviews,[2,34] Coe also includes references to work on other analytes, including lactic acid, pyruvic acid, ascorbic acid, nonprotein nitrogen, uric acid, enzymes (lactate dehydrogenase, SGOT, and SGPT), hormones, proteins and amino acids, bilirubin, carbon dioxide, calcium, magnesium, phosphorus, iodine, and osmolality. However, they will not be covered in any detail herein. The interested reader is referred to Coe's reviews for further information and for the original references. Coe also included references to findings of drugs in vitreous humor; these will be covered separately.

C. DRUGS

Many papers have been published describing the detection of drugs in vitreous humor: some have been research studies using animals as test subjects, some have been human case reports involving a single drug, and others have been more extensive reports of findings for multiple drugs. The following is not intended to be an exhaustive review of all such papers, but rather a survey including each type of report and including a range of drugs.

1. Multiple Drugs

Several papers appeared in the late 1960s and early 1970s regarding detection of drugs in vitreous humor. In one of the most extensive papers to report on drugs detected in vitreous humor, Sturner and Garriott[12] described their findings in 56 cases within a 3-year period; they included overdose cases and cases with unrelated causes of death. Their paper gives the results of drug concentrations in blood and in vitreous humor for ten cases involving propoxyphene, four cases with secobarbital, three cases with amitriptyline, eight cases with digoxin, three cases with pentazocine, two cases with amphetamine, two cases with methadone, and two cases with freon-containing aerosols, among others. Five cases involved two drugs detected in both specimens. Discussion of the vitreous humor/blood concentration ratios and the circumstances involved in some of the cases is also provided.

2. Cocaine

Cocaine has been a drug frequently detected in death investigations. Numerous publications have presented results of toxicological analyses associated with these cases. However, most of these do not include analysis of other fluids or tissues. Poklis et al.[22] did describe the disposition of cocaine in a fatality, including concentrations in blood, bile, urine, vitreous humor, and nine different solid tissues. Logan and Stafford[68] analyzed vitreous humor for cocaine and metabolites in 28 fatal cases for which blood cocaine concentrations had been determined. An examination of the data showed that, in 96% of the cases, the benzoylecgonine (BE) concentration in the vitreous humor was higher than the cocaine concentration, including cases in which BE was present but cocaine was not detected in either blood or vitreous humor. The concentrations of cocaine in blood and vitreous humor showed a correlation of $R = 0.70$, with a considerable spread. The cocaine concentration ratio in vitreous humor to blood averaged 1.61, with a range from 0.1 to 2.6; this spread would restrict any application whereby the concentration of cocaine in blood was extrapolated from that in vitreous humor. McKinney et al.[69] noted that most published data comes

from autopsy case reports for which the information of dose, prior history of use, time of administration, interval between administration and death, and post-mortem interval are not known. Juvenile swine were used as a large animal model, predose blood samples were collected, intravenous cocaine was administered, the animals were euthanized 5 min later, and blood and vitreous humor samples were collected within 3 to 5 min after death and again 8 hours after death. Analyses were done to detect cocaine, BE, and ecgonine methyl ester (EME). The mean concentration of cocaine in femoral blood (for nine animals in the study) rose about 10% over the 8-hour post-mortem interval. The mean concentration of cocaine in the vitreous humor samples was considerably less than in blood at the time of death, but it rose over 300% in 8 hours and nearly reached the blood concentration. The concentration of cocaine in vitreous humor 8 hours post-mortem was compared with the femoral blood concentration at the time of death to determine whether the former could be used to predict the latter; the results did not substantiate this theory. Although the mean vitreous humor 8-hour concentrations were not statistically different from the femoral blood concentrations at time of death, the vitreous humor result was higher than blood in some individuals and lower in others. The relative amounts of metabolites detected was of interest. BE was not detected in either blood or vitreous humor immediately after death. It was detected in blood in 4 of the 9 animals at 8 hours post-mortem. But, in vitreous humor, BE was detected in only 2 of the 9 animals 8 hours post-mortem. Conversely, EME was detected in 7 of 9 blood samples immediately after death and in all nine blood samples 8 hours later. For the vitreous humor samples, EME was detected in 3 of 9 samples immediately and in 8 of 9 samples 8 hours after death. The authors conclude by stating,

> . . . the pattern of change in cocaine concentrations was highly variable even in these controlled conditions, underscoring the caution that should be exercised in extrapolating post-mortem cocaine concentrations to antemortem values.

3. Opiates and Related Drugs

Bermejo et al.[25] noted that blood may not be available in some fatal cases or may be altered by post-mortem phenomena. They chose to compare the results of GC/mass spectrometry (MS) analyses for morphine in blood and vitreous humor. They also included results of RIA analyses of vitreous humor without and after enzymatic hydrolysis. The RIA analyses showed higher results after enzymatic hydrolysis than without. Thus, conjugates of drug and metabolites are present in the vitreous humor, and any analysis procedure used must take account of these conjugates. For ten fatal cases involving heroin, analyses of vitreous humor were performed by both RIA and GC/MS; the results revealed a strong correlation between the two methods. Jones and Pounder[70] reported the concentration of codeine (and numerous other drugs) in vitreous humor in a fatal case.

Ziminski et al.[24] investigated the comparative concentrations of morphine and of methadone in blood, vitreous humor, and several tissue samples in drug-related fatalities. Using both aqueous-based and vitreous humor-based standards, they found insignificant differences in the RIA analysis for either methadone or morphine. In 9 of 13 morphine cases, the morphine concentration in blood was considered to be in the lethal range, and morphine was present in vitreous humor. In one case, morphine was present in the vitreous humor, but not in blood. In one case, the morphine concentration in blood was high, but no morphine was detected in the vitreous humor. This latter death was certified as caused by "acute opiate intoxication." The findings in these cases support the interpretation that equilibration of morphine between blood and vitreous humor is not rapid. In cases of methadone overdose, methadone was detected in each of the 12 cases. But, the methadone concentrations in vitreous humor showed no correlation with the concentrations in other specimens, and no interpretation other than presence could be inferred from the data in vitreous humor.

Nine deaths involving oxycodone were investigated by Drummer et al.[71] The result of vitreous humor analysis is provided for only one case: blood, 1.2 mg/l and vitreous humor, 1.8 mg/l.

4. Antidepressants

One of the earlier papers to consider comparative toxicology in vitreous humor and blood was prepared by Sturner and Garriott.[12] They reported data for 56 cases, including both overdose fatalities and deaths due to other causes. The tabulation includes three cases involving amitriptyline, two with imipramine and desipramine, and one with the combination of amitriptyline and diazepam. The concentration ratio of vitreous humor to blood is reported for each case. For the three amitriptyline cases, the ratios were 0.02, 0.13, and 0.15. The authors note that the first case involved a massive ingestion with a short survival time (<3 hours), precluding time to achieve equilibrium. The higher ratios for the other two cases were associated with longer survival times (>15 and >10 hours) before death.

The question of site dependence is often raised with respect to drug concentration in blood specimens. Investigating site dependence in a multiple drug overdose fatality, Jones and Pounder[70] included vitreous humor among the many specimens analyzed. Results for imipramine, desipramine, diphenhydramine, acetaminophen, codeine, and ethanol were reported. However, the paper did not discuss vitreous humor results as a factor in any interpretation or other conclusions.

McIntyre et al.[72] reviewed 30 cases in which fluoxetine was detected; concentration ranges in blood, vitreous humor, bile, and liver were reported.

Hutchison et al.[73] reported their findings in a suicidal death after ingestion of salicylate, acetaminophen, and butriptyline. An autopsy was not performed; thus, only fluid specimens were available for analysis. Concentrations of butriptyline included: blood, 14.90 mg/l; urine, 2.96 mg/l; and vitreous humor 0.52 mg/l. The authors did not find any other fatality caused by butriptyline reported in the literature.

5. Benzodiazepines

Drummer et al.[71] in their report regarding oxycodone deaths, also included data on several benzodiazepines found in these same cases. Drugs found include diazepam, temazepam, oxazepam, and 7-aminoclonazepam. Separately, a fatality caused by flurazepam was reported by McIntyre et al.[74] Robertson and Drummer[75] have tabulated the results of benzodiazepine analyses in 14 cases, giving blood and vitreous humor concentrations and the vitreous humor/blood ratio.

6. Barbiturates, Meprobamate, and Carisoprodol

Detection of barbiturates in vitreous humor was reported as early as 1969, by Felby and Olsen.[10] Using the classic technique of recording ultraviolet absorption after pH adjustment, the presence and quantity of barbiturates was determined in blood, blood subjected to ultrafiltration, vitreous humor, and liver. They found a good correlation between the ultrafiltrate of blood and vitreous humor, but no correlation was detected when comparing whole blood and vitreous humor. Based on the finding of nearly equivalent concentrations of barbiturate in ultrafiltrate of blood and in vitreous humor, it was presumed that, in each of these cases, death had been delayed more than 5 hours after ingestion of the drug. Meprobamate was also determined in three cases by Felby and Olsen,[10] using spectrophotometric analysis after development of a chromophore with *p*-dimethylaminobenzaldehyde and antimony trichloride. The results showed similar equivalence of concentrations in blood, vitreous humor, and ultrafiltrates of blood.

Three cases reported by Backer et al.[76] provide an interesting contrast in interpretation. The three cases involve overdoses with carisoprodol, alone in one case, with verapamil in one case, and with propoxyphene in one case. Concentrations of carisoprodol and meprobamate are reported in heart blood, femoral blood, vitreous humor, and urine (one case) for these cases. The ratio of carisoprodol in femoral blood to vitreous humor was 2.08, 0.75, and 0.98 in the three cases. This suggests, using Felby's reasoning described herein, that death occurred fairly rapidly in case 1, but was delayed in cases 2 and 3. The interesting contrast occurs with the meprobamate data. The femoral blood to vitreous humor concentration ratios are 1.13, 0.40, and 0.33, respectively. The meprobamate ratios for cases 2 and 3 are not close to unity, as was suggested by Felby. Backer emphasizes the need for caution in trying to interpret the meaning of a drug concentration in a single biological specimen.

Ziminski et al.[24] used RIA analysis of vitreous humor specimens to compare concentrations of barbiturates to the concentrations in blood, liver, brain, and kidney. During method validation studies, they determined that the calibration curve for standards prepared in water was different from that of standards in vitreous humor; this suggests some effect of specimen type on the quantitation results. Their results showed that barbiturate concentrations in vitreous humor were higher in cases for which the blood concentrations were higher. Equilibration of barbiturates from blood into the vitreous humor seems to be fairly rapid, in contrast to some other drugs also included in this report (see "Opiates and Related Drugs").

In 16 medical examiner cases, for which other analyses had shown the presence of barbiturates, Ojanpera et al.[77] investigated the use of the EMIT-st system for detecting barbiturates in vitreous humor. They found positive EMIT-st results for some of the cases and negative results for others; results were only recorded as positive or negative. When the relative sensitivities toward specific barbiturates were considered, the results were interpreted to be consistent with expected results.

7. Sufentanil/Midazolam

Ferslew et al.[78] described a fatal self-administration of sufentanil and midazolam by an anesthesiologist. Analyses for midazolam were performed by GC/MS and HPLC and for sufentanil by GC/MS. Results for sufentanil are reported in blood, urine, vitreous humor, liver, and kidney; for midazolam, results are reported in blood, urine, liver, and kidney. Concentrations of each drug in blood are consistent with those reported in therapeutic anesthetic situations. However, it is noted that anesthesia, without proper care and attention, can result in death. Relative concentrations shown for sufentanil are consistent with its known pharmacokinetic profile of rapid distribution.

8. Carbofuran/Cholinesterase Activity

Ferslew et al.[79] reported a suicidal ingestion of carbofuran, a carbamate insecticide with cholinesterase-inhibiting properties. In addition to reporting the concentration of the insecticide in various specimens (plasma, serum, whole blood, erythrocytes, vitreous humor, and bile), they also examined the cholinesterase activity in each of these specimens and compared the results with normal/expected values. Because cholinesterase activity data were not previously known for vitreous humor or bile, specimens from 18 other autopsies in the same office were used to establish control values. They found that cholinesterase activity was inhibited in all specimens collected from the decedent, by proportions ranging from 74% (bile) to 99% (erythrocytes) of normal.

9. Mexiletine

Mexiletine is a class 1B antiarrhythmic drug used in the treatment of ventricular arrhythmias. Kempton et al.[80] reported the suicidal death of one individual who ingested this drug. Blood concentrations showed some site dependence, suggesting post-mortem redistribution. The concentration in vitreous humor was approximately the same as the lower blood concentration.

10. Metoprolol

Fatalities involving metoprolol have been reported by several groups: Stajic et al.,[27] Shore et al.,[81] Holzbecher et al.,[82] and Rohrig et al.[83] Concentrations in the respective cases are tabulated in Table 17–2. Stajic et al.[27] note that their results are considerably lower than those reported by Shore and Holzbecher; they offer the explanation that their patient had undergone extensive resuscitation efforts for 10 hours before death. Rohrig also notes that the concentrations in their case are above those of Stajic, but below those of the other two reports. In Rohrig's case, the patient had also ingested ethanol before death, and death was attributed to the combined effects of both ethanol and metaprolol.

11. Digoxin

The interpretation of post-mortem concentration of digoxin has been the subject of discussion for more than 20 years. Some authors assumed that post-mortem serum specimens adequately

TABLE 17–2
Metoprolol Concentrations in Fatal Overdoses

	Concentrations in mg/l or mg/kg					
Case	Blood	Vitreous humor	Bile	Liver	Urine	Ref.
1	4.7	3.3	254	6.3	194	27
2	50			120		81
3	56	42	276	260		82
4	19.8	15.1	83.1		1.6	83

reflected ante-mortem specimens. Others noted discrepancies and suggested a relationship with time interval between death and collection of the specimen. Still others reported variation depending on the site of collection of the specimen. DiMaio et al.[1] described four fatalities in which drug overdose was suspected, based on provided histories. As a control group, 35 other medical examiner cases were studied; they were chosen because of a history of taking digoxin before death. The authors make the assumption that the digoxin concentration in vitreous humor should be approximately equal to the concentration in blood after equilibration has occurred. They further suggest that individuals in whom the blood concentration was higher than the vitreous humor concentrations were still in the absorptive phase (i.e., the concentration of digoxin was still rising). Individuals in whom the vitreous humor concentration was equal to or higher than the blood were in the postabsorptive phase; furthermore, the vitreous humor concentration would be indicative of the blood concentration at some earlier time and might even be useful in estimating the elapsed time between dosing and death. The authors include the caution that the analytical method they used was subject to interference in cases of hemolyzed blood specimens; the interference caused the measured result to be lower than the correct concentration in the absence of hemolysis. Thus, the concentration at time of death should likely have been higher than the analytical result in some of their cases.

Vorpahl and Coe[23] addressed this issue by evaluating results in 27 cases; post-mortem specimens collected included blood from the left ventricular cavity and vitreous humor in all cases, as well as subclavian blood (24 cases) and femoral blood (11 cases). All patients were receiving digoxin therapeutically. Ante-mortem serum results were also available on all patients. By comparing ante-mortem and post-mortem results, Vorpahl and Coe reported that digoxin concentration in blood increased in almost all specimens after death. All of the patients in the study had received their last digoxin dose at least 6 hours before death and thus should have been in a postabsorptive phase; however, 23 of the 27 patients had vitreous humor to blood ratios <1. This contrasts sharply with the conclusions offered by DiMaio et al.[1] Vorpahl and Coe conclude that: (1) interpretation of digoxin toxicity based on an elevated post-mortem serum concentration must be made with caution; (2) a post-mortem serum digoxin concentration <2 ng/ml does seem to be good evidence against ante-mortem concentrations in the toxic range; (3) elevated vitreous humor digoxin concentrations seem to be well correlated with elevation of ante-mortem serum concentrations and indicative of toxicity; and (4) a combination of femoral venous serum and vitreous humor results provides the best basis for making a diagnosis of digoxin toxicity. Donnelly et al.[84] and Balkon and Donnelly[85] used the guinea pig as an animal model to study the kinetics of distribution of digoxin after intravenous[84] and oral[85] dosing. Their results show that the concentration in vitreous humor reaches a 1:1 ratio with blood quite rapidly (within 2 hours) after intravenous administration. However, after oral administration, vitreous humor and blood may not reach equilibrium for up to 12 hours.

In summary, the information provided by these three studies have produced three cautions that must be considered when interpreting digoxin results. First, DiMaio et al.[1] caution that hemolysis of blood specimens may cause interference that produces falsely low analytical results. Second, Vorpahl and Coe[23] show that concentrations in blood specimens increase after death. Third,

Donnelly et al.[84] and Balkon and Donnelly[85] report that the vitreous humor to blood concentration ratio is dependent on the route of administration. It should also be remembered that Balkon's studies on animals were performed under controlled conditions, including a known minimal post-mortem interval between death and specimen collection, whereas DiMaio's and Vorpahl's reports involved human death cases in which post-mortem circumstances were not under investigator control.

12. Insulin

Cohle et al.[86] used vitreous humor results for glucose and acetone, along with acetone in blood and urine in the investigation of the death of a man with a 25-year history of diabetes mellitus; his disease had been under control until 4 days before his death. A new bottle of insulin had been obtained from the pharmacy at that time. Based on the past medical history, the analytical results, and examination of the insulin bottles recently and previously used, they were able to determine that death was caused by an error in which regular insulin was dispensed instead of the NPH insulin that the patient had been using.

13. Probenecid

McIntyre et al.[87] described the analytical results obtained during the investigation of a suicidal death involving probenecid and ethanol. HPLC was used for the analysis. The concentration of probenecid in serum was approximately five times greater than the reported peak plasma concentration after therapeutic dosing. Concentrations in other specimens (vitreous humor, blood, liver, bile, urine, and gastric contents) are also reported. No other fatalities caused by probenecid were known to the authors.

14. Colchicine

McIntyre et al.[88] described a case of death after colchicine poisoning. Ante-mortem hospital findings, autopsy details, and toxicology laboratory results are reported. Using HPLC with a photodiode array detector, colchicine was detected in the initial hospital serum specimen and in the post-mortem blood specimen. However, it was not detected in vitreous humor or in several other specimens.

15. Sertraline, Meclobemide, and Pimozide

A death involving the combination of sertraline, meclobemide, and pimozide has been reported by McIntyre et al.[89] Meclobemide is a monoamine oxidase inhibitor type A and is used as an antidepressant. Sertraline is another antidepressant, structurally unrelated to the tricyclic, bicyclic, tetracyclic, or monoamine oxidase inhibitor drugs. Pimozide is an antipsychotic of the diphenylbutylpiperidine series. Using HPLC analyses, concentrations for all three drugs were determined in ante-mortem and post-mortem femoral blood specimens; concentrations for meclobemide and sertraline are also reported in liver, bile, and vitreous humor.

16. Gentamicin

In one of the rare clinical studies involving analysis of vitreous humor in humans, Rubinstein et al.[90] studied the efficacy of various methods of administration of gentamicin for treatment of bacterial endophthalmitis. Twenty-two patients received gentamicin before undergoing ocular surgery. Vitreous humor specimens were obtained 15 to 20 min after the beginning of surgery. Intramuscular administration resulted in serum gentamicin concentrations ranging from 1.6 to 11 μg/ml, with a mean of 5.7 ± 3.6 μg/ml, but did not produce any detectable gentamicin in the vitreous humor of the ten patients in this group. Of the 12 patients to whom the drug was administered subconjunctivally, only three showed any gentamicin in the vitreous humor; concentration in serum had a mean of 2.16 ± 2.52 μg/ml. The authors acknowledge that the patients had severe disturbances in the vasculature around the eye and also that the short time interval before specimen collection may have contributed to the lack of positive findings.

17. Animal Studies

Most forensic toxicologists are concerned only with data from humans and involving drugs frequently encountered. However, it must be recognized that "rarely encountered" does not equate to never encountered. Therefore, Table 17–3 (Refs. 91–96 contained therein) lists several studies of drugs seen less frequently in forensic practice and animal studies. Thus, readers will have references to analytical procedures that have been worked out previously in case they face the need to perform analysis for one of these drugs.

D. METABOLITES DETECTED AND PARENT/METABOLITE RATIOS

Toxicologists commonly acknowledge that the metabolism of some drugs will have an impact on their analytical protocol. Benzodiazepines, cocaine, and opiates are three drug groups for which knowledge of metabolism will influence the analysis procedures to be used and any interpretation offered regarding the results. Logan and Stafford[68] reported that, for 28 cases studied, BE was higher than cocaine in 96% of the samples analyzed. Especially significant was the finding that BE was present in some specimens when cocaine was not detected (this applies to both vitreous humor and blood specimens). In their animal experimental study, McKinney et al.[69] showed that the concentrations of cocaine, BE, and EME changed over the 8-hour post-mortem interval of the study. Bermejo et al.[25] showed that conjugates of opiates are also present in vitreous humor specimens. Other previously cited papers did not include comparative studies such as these. However, it is obvious that toxicologists must be cognizant of the patterns and time course of metabolism of various drugs when planning the analytical scheme to be used. Choice of inadequate specimen preparation (lack of hydrolysis of opiate conjugates) or analysis for parent only may prevent the toxicologist from finding significant metabolites; the laboratory report thus produced will be insufficient because of these omissions. Furthermore, any interpretation of results must take into account the analytical procedures used and whether or not such procedures would have detected the various metabolites that might have been present in the particular case.

V. CONCLUSIONS

It is apparent that vitreous humor is a specimen that contains much information that is of use to the forensic toxicologist and to the ultimate users of the toxicology laboratory reports. Because the eye is fairly isolated from other portions of the body, vitreous humor specimens are often available even

TABLE 17–3
Drugs Studied in Animals

Drug	Animal	Analysis procedure	Ref.
Quinine	Rabbit	Fluorescence	91
Salicylic acid	Rabbit	Fluorescence	91
p-Aminosalicylic acid	Rabbit	Fluorescence	91
Salicyluric acid	Rabbit	Fluorescence	91
Barbital	Rabbit	Spectrophotometric	91
Lithium	Rabbit	NA[a]	91
Digoxin	Dog	Scintillation	92
Acetazolamide	Rabbit	HPLC	93
Timolol	Rabbit, monkey	HPLC	94
Trimethylpsoralen	Guinea pig	HPLC	95
Aztreonam	Rabbit	Agar diffusion	96

[a] NA, not available.

when other specimens are not. Furthermore, the protection of the eye reduces the incidence of contamination of the vitreous humor, thus making any analytical results more reliable. Although direct conversion of analytical results from post-mortem vitreous humor analyses to the corresponding ante-mortem serum or blood values cannot be made, still the relationships can provide a valid basis for interpretative opinions in many circumstances. Because of availability, reliability, and interpretative significance, the vitreous humor is a specimen that should be collected and submitted to the toxicology laboratory with each forensic case.

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Chapter 18

SALIVA DRUG ANALYSIS

Edward J. Cone and Amanda J. Jenkins

CONTENTS

I. INTRODUCTION

Human drug exposure can occur through a variety of different means. Therapeutic drugs are often administered orally, intravenously, intramuscularly, or subcutaneously. Drugs of abuse are normally self-administered by the oral, smoked, intravenous, and intranasal routes. However, esoteric routes are sometimes used, such as topical application, and also by rectal, vaginal, and sublingual administration. In addition to active use, passive drug exposure can occur in situations in which an individual is in contact with drugs, either knowingly or unknowingly. The most common form of

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TABLE 18–1
Comparison of Saliva Testing with Blood and Urine Testing for Drugs of Abuse

	Saliva	Blood	Urine
Sample collection	Noninvasive	Highly invasive	Intrusion of privacy
Principal analyte	Parent drug	Parent drug or metabolites	Metabolites
Analyte concentration	Low	Low to moderate	Moderate to high
Interpretation	1. Can be used to determine pharmacokinetic parameters 2. Potential correlation with impairment 3. Used to estimate blood levels and free drug fraction	1. Can be used to determine pharmacokinetic parameters 2. Potential correlation with impairment	1. Limited use in pharmacokinetics 2. No correlation with impairment 3. Cannot be used to estimate blood levels
Potential problems	1. Contamination from smoke, intranasal, and oral administrations 2. Changes in pH during collection may change S/P ratio	1. Limited sample availability	1. Possibility of adulteration 2. Changes in pH of urine during storage 3. Drug excretion influenced by pH

passive exposure is via smoke inhalation or oral ingestion; however, numerous other routes of exposure have been documented.[1]

Data regarding the nature of drug exposure can be obtained from chemical testing of biological specimens. The choice of which biological specimen to test is critical because each type of specimen may impart different chemical and pharmacodynamic information. For example, the presence of benzoylecgonine in urine can be interpreted as evidence of recent cocaine exposure, but cannot be interpreted to mean that the subject was under the influence of cocaine at the time of sampling. In contrast, the presence of cocaine in saliva or blood can reasonably be interpreted as an indication of both recent use and a high likelihood that the subject was experiencing pharmacological effects when the sample was obtained. Therefore, saliva and blood testing offer specific advantages over urine testing if the information desired relates to the pharmacological state of the individual at the time of testing.

It is generally accepted that chemical testing of biological fluids is the most objective means of diagnosing drug use. The presence of parent drug or metabolite in a biological specimen may be used as evidence of recent exposure. In some forensic circumstances, a qualitative result demonstrating the presence or absence of drug is sufficient evidence; whereas, in other situations a quantitative result is needed. In the latter case, the relationship between drug concentrations in biological fluids to pharmacological effects is critical. As shown in Table 18–1, saliva has specific advantages over both blood and urine in being readily accessible for sampling and is more amenable to pharmacological interpretation. Despite these advantages, saliva drug testing for forensic purposes has been slow to develop as a mature science, compared with other means of drug testing.

[1] Adapted from Saliva Testing for Drugs of Abuse in Saliva as a Diagnostic Fluid, *Ann. New York Acad. Sci.*, 694:91-127, 1993.

A. COLLECTION TECHNIQUES

Saliva is produced by the salivary glands: the parotid, submandibular, and sublingual. Various collection techniques have been used to collect mixed saliva. Saliva may be collected by tilting the head forward and allowing the saliva to freely flow from the mouth into a container, aspirating the saliva through a tube using a vacuum pump, or by placing a cotton swab in the mouth and allowing saliva to be absorbed over a period of time or until saturated. For the purposes of investigational studies, saliva secretion is usually increased. Flow may be stimulated by chewing on a piece of Teflon® or a clean rubber band. Substances such as Parafilm® should be avoided, because they may absorb highly lipophilic drugs. The individual should allow saliva to accumulate in the mouth and then expectorate into a suitable container. Bubble production should be minimized, because these may alter pH. Alternatively, citric acid crystals or candy may be used to increase saliva flow. Changes in saliva flow will change saliva pH, and this may affect the concentration of drugs found in the saliva. To collect saliva from a particular gland, the individual ducts must be isolated and flow collected with special devices or by cannulation. Again, saliva flow is usually stimulated before collection.

The literature on saliva testing is expanding at a substantial rate, which is indicative of the increasing interest in this unique biological fluid. Several reviews already have appeared on the use of saliva for general drug testing.[2–4] This review describes the current state of knowledge regarding the disposition of licit and illicit drugs in saliva.

II. ALCOHOL

Ethanol is a central nervous system (CNS) depressant. Loss of inhibition may appear as a stimulating effect. Psychic changes include expansive behavior, mood swings, and sensory and motor disturbances. As intoxication increases, general impairment of the nervous system occurs. The effects of ethanol on the CNS are generally considered to be proportional to its concentration in blood.[5] Chronic use produces physical dependence, with potential life-threatening withdrawal symptoms upon discontinuation. Ethanol is typically used orally and is rapidly absorbed from all parts of the gastrointestinal system. It is distributed fairly uniformly throughout all parts of the body according to body water content. The majority of ethanol is metabolized in the liver (90 to 95%) mainly by the enzyme alcohol dehydrogenase, producing acetaldehyde. Further enzymatic metabolism produces acetic acid. The mixed-function oxidase system is considered to exert a minimal role in ethanol metabolism under normal circumstances. The remainder is excreted unchanged in breath, urine, sweat, and feces.[6] Ethanol testing can be performed by analysis of blood, breath, urine, sweat, or saliva. Because ethanol is metabolized at a relatively constant rate of 0.015 to 0.018 g/dl/hr, the detection time is highly dependent on initial concentration. A variety of assays have been devised to measure ethanol in biological fluids, including chemical, enzymatic, and instrumental methods.[7–9] Recently, a number of rapid on-site tests for ethanol in saliva and other biological fluids have been marketed.[10–13]

The distribution of ethanol in the body is considered to occur by passive diffusion. Therefore, under equilibrium conditions, ethanol content will be dependent on the water content of the fluid or tissue being measured. Therefore, the content in saliva will be higher than that found in blood or serum. On a theoretical basis, the saliva-to-blood (S/B) ethanol ratio should be 1.17 if saliva and whole blood contain 994 and 850 g of water, respectively.[14,15] Generally, lower ratios have been found in the postabsorption phase. Jones[16] found a mean S/B ratio of 1.082 ($N = 336$) between 60 to 360 min after the start of drinking. This ratio was remarkably constant throughout the absorption, distribution, and elimination phases of ethanol metabolism. DiGregorio et al.[17] reported a saliva-to-plasma (S/P) ratio of 1.04 (range = 0.95–1.13) between 15 to 120 min after ethanol administration. Haeckel and Bucklitsch[18] found an overall mean S/P ratio of 1.032 in the postabsorption phase. In the latter study, ethanol concentrations were almost identical in unstimulated and stimulated (citric acid) saliva. Although S/P ratios seemed to decrease over time, corrections for differences in water content of samples indicated that S/P ratios during the elimination phase were constant. The study included a comparison of ethanol concentrations in saliva vs. capillary and venous blood. They

found that ethanol concentrations in saliva paralleled capillary blood more accurately than venous blood concentrations. Assuming that capillary blood was a better measure of ethanol exposure to brain cells, the authors concluded that saliva was at least as well suited as venous or capillary blood to reflect the intoxication state of an individual in the postabsorption state.

III. SYMPATHOMIMETIC AMINES

Amphetamines are sympathomimetic amines with CNS stimulant activity similar to cocaine, but with a longer half-life. The effects of moderate doses include increased blood pressure, mood elevation, increased energy and alertness, and decreased appetite.[5] Higher doses and prolonged use are followed by mental depression and fatigue. Amphetamine is generally abused by the oral or intravenous route. Methamphetamine is abused by the oral, intravenous, and smoking routes. Amphetamine is metabolized by hydroxylation of the aromatic ring to *p*-hydroxyamphetamine and deamination to benzyl methyl ketone followed by oxidation to benzoic acid. Methamphetamine is partially metabolized to amphetamine, which in turn is further metabolized. The relative amounts of amphetamine and methamphetamine excreted in urine are highly dependent on urinary pH conditions. Under normal conditions, approximately 30% of amphetamine and 40% of methamphetamine are excreted in urine unchanged.[6] Small amounts of amphetamine (4 to 7%) are excreted as a metabolite with methamphetamine. Amphetamine and methamphetamine can be detected in urine at a cutoff concentration of 1,000 ng/ml by immunoassay and at a cutoff of 500 ng/ml by gas chromatography/mass spectrometry (GC/MS).[19] A requirement, imposed by the U.S. Department of Health and Human Services, permits the reporting of methamphetamine-positive results in employee urine drug testing only if accompanied by the presence of 200 ng/ml of amphetamine. Detection of amphetamine and methamphetamine use is generally successful for only 24 to 48 hr after drug exposure, although the detection period after chronic use may be extended for several days. Fluctuating urinary pH can produce a pattern of alternating negative and positive testing results.

Amphetamine has been identified in saliva after the administration of *d*-amphetamine, *l*-amphetamine, or *d,l*-amphetamine.[20,21] Wan *et al.*[21] reported that saliva amphetamine concentrations exceeded plasma concentrations by a factor of 2 to 3 and were detectable for 48 hr after drug administration. In that study, subjects were administered 10 mg of amphetamine hydrochloride orally in gelatin capsules after fasting overnight. Subjects had been pretreated with sodium carbonate or ammonium chloride to induce alkaline or acidic urinary pH conditions. Saliva was collected by the subject chewing on a piece of Teflon® to induce salivation. Simultaneous blood samples were also collected. The samples were derivatized with a chiral reagent and analyzed by GC/MS. This procedure allowed resolution of the optical isomers of amphetamine. The *d*-isomer was eliminated more rapidly than the *l*-isomer, and the rate of excretion was faster under acidic conditions. Plasma protein binding was 16% for both isomers. Salivary pH was relatively constant and unaffected by pretreatment with sodium carbonate or ammonium chloride. S/P (total) ratios were somewhat higher during the absorption phase, possibly caused by contamination of the oral cavity during drug administration. Oral contamination by drug is recognized to be a problem for orally administered drugs and smoked drugs. During the postabsorption phase, amphetamine S/P (total) ratios were relatively constant and averaged 2.76, compared with the theoretical value of 2.62. The authors concluded that the use of saliva amphetamine measurements were advantageous over urinary rate measures for estimation of pharmacokinetic parameters, provided that only postabsorption saliva data were used for curve-fitting.[21] Amphetamine was also detected in saliva in a forensic investigation. Smith[22] measured amphetamine by radioimmunoassay in saliva and in saliva stains on a cigarette from a subject undergoing amphetamine therapy. The concentration of amphetamine in saliva was similar to that found in whole blood and semen from the same subject.

Methamphetamine has been detected in mouse salivary glands by immunohistochemical methods[23] and in human saliva by mass fragmentography.[24] Suzuki et al.[24] reported the occur-

rence of methamphetamine in hair, nails, sweat, and saliva of 25 methamphetamine abusers. Methamphetamine was detectable in 11 of 15 hair samples, 13 of 20 nail samples, 4 of 8 sweat samples, and 3 of 19 available saliva samples. Methamphetamine was detected for up to 2 days in saliva samples, 18 days in hair, and 45 days in nails after the last dose, as reported by the methamphetamine users.

Other sympathomimetic amines have also been detected in saliva. Ephedrine, amphepramone, and amphetamine were detected by thin-layer chromatography in saliva after subjects were administered single oral doses.[25] Although the sensitivity of this detection method was low (approximately 1 μg/ml), ephedrine was detected in all subjects for 8 hr. Amphepramone and metabolites were detected in the saliva of some subjects, and amphetamine was found in the saliva of two subjects between 2 and 12 hr. In a study by Turner et al.[26] using fluorescence polarization immunoassay, saliva and urine samples were found to test positive (100 ng/ml cutoff for amphetamine) for 72 hr after administration of 25 mg phentermine. Ephedrine (18 mg) was detectable for at least 24 hr in both saliva and urine. Phenylpropanolamine (25 mg) was detectable for 2 hr in saliva and for 48 hr in urine.

IV. BARBITURATES

Barbiturates can produce a variety of CNS mood changes, including excitation, sedation, hypnosis, anesthesia, and coma. Phenobarbital and mephobarbital are effective anticonvulsants at subhypnotic doses. Barbiturates are indicated for the production of sedation, hypnosis, preanesthesia, and long-term anticonvulsant therapy. Barbiturates can produce euphoriant effects comparable with those of morphine. They may be habit-forming, producing physical and psychological dependence with severe withdrawal symptoms upon discontinuation of use. They are well absorbed and are commonly self-administered by the oral route. In contrast to phenobarbital and barbital that undergo negligible metabolism, amobarbital, secobarbital, and pentobarbital undergo extensive metabolism, usually by oxidation of the side-chain substituents. Only 1 to 5% of the dose is excreted unchanged in urine. Barbiturates can be detected in urine at a cutoff concentration of 300 ng/ml (secobarbital equivalents) by immunoassay and GC/MS. The detection times for the short-acting barbiturates (e.g., secobarbital) may be as little as 24 hr and as long as 2 to 3 weeks for long-acting barbiturates, such as phenobarbital.

Amobarbital has been measured in human saliva by GC[27,28] and high–performance liquid chromatography.[29] Inaba and Kalow[27] reported high linear correlation ($r = 0.993$) between saliva and serum concentrations of amobarbital after an oral dose of 120 mg in five normal adults. Saliva concentrations averaged 36.1% of serum concentrations and were detectable for approximately 48 hr. The observed mean (±SD) saliva-to-serum (S/S) ratio was 0.35 ± 0.03, with mean serum protein binding (% unbound) of 42.4 ± 1.5. Barbital was measured in saliva by Ogata et al.[30] after an oral dose of 114 mg to healthy male volunteers. A significant relationship was found between barbital concentrations in serum and saliva and urinary excretion rate. The S/S ratio was 0.999.

Hexobarbital was measured in saliva and plasma or serum of healthy subjects after oral administration.[31,32] Van der Graaff et al.[31] reported that drug was detectable in saliva and plasma for approximately 12 hr. Mean S/P (total) ratios ± SEM were 0.34 ± 0.03, although there was a tendency for the ratio to increase as a function of time. Half-lives calculated from saliva data (3.3 hr) were similar to those calculated from plasma (3.2 hr). It was concluded that hexobarbital elimination half-lives could be determined accurately from saliva concentrations. Pentobarbital was measured in unstimulated mixed saliva of healthy subjects by GC after the oral administration of a single 100-mg dose.[28] Corresponding blood concentrations were not measured. The terminal half-life of pentobarbital determined from saliva concentrations was approximately 18 hr. Blom and Guelen[33] cited the S/P (total) ratio of pentobarbital as 0.36.

Phenobarbital is a widely prescribed anticonvulsant. In 1966, Borzelleca and Doyle[34] reported that a series of drugs, including phenobarbital, were excreted in the saliva of dogs treated with acute

intravenous doses of drug. They observed that saliva phenobarbital concentrations seemed to be dependent on plasma concentrations. Subsequent work by numerous investigators have extended these findings to humans. Cook et al.[35] measured phenobarbital in the saliva and plasma of epileptic patients and found a highly significant correlation ($r = 0.98$); the S/P (total) ratio was approximately 0.3. In addition, they examined saliva extracts by mass spectrometry and found no evidence of metabolites in saliva even in acid-hydrolyzed samples. Horning et al.[36] determined parotid S/P ratios for phenobarbital by two different analytical methods. S/P (total) ratios by GC/MS were 0.31 to 0.37 and by enzyme immunoassay were 0.32. Although S/P ratios were similar by both techniques, the enzyme immunoassay consistently produced higher absolute values, probably as a result of cross-reactivity with phenobarbital metabolites in plasma. Numerous other S/P or S/S ratios for phenobarbital have been reported, including: in children under the age of 2 ($N = 19$), S/P (total) = 0.45;[37] epileptic young people aged 9 to 18 ($N = 15$), S/S (total) = 0.285;[38] epileptic children aged 5 months to 18 years ($N = 121$), S/P (total) = 0.30 by GC and 0.31 by enzyme immunoassay;[39] adults aged 17 to 65 ($N = 11$), S/P (total) = 0.41;[40] patients undergoing pneumoencephalography ($N = 48$), S/S (total) = 0.32;[33] and epileptic patients aged 9 to 70 ($N = 29$), S/P (total) = 0.33.[41] An interesting example of forensic testing for phenobarbital was reported by Smith and Pomposini.[42] They detected drug by radioimmunoassay in saliva stains on a cigarette from an individual maintained on long-term phenobarbital therapy. Drug was also detected in bloodstains, seminal stains, perspiration stains, and hair from the same individual.

Secobarbital was detected in the saliva of healthy subjects after a single oral dose of 50 mg.[43] The mean experimental S/P (total) ratio ± SD was 0.30 ± 0.04, with a range of 0.24 to 0.38. The predicted S/P (total) for secobarbital was 0.39.

Other nonbarbiturate-sedative hypnotics have been detected in saliva. Sharp et al.[43] detected methaqualone in saliva samples of healthy subjects after a single oral dose of 250 mg. The mean experimental S/P (total) ratio ± SD was 0.11 ± 0.02. Peat et al.[44] reported detection of methaqualone and its major hydroxy metabolite in saliva after oral administration. Initially, the metabolite was present in much lower concentrations (0 to 4 hr), but matched or exceeded concentrations of the parent compound at later times (4 to 24 hr). Saliva concentrations of methaqualone and metabolite were approximately 10% of those observed in plasma. Another sedative, meprobamate, was reported to be present in saliva in nearly equal concentrations as found plasma after oral administration.[45] Chloral hydrate and metabolites have been detected in horse saliva after chloral hydrate doping.[46] In this study, the concentration of chloral hydrate and the metabolite, trichloroethanol, in saliva were approximately equal to plasma concentrations, whereas saliva trichloroacetic acid concentrations were much lower.

V. BENZODIAZEPINES

Benzodiazepines can produce a variety of effects, such as sedation and hypnosis, and also possess muscle relaxant, anxiolytic, and anticonvulsant properties. All of the benzodiazepines, with the exception of clorazepate, are completely absorbed when administered orally; clorazepate is decarboxylated in gastric juice to *N*-desmethyldiazepam, which is subsequently absorbed.[5] This class of drugs is commonly administered by the oral route and undergoes extensive metabolism by numerous pathways, including oxidation, reduction, and conjugation. Some active metabolites have longer elimination half-lives than the parent compounds; thus, the duration of action of many benzodiazepines is not directly related to the half-life of the parent drug. Only traces of parent compound are usually found in urine. Benzodiazepines can be detected in urine at a cutoff concentration of 300 ng/ml (oxazepam equivalents) by immunoassay, high-performance liquid chromatography, and GC/MS. Detection times are highly dependent on drug and dosing frequency. Some benzodiazepines, such as flurazepam, may be difficult to detect because they are present in very low concentrations; whereas others, like diazepam, may be detectable in urine for several weeks after chronic use.

Diazepam is a long-acting lipophilic benzodiazepine that is primarily undissociated in biological fluids. As a result of extensive protein binding (96 to 99%), only a small percentage of total plasma diazepam is found in saliva. Concentrations are found in the 0 to 20 ng/ml range. Sensitive assays must therefore be used for detection. Numerous techniques have been used to measure diazepam and other benzodiazepines in saliva, including radioimmunoassay,[47] radioreceptor assay,[48] high-performance liquid chromatography,[49] capillary GC with nitrogen–phosphorus detection,[50] and GC with electron capture detection.[51–53]

After an oral dose of 10 mg of diazepam, DiGregorio et al.[51] reported that drug concentrations peaked at 0.75 hr in plasma, parotid saliva, and mixed saliva. Concentrations in the range of 1 to 6 ng/ml were achieved over an 8-hr period after dosing. The mean parotid S/P (total) ratio (±SE) was 0.035 ± 0.0047, and the mean mixed S/P (total) ratio (±SE) was 0.029 ± 0.0048. Highly significant correlations were found between plasma diazepam concentrations, and both parotid and mixed saliva concentrations. Similar correlations between plasma diazepam concentrations and saliva were found by de Gier et al.[53] Hallstrom et al.[52] also reported similar correlations between saliva diazepam concentrations, and both plasma and cerebrospinal fluid. In contrast to the observation by DiGregorio et al.[51] of similar peak times, Giles et al.[54] reported that peak plasma diazepam concentrations occurred at 60 min, whereas peak saliva concentrations occurred at 100 min. It is likely that the time delay between drug appearance in plasma and saliva represented the time required for passive diffusion from plasma to saliva and for equilibrium to be established between these fluids.

A major active metabolite of diazepam, *N*-desmethyldiazepam, is also found in saliva after administration of diazepam. After an acute dose of 0.143 mg/kg diazepam, Giles et al.[54] reported slowly increasing *N*-desmethyldiazepam concentrations over time, reaching a maximum concentration of approximately 1 ng/ml, 24 hr after dosing. After chronic diazepam dosing in a group of outpatients who participated in a study of driving performance, de Gier et al.[55] found saliva concentrations of *N*-desmethyldiazepam ranging from 1.2 to 23.0 ng/ml, approximately equivalent to diazepam concentrations. In this study, saliva and plasma concentrations of diazepam and *N*-desmethyldiazepam were highly correlated. Mean S/P (total) ratios ± SD for diazepam and *N*-desmethyldiazepam were 0.013 ± 0.002 and 0.018 ± 0.004, respectively. The authors noted that, although patients showed impaired driving performance, there was no correlation between plasma or saliva concentrations of diazepam or its metabolite and performance decrement. In a study of diazepam and *N*-desmethyldiazepam, concentrations in the saliva of chronically dosed hospital inpatients, Giles et al.[56] found a low correlation between saliva concentrations and dose. Because of the wide intersubject variability, individual saliva drug concentrations were poor predictors of dose.

Clorazepate is converted to *N*-desmethyldiazepam in the acidic conditions of the stomach.[57] After an acute 15-mg oral dose of clorazepate, Hallstrom et al.[52] reported detection of approximately 5 to 20 ng/ml of *N*-desmethyldiazepam in saliva. S/P (total) ratios averaged 5.78 (range = 2.33 to 6.65). Another benzodiazepine, chlordiazepoxide, was reported to be detectable by radioimmunoassay in saliva after acute and multiple dosing.[58] Saliva and plasma concentrations were highly correlated and were detectable for 30 to 60 hr. The S/P (total) had a mean value of approximately 0.03. Drug half-lives determined from plasma and saliva were equivalent.

Nitrazepam was reported by Kangas et al.[59] to be excreted in saliva after a 5-mg oral dose. The concentration of nitrazepam in saliva correlated significantly with serum concentrations; however, saliva concentrations were substantially lower than free serum concentrations. This finding led the authors to conclude that saliva testing for nitrazepam was of little clinical value. In a later study, Hart et al.[60] also found that salivary nitrazepam concentrations were lower than free serum concentrations. Eventually, the discrepancy between saliva and plasma was linked to the instability of nitrazepam in saliva. It was found that nitrazepam in saliva was rapidly converted to 7-aminonitrazepam. The conversion rate, however, was strongly dependent on the composition of individual subject's saliva.[61] Another benzodiazepine, clonazepam, also declined in concentration upon storage in saliva.

VI. CAFFEINE

Caffeine is one of several xanthine alkaloids that occur naturally in coffee, tea, kola nuts, and cocoa beans. Caffeine is a potent stimulator of the CNS and a smooth muscle relaxant (notably bronchial muscle), cardiac muscle stimulant, and diuretic. Rapid absorption occurs after oral administration, followed by distribution into all body compartments. Caffeine is extensively metabolized by the liver, and less than 5% is excreted unchanged in urine. It can be detected in urine by immunoassay, ultraviolet spectrophotometry, GC, GC/MS, and high-performance liquid chromatography. Single doses of caffeine may be detected for 24 to 48 hr, whereas chronic dosing results in somewhat longer detection times.

Caffeine is consumed by a large segment of the population in beverages (coffee, tea, soft drinks, and cocoa), food (chocolate), and medicines (cold medications and analgesics). Caffeine is also one of the major ingredients found in "look-alike" drugs that are available in the U.S. Look-alike capsules and tablets are preparations that resemble controlled substances and are sold to unwitting drug users in the illicit drug market. Caffeine rapidly appears in saliva after oral and intravenous doses.[62] It is a weak base and the percentage of bound fraction in plasma has been reported to be 26%[62] and 40%.[63] S/P S/S (total) ratios for caffeine have been reported to range from 0.55 to 1.02.[36,64–69] Haeckel[67] administered pure caffeine (350 mg) by the oral route to six healthy, male subjects. Capillary blood was drawn from hyperemised fingertips and was considered to be closer in composition to arterial than venous blood. During the absorption and distribution phases, the concentration of caffeine was higher in capillary than in venous blood. Saliva caffeine concentrations during this period accurately reflected the change in concentration occurring in capillary blood. The high blood flow to salivary glands[70] allowed equilibrium to be established quickly between saliva and arterial blood. Consequently, saliva caffeine concentrations were considered to represent more accurately drug concentrations in the central compartment than did venous blood caffeine concentrations.

Determination of caffeine in saliva has been suggested to be useful for the assessment of hepatic function. Setchell et al.[71] and Wahllander et al.[68] administered oral doses of caffeine, and then measured caffeine in saliva and serum periodically over the subsequent 12 to 24 hr. Both groups reported that half-lives and clearance rates of caffeine could be determined as accurately from saliva as serum. They found that saliva caffeine concentrations could be used to distinguish cholestatic and cirrhosis patients from control subjects. Wahllander et al.[68] indicated that measurement of overnight caffeine clearance in saliva might be a useful method for assessing progression and prognosis of liver disease. Apparently, there have been no attempts to measure caffeine saliva concentrations for forensic purposes; however, the chemical stability of caffeine, its abundance in saliva, and ease of measurement[69,72] present many opportunities for its detection in a variety of circumstances.

VII. COCAINE

Cocaine is obtained from the leaves of *Erythroxylum coca* and other species grown in South America. Cocaine hydrochloride is used topically as a local anesthetic for mucous membranes of the oral, laryngeal, and nasal cavities. It has also been used in ophthalmology, but has generally been replaced by other agents with fewer toxic side-effects. Cocaine produces CNS stimulation that is manifested by a feeling of well-being and euphoria. Absorption occurs from all sites of application, including mucous membranes, gastrointestinal tract, and the lungs.[5] Illicit cocaine is sold as cocaine hydrochloride for oral, intranasal, and intravenous use and cocaine base ("freebase" and "crack") for smoking. The drug is hydrolyzed by serum esterases, liver and chemical action to two major metabolites, benzoylecgonine and ecgonine methyl ester, plus a number of minor metabolites. After cocaine use, urine contains less than 10% of unchanged cocaine and 30 to 40% each of the two major metabolites. Cocaine metabolite (benzoylecgonine) can be detected in urine at a cutoff concentration of 300 ng/ml by immunoassay and at a cutoff concentration of 150 ng/ml by GC/MS.

Detection times of benzoylecgonine in urine after administration of single euphorigenic doses of cocaine vary from 24 to 48 hr. With higher doses or chronic use, detection times are slightly increased.

The excretion of cocaine in saliva was first reported by Inaba et al.[73] Radiolabeled cocaine was administered by the oral route, and radioactivity in saliva was measured. Peel et al.[74] also reported the detection of cocaine in a single saliva sample in a survey of impaired drivers. Thompson et al.[75] confirmed the presence of cocaine in saliva by GC/MS after single intravenous doses of cocaine hydrochloride to volunteer subjects. S/P (total) ratios for one subject across time averaged 1.26, with a range of 0.5 to 2.96. Ferko et al.[76] reported similar parotid S/P ratios for cocaine in the rat after intravenous administration of various doses. In the latter study, they also reported detection of benzoylecgonine in amounts approximately equivalent to cocaine. Salivary cocaine was highly correlated with plasma concentrations in contrast to benzoylecgonine concentrations.

Cone et al.[77] studied the relationship of saliva cocaine to plasma concentrations, and behavioral and physiological effects after intravenous drug administration to human subjects. Significant correlations were found between saliva cocaine and plasma cocaine ($r = 0.89$, $p < 0.01$), subjective effects ("Feel Drug" scale, $r = 0.74$, $p < 0.01$; "Goodness" scale, $r = 0.54$, $p < 0.05$; and "Rush" scale, $r = 0.55$, $p < 0.05$), and physiological effects (pulse, $r = 0.76$, $p < 0.01$). Cone et al.[77] speculated that, "It may be that the cocaine addict attempts to titrate blood concentrations (and thereby drug concentrations at effector sites) for the production of feelings of rush and euphoric high and the prevention of dysphoric effects and craving." They further indicated that the observation of a significant correlation of saliva cocaine concentrations with plasma concentrations and also with behavioral effects provided the opportunity for development of a new, noninvasive test for cocaine abuse.

Cocaine is self-administered by a variety of routes of administration. Unfortunately, little information has been available on the effects of different routes of administration on cocaine and metabolite concentrations in saliva. Recently, the author (Cone) conducted a series of studies on cocaine concentrations in saliva and plasma after intravenous, smoking and intranasal cocaine administration. Plasma and saliva samples were collected periodically after drug and were analyzed by GC/MS. Cocaine, benzoylecgonine, and ecgonine methyl ester concentrations are shown in Table 18–2. Cocaine was the major analyte in saliva by all routes of administration. Benzoylecgonine and ecgonine methyl ester generally were present in minor amounts, and their concentrations usually peaked later than cocaine. Cocaine contamination of the oral cavity after the smoking and intranasal routes of administration was anticipated; however, the magnitude of contamination by the intranasal route was surprising. The changes in S/P (total) ratios over time in this study are shown in Figure 18–1. Contamination of the oral cavity produced elevated S/P (total) ratios in the early period after drug administration. After approximately 3 hr, S/P (total) ratios were equivalent by the three routes of administration. Jenkins et al.[78] compared cocaine concentrations in saliva with concentrations in plasma after smoked and intravenous drug administration to seven male volunteers. Peak saliva cocaine concentrations after intravenous administration of 44.8 mg cocaine hydrochloride ranged from 428 to 1,927 ng/ml; and after smoking an equivalent dose, ranged from 15,000 to >500,000 ng/ml ($N = 7$). Contamination of the oral cavity after smoking was responsible for the elevated cocaine concentrations in saliva in the early time period after cocaine administration. The mean saliva/plasma cocaine concentration ratios for the time points between 2 to 60 min, after smoking 40-mg cocaine base, were >10 compared with mean ratios <6 between 2 min and 24 hr after intravenous administration. Detection times (limit of detection = 1 ng/ml) were longer in saliva than plasma for both routes of drug administration, with an average detection time in saliva of 446 min after smoking and 514 min after intravenous administration, compared with 240 and 377 min in plasma, respectively. Anhydroecgonine methyl ester (AEME), a pyrolysis product of cocaine, was also detected in saliva after smoking. Peak AEME concentrations were achieved at 2 min and ranged from 558 to

TABLE 18–2
Saliva and Plasma Concentrations and S/P (Total) Ratios of Cocaine and Metabolites After Acute Doses of Cocaine to a Single Human Subject by the Intravenous, Smoking, and Intranasal Routes of Administration

	Cocaine			Benzoylecgonine			Ecgonine methyl ester		
Minutes	Saliva (ng/ml)	Plasma (ng/ml)	S/P (total)	Saliva (ng/ml)	Plasma (ng/ml)	S/P (total)	Saliva (ng/ml)	Plasma (ng/ml)	S/P (total)
				Intravenous Cocaine (25 mg)					
5	530.9	161.6	3.3	0.0	0.0	—	0.0	0.0	—
10	114.5	153.7	0.7	0.0	3.7	0.0	0.0	0.0	—
15	287.7	127.3	2.3	0.0	24.6	0.0	0.0	0.0	—
20	85.8	111.8	0.8	0.0	45.9	0.0	0.0	0.0	—
30	108.3	83.2	1.3	5.6	79.1	0.1	3.7	0.0	—
45	233.1	76.0	3.1	22.0	93.4	0.2	15.7	0.0	—
60	107.4	58.4	1.8	16.9	115.6	0.1	20.3	1.2	16.9
90	95.5	43.1	2.2	22.0	128.1	0.2	17.8	2.5	7.0
120	28.2	32.5	0.9	56.2	134.0	0.4	38.9	3.1	12.5
180	63.7	17.4	3.7	18.1	135.1	0.1	16.4	0.0	—
240	31.0	10.4	3.0	29.0	130.4	0.2	19.3	0.0	—
360	9.2	2.6	3.5	16.2	119.8	0.1	11.4	0.0	—
720	0.0	0.0	—	10.5	53.9	0.2	0.0	0.0	—
				Smoked Cocaine ("Crack") (42 mg)					
5	3,404.0	198.1	17.2	0.0	6.7	0.0	0.0	0.0	—
10	2,082.3	146.7	14.2	0.0	45.0	0.0	0.0	0.0	—
15	NA[a]	210.8	—	0.0	16.5	0.0	0.0	0.0	—
20	407.8	NA	—	0.0	NA	—	0.0	NA	—
30	278.6	128.7	2.2	0.0	83.0	0.0	0.0	0.0	—
45	332.1	102.6	3.2	20.0	125.0	0.2	15.7	1.9	8.5
60	240.5	82.1	2.9	25.3	135.5	0.2	23.5	4.0	5.8
90	109.1	50.9	2.1	19.6	159.5	0.1	17.8	8.1	2.2
120	165.3	45.7	3.6	46.3	164.5	0.3	28.6	7.5	3.8
180	40.5	27.6	1.5	18.5	162.5	0.1	14.0	6.6	2.1
240	24.1	15.7	1.5	19.4	152.0	0.1	0.0	3.9	0.0
360	32.2	12.5	2.6	41.5	129.0	0.3	26.3	0.0	—
720	0.0	0.0	—	0.0	65.3	0.0	0.0	0.0	—
				Intranasal Cocaine (42 mg)					
5	58,250.0	4.4	13,238.6	0.0	0.0	—	94.2	0.0	—
10	112,800.0	16.7	6,754.5	0.0	0.0	—	316.0	0.0	—
15	85,170.0	27.0	3,154.4	0.0	0.0	—	250.4	0.0	—
20	119,700.0	32.6	3,671.8	0.0	0.0	—	427.0	0.0	—
30	38,310.0	42.6	899.3	0.0	9.8	0.0	123.3	0.0	—
45	540,800.0	48.7	11,104.7	0.0	57.9	0.0	95.1	0.0	—
60	NA	50.8	—	0.0	96.2	0.0	74.4	7.5	9.9
90	145.5	18.2	8.0	34.1	117.1	0.3	19.0	3.4	5.6
120	156.9	10.6	14.8	50.7	122.9	0.4	32.7	7.5	4.3
180	25.0	38.0	0.7	38.5	103.6	0.4	20.6	2.7	7.6
240	72.1	31.0	2.3	49.4	118.3	0.4	29.2	2.7	10.7
360	21.7	2.0	10.9	47.3	104.4	0.5	25.6	0.0	—
720	0.0	0.0	—	22.4	NA	—	0.0	0.0	—

[a] NA, sample not analyzed.

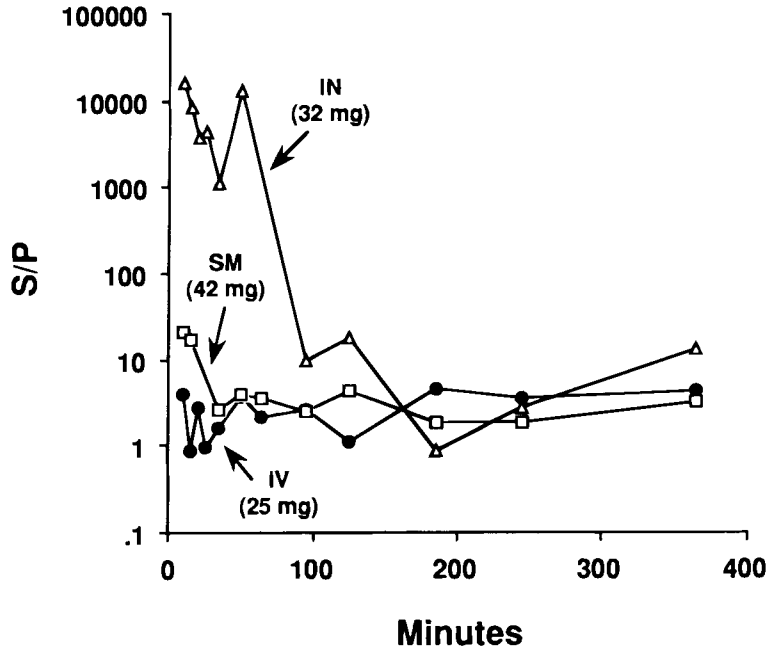

FIGURE 18–1. Changes in cocaine S/P (total) ratios over time in a single human subject after cocaine administration by the intravenous (IV), smoked (SM), and intranasal (IN) routes.

4,374 ng/ml (N = 7). Figure 18–2 illustrates the mean concentrations of AEME, cocaine, and metabolites detected in saliva after smoking 40-mg cocaine base.

In a study by Kato et al.,[79] the effect of changes in saliva flow rate on cocaine excretion in saliva was evaluated. As a result of cocaine's sensitivity to changes in pH, it was expected that increases in saliva flow rate would substantially decrease the concentration of cocaine and metabolites in saliva. Collection of unstimulated saliva after intravenous administration resulted in excretion of higher concentrations of cocaine than when saliva flow was stimulated with a piece of sour candy. Figure 18–3 illustrates the excretion of cocaine in saliva of two experienced cocaine users over a period of 6 hr. During this time, a 25-mg dose of intravenous cocaine was administered under controlled conditions every 2 hr. The ratio of the area under the curves for unstimulated saliva cocaine vs. stimulated saliva cocaine for Subjects K and L were 9.45 and 3.62, respectively, indicating that there was an average 6.5-fold reduction in cocaine excretion as a result of stimulation of saliva flow. Although the pH of saliva was not measured in this study, the observed differences were likely caused by the differences in pH between unstimulated and stimulated saliva. It is known that salivary pH is dependent on flow rate and that pH increases as flow increases.[80] Because cocaine is a weak base with a pKa of 8.6,[81] the concentration in saliva is highly dependent on salivary pH and consequently salivary flow. Indeed, estimation of cocaine excretion in saliva at pH values of 5.9 (resting saliva pH) and 7.8 (citric acid stimulation)[80] provides a maximal theoretical ratio of 68.8. Therefore, the average ratio for unstimulated to stimulated cocaine concentrations in saliva of 6.5 was clearly in line with the estimates of pH effects on cocaine distribution in saliva.

The findings that cocaine administration by the intranasal and smoking routes produced elevated S/P (total) ratios and that changes in collection conditions substantially altered the concentration of cocaine in saliva are significant. Obviously, these influences weaken the possible use of saliva cocaine concentrations as a correlate of behavioral effects. Nonetheless, this does not preclude use of saliva in forensic testing for evidence of recent use. The temporal patterns of cocaine in saliva after all routes of administration were equivalent.

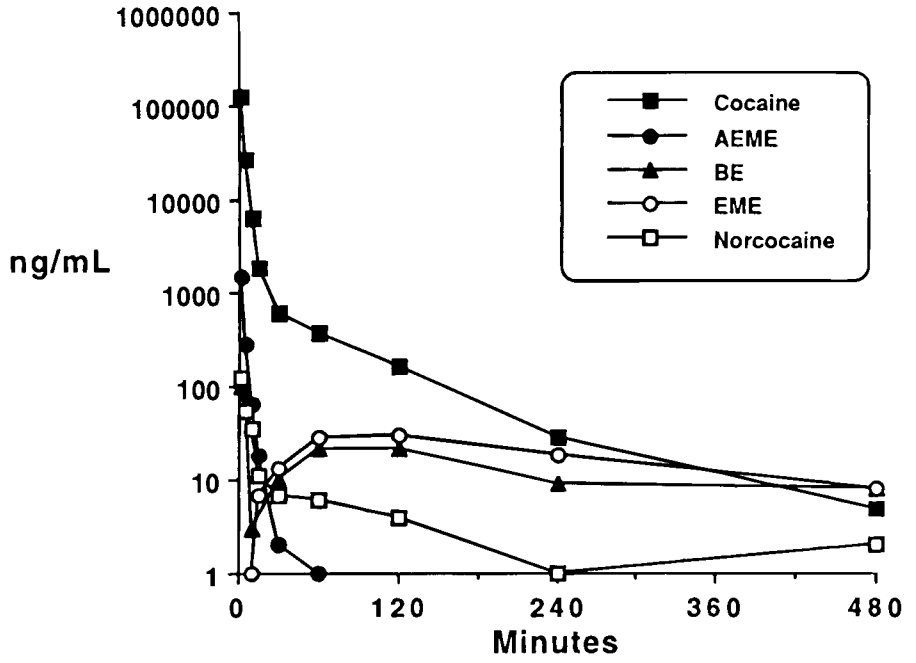

FIGURE 18–2. Mean concentrations ($N = 7$) of AEME, cocaine, and metabolites in saliva after smoking 40-mg cocaine base. BE, benzoylecgonine; EME, ecgonine methyl ester. (From Jenkins, A. J. et al., *J. Anal. Toxicol.*, 19, 359–374, 1995. With permission.)

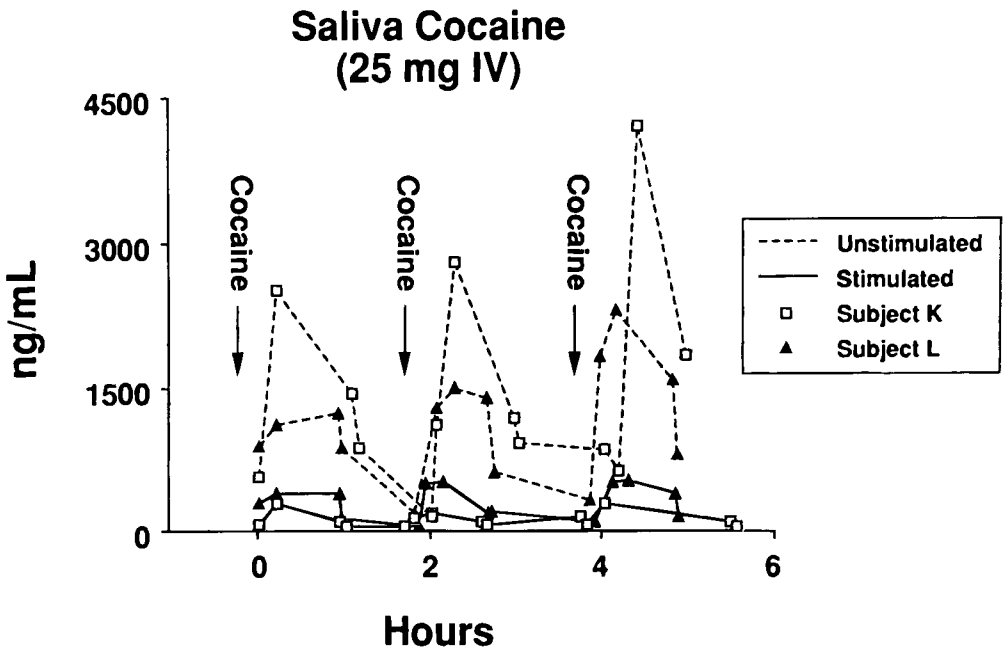

FIGURE 18–3. Effects of changes in saliva flow rate on cocaine concentration in the saliva of two human subjects. Saliva flow was stimulated with sour candy.

VIII. INHALANTS

Inhalants include a wide range of chemical substances, but are generally classified into two broad categories: anesthetic gases and volatile hydrocarbons. The anesthetic gases include ether, chloroform, cyclopropane, ethylene, and nitrous oxide. Volatile hydrocarbons include many household items, consisting primarily of petroleum products, such as gasoline, paint thinner, lighter fluid, glue, cleaning solutions, nail polish products, and organic solvents like acetone, ethyl acetate, isopropyl alcohol, carbon tetrachloride, naphtha, and amyl and butyl nitrite. These products all share one feature in common: they are highly volatile at room temperature and may be easily inhaled. Inhalants are readily absorbed by the lungs and are rapidly distributed throughout the body. Many are fat soluble and quickly accumulate in adipose tissues. At low doses, they cause giddiness, excitement, loss of coordination, confusion, and disorientation. The duration of effects are usually short-lived, lasting less than 1 hr. At higher doses, the cardiovascular and respiratory systems are depressed, which may result in delirium, coma, and death. Some inhalants produce psychedelic effects. Many inhalants are eliminated unchanged, primarily through the lungs, whereas a few are extensively metabolized and excreted in urine. Because of their physical and chemical properties and the diversity of inhalants, the analysis of biological samples for their presence is performed primarily by GC and GC/MS.

Inhalants in saliva have received relatively little study, although there are numerous reports of detection by headspace techniques of volatiles in saliva for metabolic profiling studies.[82–84] Ramsey and Flanagan[85] used headspace techniques for the detection of volatile organic compounds in blood as an aid in the diagnosis of solvent abuse. The method was useful for the detection of bromochlorodifluoromethane, *n*-butane, carbon tetrachloride, chlorobutanol, cryofluorane (Halon 114), dichlorodifluoromethane (Halon 12), ethyl acetate, halothane, isobutane, isopropanol, isopropyl nitrate, methyl ethyl ketone, propane, tetrachloroethylene, and trichlorofluoromethane (Halon 11) in blood specimens obtained from patients suspected of abusing inhalants. However, when saliva specimens were analyzed from selected patients for the same volatile components, the investigators had little success. Their failure to detect inhalants in saliva led them to conclude that saliva was not likely to yield useful information regarding inhalant abuse. In contrast, Tomita[86] studied the excretion of a variety of organic solvents in rat saliva after intraperitoneal administration and found excellent correlation of saliva concentrations with blood concentrations for a number of organic solvents. The correlation coefficient for isopropanol in saliva vs. blood was 0.96. Acetone was also present in saliva and blood as a metabolite after isopropanol administration; again, saliva concentrations were correlated with plasma concentrations ($r = 0.81$). The average S/B (total) ratios for isopropanol and acetone (metabolite) were 0.90 and 0.83, respectively. Other solvents studied in the rat had mean S/B (total) ratios as follows: ethyl alcohol, 1.37; *n*-propyl alcohol, 0.61; acetone, 0.60; methyl ethyl ketone, 0.56; ethyl acetate, 1.12 (for the metabolite ethyl alcohol); benzene, 0.12; toluene, 0.02; xylene, 0.00; and dichloromethane, 0.04. These studies indicated that, as the lipid solubility of the solvent increased, the amount of the compound detected in saliva decreased. The author concluded that saliva testing of human subjects could prove useful in the evaluation of levels of exposure to several organic solvents.

IX. LSD

Lysergic acid diethylamide (LSD) is a potent psychotomimetic drug that induces states of altered feeling, perception, and thought. There is heightened awareness of sensory input accompanied by sympathomimetic changes such as pupillary dilation, increased blood pressure, tachycardia, hyperreflexia, tremor, nausea, piloerection, muscular weakness, and increased body temperature.[5,87] LSD is commonly administered by the sublingual or oral route and is rapidly absorbed. In labora-

tory animals, LSD is oxidatively metabolized to a variety of metabolites; however, only limited information is available on the human metabolism of LSD.[88] Recently, LSD and three metabolites, *N*-desmethyl LSD and two hydroxy metabolites, were identified in urine from an LSD user.[89] Cumulative urinary profiles indicated that the amount of LSD and *N*-desmethyl LSD excreted in urine represented 0.9 and 1.2% of the dose, respectively. LSD can be detected in urine at a cutoff of 1 ng/ml by radioimmunoassay and by GC/MS at a detection limit of 0.05 ng/ml. LSD and *N*-desmethyl LSD were detectable in urine for 22 and 72 hr, respectively, after a single oral dose of LSD (1 μg/kg).[89]

LSD excretion in saliva has not been studied; however, several reports have appeared on the detection of LSD in blood[90–92] and urine[89,93] after administration to human subjects. Aghajanian and Bing[90] and Upshall and Wailling[91] used spectrophotofluorimetry to measure LSD in plasma after single doses of LSD to human subjects. They reported that LSD concentrations in plasma ranged from 1 to 10 ng/ml after intravenous and oral dosing. Plasma concentrations of LSD were detectable for 5 to 8 hr. Because LSD is a weak base with a pKa of 7.8, the theoretical S/P (total) ratio for LSD (assuming saliva pH = 6.8) is estimated to be 1.41. Consequently, it can be assumed that saliva concentrations of LSD would be maximal in the range of 0.3 to 3.1 ng/ml and demonstrate comparable detection times with those observed for plasma.

X. MARIJUANA

Marijuana use may produce sedation, euphoria, hallucinations, and temporal distortion. It is defined in Federal Schedule I of the Controlled Substances Act as, "all parts of the plant *Cannabis sativa,* whether growing or not, the seeds . . ., the resin . . ., and every compound, salt, derivative . . ., or preparation of such plant . . . but shall not include the mature stalks of such a plant,. . . ." There are purported to be two other species of cannabis, namely, indica and ruderalis. The main psychoactive compound, Δ^9-tetrahydrocannabinol (THC), constitutes 0.5 to 15% of plant weight. At low doses, marijuana or THC produces effects on mood, memory, motor coordination, cognitive ability, increases heart rate and systolic blood pressure, conjunctival injection (reddening of eye rims), and occasional orthostatic hypotension.[5,94] At higher doses, cognitive processes become confused, and paranoid feelings and delusions may occur. At very high doses, a toxic psychosis with frank hallucinations may result. Marijuana is usually smoked, but may be ingested orally. THC is first biotransformed to an active metabolite, 11-hydroxy-Δ^9-THC, which in turn is rapidly converted to an inactive metabolite, 11-nor-9-carboxy-Δ^9-THC (THCCOOH). Although many other metabolites of marijuana are excreted in urine, it is the carboxy metabolite that serves as the target analyte for urine drug testing. This metabolite is excreted in urine primarily as a glucuronide conjugate. Hydrolysis procedures are used to remove the glucuronide group before confirmation analysis. Cannabinoids may be detected in urine at cutoff concentrations of 20, 50, and 100 ng/ml (THCCOOH equivalents) by immunoassay. Most immunoassays are selective for THCCOOH, but cross-react with many other marijuana metabolites. THCCOOH is confirmed in urine at a cutoff concentration of 15 ng/ml by GC/MS. Acute marijuana use (one or two joints) is detectable for 1 to 3 days; moderate use (more than two joints) is detectable for 3 to 7 days; and chronic use (daily use) is detectable in some cases for more than 30 days.[93]

THC has been detected in saliva after smoking of marijuana cigarettes,[95–99] tobacco cigarettes containing THC,[100] and hashish.[101,102] The presence of THC in saliva is caused by contamination of the oral cavity during the smoking process. One report indicated that THC could not be detected in saliva after an intravenous dose to human subjects;[103] however, another report indicated that small amounts of radiolabeled THC were detected in the saliva of monkeys after intravenous injection.[104] Ohlsson et al.[105] reported that only extremely low concentrations of cannabidiol, a THC derivative, were excreted in saliva after intravenous administration of a 20-mg dose of cannabidiol to five male human subjects.

THC detection times in saliva are variable and range from 2 to 10 hr. Huestis et al.[106] found that THC detection times, determined by radioimmunoassay, for four subjects averaged 6 hr after smoking a single 1.75% THC marijuana cigarette and 10 hr after a 3.55% THC marijuana cigarette (Figure 18–4) in comparison with an average detection time in plasma of approximately 5 hr (determined by GC/MS). In the same study, saliva samples collected over a 7-day period after marijuana administration were analyzed by GC/MS for the presence of THC metabolites. Neither THCCOOH or 11-hydroxyΔ^9-THC were detected in any sample in concentrations above the detection limit of the assay (approximately 0.5 ng/ml for each analyte). In contrast to these findings, Schramm et al.[2] reported detection by thermospray mass spectrometry of small amounts of THCCOOH, 11-hydroxyΔ^9-THC, and cannabidiol in a single saliva sample collected as an ultrafiltrate, from a marijuana smoker. No detection limits were reported for the assay, and the amount of metabolite present was difficult to determine from the data presented in the article.

Saliva concentrations of THC generally vary over a wide range, from 50 to 1,000 ng/ml shortly after marijuana exposure. Thompson and Cone[99] reported that THC was detectable immediately after smoking in very high concentrations, followed by a rapid decline over the first hour. Thereafter, concentrations declined gradually and seemed to mimic plasma concentrations. Other authors have reported similar saliva results.[96–98] Huestis et al.[106] reported a significant correlation of saliva THC concentrations with plasma concentrations ($p < 0.01$) in male marijuana users following the smoking of a single marijuana cigarette of different strengths (1.75 and 3.55% THC). Figure 18–5 illustrates the mean S/P (total) ratios ($N = 4$) over a period of 4 hr. The S/P ratio (total) declined rapidly over the first 20 min, followed by a gradual increase over the next 4 hr.

Correlations of saliva THC concentrations with behavior and physiological measures have also been reported. Huestis et al.[106] reported a highly significant correlation ($p < 0.01$) between mean saliva THC concentrations and several subjective, performance, and physiological measures of drug effect, namely, the "Feel Drug" scale, Digit Symbol Substitution Test, and heart rate. However, when individual saliva data were correlated with concurrent measures, the correlations were not significant, leading the authors to conclude that predictions of performance effects from a single THC saliva test would be unreliable as a result of high individual variability. In contrast, Menkes et al.[97]

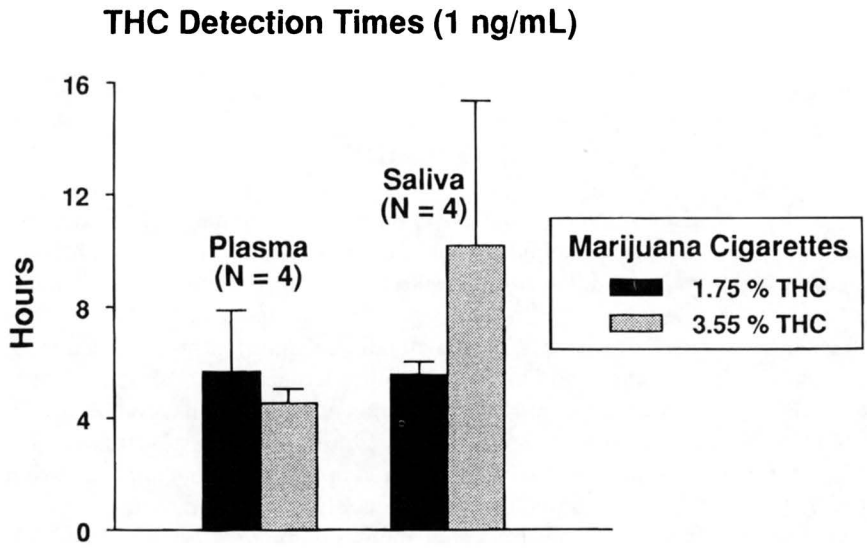

FIGURE 18–4. Detection times of THC in plasma and saliva of four human subjects after smoking of a marijuana cigarette. Bars represent the SEM ($N = 4$) for each dose and route. THC was measured in blood by GC/MS and in saliva by radioimmunoassay.

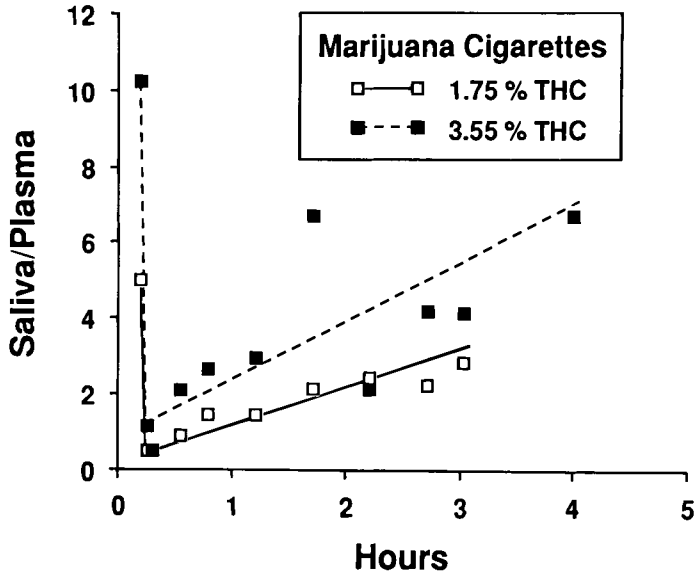

FIGURE 18–5. Changes in THC S/P (total) ratios over time for four human subjects after smoking a marijuana cigarette.

reported significant correlations for within-subject data with log–THC saliva concentrations vs. subjective intoxication measures and with heart rate changes. They suggested that saliva THC concentration can be a sensitive index of recent cannabis smoking and was more closely linked than either urine or blood cannabinoid concentrations.

Saliva testing for THC for forensic purposes was reported by Peel et al.[74] A total of 56 saliva samples were obtained from drivers suspected of impaired driving. Samples were initially screened by a modified immunoassay for cannabinoids in blood. Positives were confirmed for THCCOOH by GC/MS. Alcohol was the most common drug detected, followed by cannabinoids and diazepam. Six saliva samples were positive for cannabinoids. Three of the six drivers admitted use of the drug sometime before their arrest, whereas the other three drivers denied use of marijuana. The authors concluded that saliva testing was versatile enough to serve as a noninvasive test for detection of drug use in impaired drivers.

XI. OPIOIDS

Opium, the dried exudate of the poppy *Papaver somniferum,* contains morphine, codeine, and other phenanthrene and benzylisoquinoline alkaloids. Many synthetic and semisynthetic derivatives of morphine have been synthesized that comprise the family of compounds termed "opioids." This term refers to all compounds with morphine-like agonist and antagonist activity. Opioid effects are diverse and include analgesia, drowsiness, mental clouding, reduced gastrointestinal motility, miosis, nausea, vomiting, and respiratory depression.[5] Tolerance and physical dependence develops with repeated use, and withdrawal symptoms appear soon after discontinuation of use. Heroin, the diacetyl derivative of morphine, is more lipid soluble and, consequently, penetrates the CNS faster than morphine. Heroin is typically abused by the intravenous route, although it is occasionally self-administered by the subcutaneous, intramuscular, intranasal, and smoked routes. Opioids are readily absorbed from the gastrointestinal tract, but many compounds, such as morphine, suffer significant first-pass effects. Heroin undergoes rapid hydrolysis in blood to the active metabolite, 6-acetylmorphine that in turn is hydrolyzed to morphine. Only traces of heroin are found in urine. After a 6.0-mg intramuscular dose of heroin, the mean percentage doses of heroin metabolites

excreted in urine were as follows: 6-acetylmorphine, 0.5%; free morphine, 5.2%; and conjugated morphine, 58.2%.[107] Opioids can be detected in urine at a cutoff concentration of 300 ng/ml (morphine equivalents) by immunoassay. Morphine and codeine can be confirmed at this same cutoff by GC/MS. Single doses of heroin can be detected for 24 to 48 hr as total morphine; however, 6-acetylmorphine can only be detected for 2 to 8 hr.[107] Multiple doses can be detected for slightly longer periods of time.

Heroin's chemical and metabolic instability limited early dispositional studies to the measurement of morphine in various body fluids. Leute et al.[108] reported detection of morphine by spin immunoassay in the saliva of patients undergoing methadone maintenance. Saliva concentrations of morphine were poorly correlated with urine concentrations. Gorodetzky and Kullberg[109] evaluated three different immunoassays for detection of morphine in plasma and saliva after heroin administration. They found that 5- and 10-mg doses of heroin could be detected, with high probability for 2 to 4 hr in plasma and 1 to 2 hr in saliva. Lower doses were not consistently detected at any sampling time. Goldberger et al.[110] developed a GC/MS assay for heroin and its metabolites in biological fluids, including saliva. Heroin, 6-acetylmorphine, and morphine were readily detected in plasma and saliva after intranasal administration. The estimated half-life of heroin in plasma from these studies was 6.3 min. Figure 18–6 illustrates the detection of heroin and metabolites in saliva and plasma after a 12-mg intranasal dose of heroin hydrochloride. Saliva concentrations were highly elevated over plasma concentrations through the first hour and continued to be elevated throughout the remaining detection period as a result of contamination of the oral cavity from intranasal administration of drug. However, both saliva and plasma had substantially shorter detection periods (4 to 8 hr) than urine (24 to 72 hr).[107,111] Recently, Jenkins et al.[78] used a similar assay to measure heroin and metabolites in saliva after smoked and intravenous heroin administration. Peak heroin concentrations in saliva ranged from 3,000 to >20,000 ng/ml after smoking 2.6 and 5.2 mg heroin base, respectively.

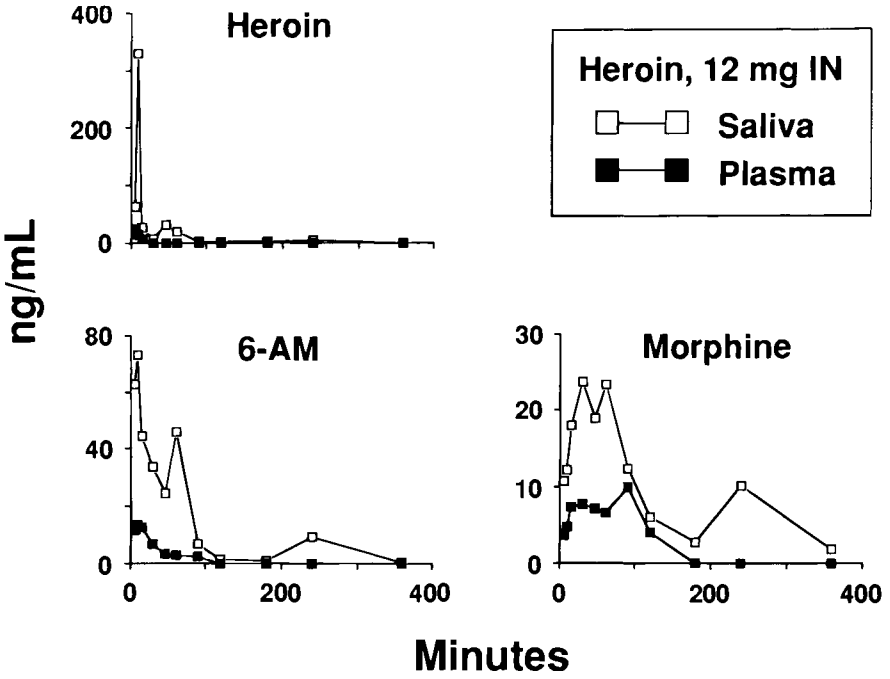

FIGURE 18–6. Concentrations of heroin and metabolites in saliva and plasma of a single human subject after administration of heroin by the intranasal (IN) route. 6-AM, 6-acetylmorphine.

Heroin saliva concentrations were much lower after intravenous administration, with a peak concentration of 30 ng/ml after 12 mg heroin hydrochloride. Contamination of the oral cavity during smoking was responsible for the high S/B ratios in the early period after drug administration. When saliva and blood heroin concentrations were >1 ng/ml, S/B ratios were >5. In contrast, S/B ratios after intravenous administration were <2. Because plasma and saliva drug detection times correspond more closely than urine to the time course of heroin-induced effects, saliva or plasma tests could add unique information in forensic investigations of cases concerned with drug-induced impairment.

Morphine is less lipid soluble than heroin and penetrates the CNS more slowly. There also seems to be a delay in the appearance of morphine in saliva. Figure 18–7 illustrates the hysteresis between plasma and saliva concentrations of morphine after 10-and 20-mg intramuscular doses of morphine sulfate. Individual data points represent mean determinations ($N = 6$) for morphine by a radioimmunoassay specific for free morphine. The same data are illustrated as S/P (total) ratios over time in Figure 18–8. It is clear that equilibrium was not reached between saliva and plasma for approximately 45 min after dosing. Saliva and plasma concentrations of morphine were detected by radioimmunoassay (cutoff = 1 ng/ml) for approximately 12 hr. After chronic dosing, Gorodetzky and Kullberg[109] reported a high probability of detecting morphine in plasma for at least 6 hr and in saliva for 3 to 4 hr after the last morphine dose. Detection times were shorter in the latter study because of the lower sensitivity of the immunoassay.

Codeine has been detected in saliva after oral[43] and intramuscular[111] administration. Sharp et al.[43] reported the mean S/P (total) ratio for codeine of 3.3 for three subjects after an oral dose of 30 mg, which corresponded to the predicted S/P (total) of 3.57. Cone[111] reported peak concentrations of codeine in saliva of 307.6 ng/ml and 183.9 ng/ml occurring at 0.5 to 0.75 hr, respectively, after doses of 120 and 60 mg of codeine. Plasma concentrations in the same subjects peaked at 0.25 to 0.5 hr at concentrations of 272.4 and 212.4 ng/ml, respectively. By 24 hr, concentrations in both saliva and plasma were in the range of 1 to 4 ng/ml. By 36 hr, saliva concentrations were below assay sensitivity. Figure 18–9 illustrates the time profile of codeine in saliva, plasma, urine, and hair for one subject after the administration of 120- and 60-mg intramuscular doses of codeine. The

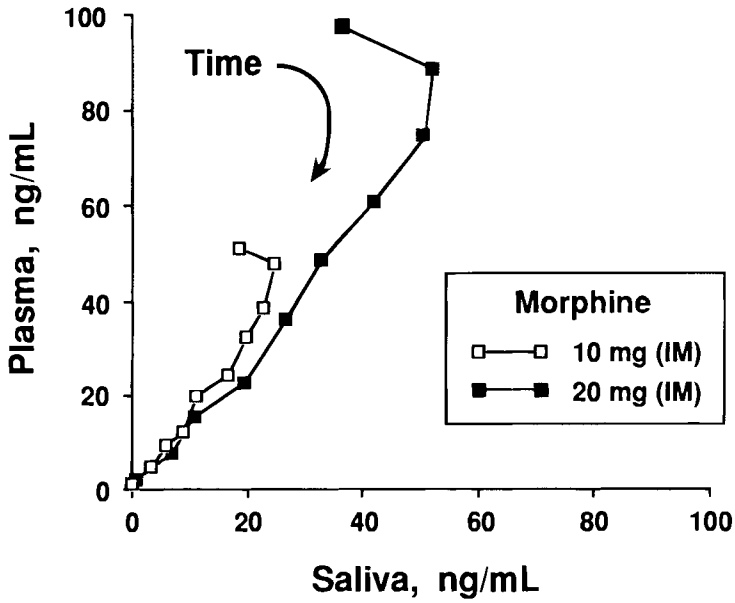

FIGURE 18–7. Hysteresis plot of mean morphine concentrations in saliva and plasma of human subjects ($N = 6$) after intramuscular (IM) administration of morphine.

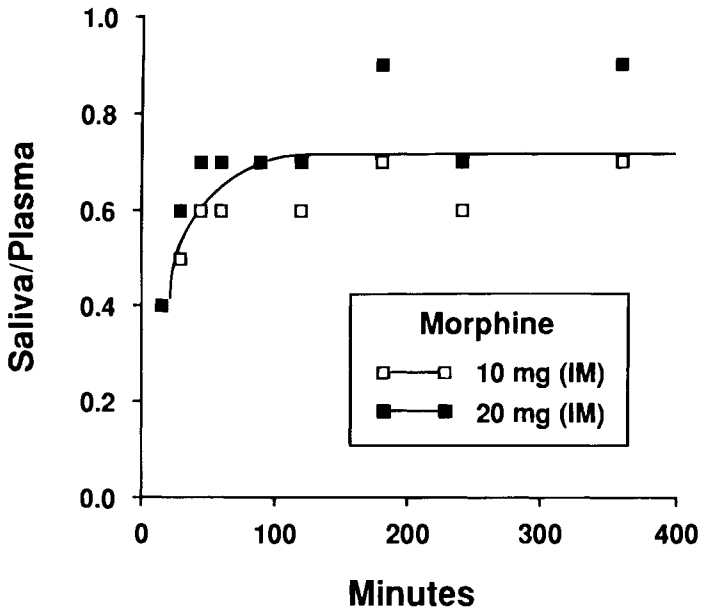

FIGURE 18–8. Changes in mean morphine S/P (total) ratios over time for human subjects ($N = 6$) after intramuscular (IM) administration of morphine.

various biological fluids and tissues demonstrated differing timelines for the detection of drug. For codeine, detection times for different biological fluids were as follows: hair ≫ urine > plasma > saliva.

Pholcodine is a codeine-like antitussive that is readily available in many countries. Chen et al.[112] reported its detection by high-performance liquid chromatography in saliva and plasma of a young male volunteer after oral administration of 60 mg of pholcodine. Saliva contained approximately three times the amount of pholcodine found in plasma, and this ratio seemed to be relatively stable during the elimination phase. Urine concentrations of drug were substantially higher than saliva and plasma concentrations. Terminal half-lives calculated from the three biological fluids were similar and averaged 48.6 hr.

Hydromorphone is a potent semisynthetic derivative of morphine that is abused by heroin addicts. It is excreted in saliva in a pattern similar to that observed for morphine. Ritschel et al.[113] reported that saliva hydromorphone concentrations were lower than plasma concentrations immediately after intravenous drug administration. After achieving peak concentrations, saliva concentrations declined in a similar manner to hydromorphone plasma concentrations. The S/P (total) ratio was lower (0.25) in the beginning, then attained a maximum of 2.32, followed by a constant ratio of approximately 1.0 in the elimination phase. The authors concluded that saliva was not useful for the estimation of pharmacokinetic parameters because of the lack of constant S/P (total) ratios during the distribution phase; however, estimations of terminal elimination half-life based on plasma ($t_{1/2}$ = 2.36 ± 0.58 hr) or saliva ($t_{1/2}$ = 2.12 ± 0.93 hr) data were similar.

Buprenorphine is a partial agonist of morphine that has been proposed for use as a therapeutic modality for the treatment of heroin addiction. In a comprehensive assessment of the acute and chronic effects of buprenorphine,[114,115] saliva and plasma concentrations were measured after sublingual and intramuscular administration. Cone et al.[116] reported that saliva concentrations after intramuscular administration were substantially less than plasma concentrations (S/P [total] ratio = 0.05 to 0.41), whereas they were highly elevated during the first 12 hr after sublingual dosing. Figure 18–10 illustrates saliva and plasma concentrations of buprenorphine by radioimmunoassay

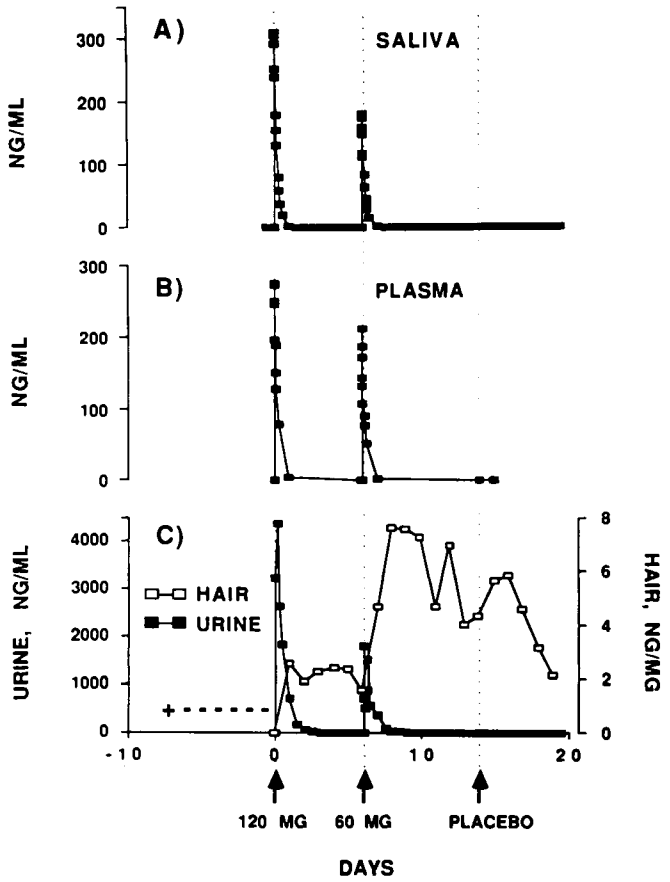

FIGURE 18–9. Time profile of codeine in (A) saliva, (B) plasma, and (C) hair (beard) and urine of a single subject after codeine administration (120 mg and 60 mg) and placebo administration. (C) Daily enzyme multiplied immunoassay technique-drugs of abuse test results for urinary opiates (+, positive; –, negative, 300 ng/ml cutoff) for the subject before entering the study protocol. (From Cone, E. J., *J. Ann. Toxicology*, 14, 1–7, 1990. With Permission.)

from a subject following a 1.0-mg dose by the intramuscular and sublingual routes of administration. The elevated concentrations after sublingual buprenorphine may be the result of a "shallow depot" of drug in the oral cavity.

Although buprenorphine S/P (total) concentrations were highly distorted from contamination by the sublingual route of administration, it seemed that measurement of saliva concentrations would be useful in monitoring treatment of heroin addicts maintained on sublingually administered buprenorphine. Figure 18–11 illustrates saliva concentrations determined for a heroin subject who was slowly inducted on sublingual buprenorphine over a 3-day period. This was followed by daily maintenance for a period of 15 days, then dosing every other day for a period of 16 days, whereupon drug administration was discontinued. Saliva measurements were obtained at 0700 hr before the subject's daily dose (trough concentration) and at 1200 hr (5 hr after dosing). Because of the blind conditions of the study, saliva collections were also obtained at 0700 and 1200 hr on days between buprenorphine doses (alternate dosing phase) when placebo was administered. The sawtooth pattern observed in Figure 18–11 was the result of contamination immediately after dosing, followed by a clearing of drug from the oral cavity. By 24 hr, buprenorphine saliva concentrations were in the 1 to 10 ng/ml range. During the alternate-day dosing, saliva concentrations were lower and reflected the change in dosing pattern. On one occasion, the subject missed an active dose, as

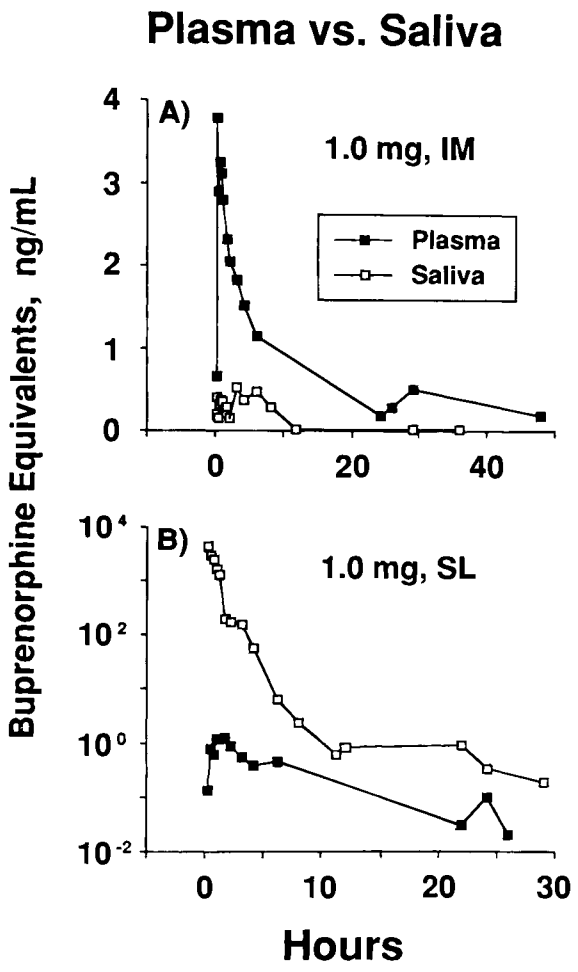

FIGURE 18–10. Concentrations of buprenorphine in plasma and saliva of a single human subject after sublingual (SL) and intramuscular (IM) administration of buprenorphine. Buprenorphine concentrations (equivalents) were measured by radioimmunoassay.

shown in Figure 18–11. Saliva testing for buprenorphine effectively revealed the missed dose. Upon discontinuation of buprenorphine, saliva concentrations declined to the level of assay sensitivity over a period of 5 days. Interestingly, saliva trough concentrations (collected before buprenorphine or placebo) were similar to plasma concentrations. Figure 18–12 illustrates saliva and plasma concentrations for Subject G (Figure 18–12A) and Subject I (Figure 18–12B), who participated in the same study, but received daily dosing throughout the study. These data suggested that the shallow drug depot in the oral cavity produced by sublingual buprenorphine contributed to the active pool of drug circulating in blood.

Meperidine was measured in the saliva of one subject after a single 80-mg oral dose (Muchlow et al.[40]). The study was designed to examine the effect of saliva flow and pH changes on S/P ratios of drugs primarily ionized at normal plasma pH (e.g., meperidine), compared with drugs primarily nonionized at normal plasma pH (e.g., antipyrine). Chewing waxed film was used to increase saliva flow. For meperidine, at a moderate saliva flow rate of 0.65 ml/min, the saliva pH was 6.7 and the S/P (total) ratio was 2.6. When the subject chewed vigorously on the waxed film, the flow rate increased to 2.77 ml/min and the pH increased to 7.3. The resultant S/P (total) ratio declined to 1.37. In contrast, antipyrine S/P (total) ratios measured under similar conditions did not change with alter-

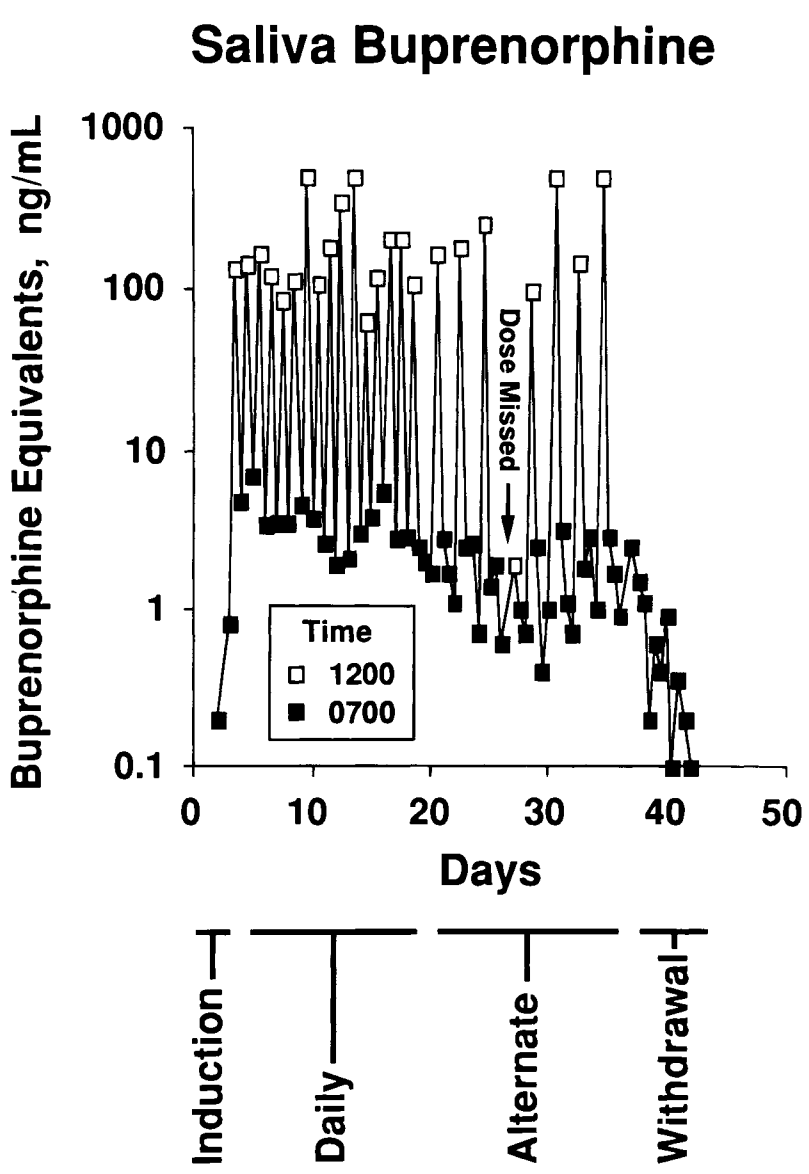

FIGURE 18–11. Concentrations of buprenorphine in saliva of a subject during induction, maintenance, and withdrawal from buprenorphine. Buprenorphine was administered by the sublingual route. During the induction period, the dose of buprenorphine was increased to 8 mg and maintained there throughout daily and alternate-day dosing periods. Saliva samples were collected just before buprenorphine administration (0700 hr) and approximately 5 hr after dosing (1200 hr).

ations in saliva flow rate and pH. It was concluded that saliva pH was the chief variable determining the saliva concentration of ionizable drugs.

Methadone is used extensively in maintenance therapy for heroin addicts. It has been detected in mixed saliva of humans after acute and chronic dosing[117,118] and in parotid rat saliva.[119] Lynn et al.[117] reported that unstimulated saliva concentrations exceeded whole blood concentrations of methadone by a factor of 3 to 10 after intramuscular administration. In contrast, Kang and Abbott[118] reported a mean S/P (total) ratio of 0.51 in two patients on maintenance doses of 30 and 90 mg/day of methadone. In the latter case, subjects rinsed their mouth with water before saliva sampling. Differences in blood and saliva collection conditions may account for the discrepancy between these two studies.

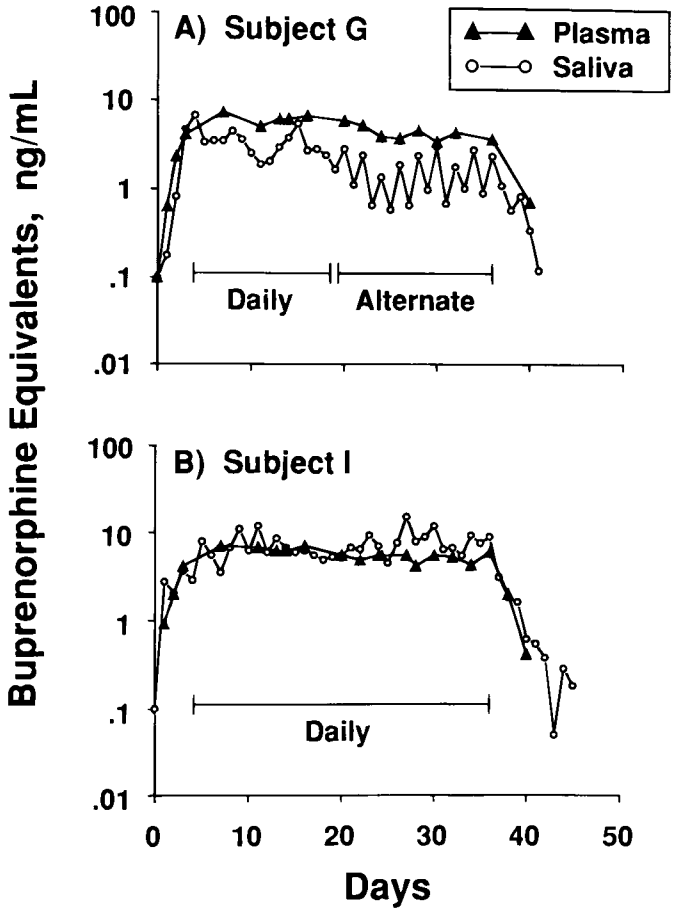

FIGURE 18–12. Concentrations of buprenorphine in plasma and saliva of two subjects obtained before sublingual buprenorphine dosing (0700 hr). Subject G (A) participated in a daily dosing schedule of buprenorphine, followed by an alternate-day schedule. Subject I (B) participated in a daily dosing schedule throughout the treatment study.

XII. PHENCYCLIDINE

Phencyclidine (PCP; 1-[1-phenylcyclohexyl]piperidine) is a dissociative anesthetic with a wide range of pharmacological actions, including CNS stimulant, depressant, hallucinogenic, and analgesic actions.[120] It is well absorbed by all routes and is commonly ingested or smoked with marijuana, or after impregnating parsley flakes with PCP in solution. However, it may also be administered orally, intranasally, or by injection. PCP is initially metabolized by hydroxylation, with further oxidative metabolism resulting in the formation of polar inactive metabolites. A relatively small portion (4 to 19%) of the original dose is excreted unchanged in the urine. PCP can be detected in urine by immunoassay and by GC/MS at a cutoff concentration of 25 ng/ml. Detection time in urine is dependent on pattern of usage. Single doses of PCP may be detected for as long as 5 to 8 days, whereas chronic users may remain positive for as long as 30 days.

PCP has been detected in saliva of laboratory animals[121] and humans.[122,123] S/S (total) ratios in rats ranged from 0.4 to 3.0, at a salivary pH of 8 to 9. PCP concentrations were determined by GC with nitrogen–phosphorus detection. In 100 emergency department patients suspected of PCP intoxication, 74 saliva samples and 75 of the paired serum samples were positive for PCP by radioimmunoassay.[122] In a kinetic study of PCP in healthy male volunteers, Cook et al.[123] reported the excretion of radiolabeled PCP in saliva after administration of small (subeffective) doses by the

intravenous and oral routes. Mixed saliva samples were obtained with each blood sample. During collection, saliva pH averaged 6.70 ± 0.17 (SD). PCP binding in plasma and saliva averaged 60.2% and 7.0%, respectively. Saliva drug concentrations tended to be higher than plasma concentrations, with an average S/P (total) ratio of 2.4, compared with the predicted ratio of 3.93. Although saliva PCP was highly correlated with plasma concentrations ($r = 0.921$), both inter- and intrasubject variability was high. The authors concluded that measurement of PCP in saliva could be useful for diagnostic purposes, but was not sufficiently accurate to predict specific plasma concentrations.

XIII. TOBACCO

Nicotine is an alkaloid that constitutes approximately 0.5 to 8.0% by weight of the tobacco plant, *Nicotiana tabacum.* The average cigarette in the U.S. contains 1.5% nicotine. Nicotine is both a transient CNS stimulant and a depressant. Initial exposure causes dizziness, nausea, vomiting, headaches, and dysphoria. Heart rate and blood pressure are increased. With repeated use, acute and chronic tolerance develops to many of the effects of nicotine.[124] Nicotine is rapidly absorbed in the unionized form during smoking and oral use, and can also be absorbed through the skin. The primary metabolites of nicotine are nicotine-1′-*N*-oxide and cotinine. Approximately 5 to 10% of a nicotine dose is excreted unchanged in urine, with 4% excreted as nicotine-1′-*N*-oxide and 17% as cotinine. Nicotine and cotinine can be detected in urine by immunoassay, GC, and GC/MS. Nicotine is excreted rapidly, whereas cotinine has a longer half-life and remains detectable for several days.

Nicotine is excreted in saliva, but is not considered to be a reliable marker of tobacco smoke exposure because of its short half-life (approximately 2 hr). In contrast, cotinine, the major metabolite of nicotine, has a half-life of approximately 17 hr.[125] Cotinine appears rapidly in both saliva and plasma after nicotine administration and saliva concentrations generally exceed corresponding plasma levels. Curvall et al.[126] reported that saliva cotinine concentrations were highly correlated with plasma concentrations ($r = 0.99$), with a S/P (total) ratio of 1.2 to 1.4. Furthermore, Curvall and Enzell[127] determined that saliva nicotine concentrations did not reflect actual uptake of nicotine as accurately as saliva or plasma cotinine levels. Jarvis et al.[128] compared 11 different tests for their ability to categorize smokers and nonsmokers correctly. Saliva cotinine measurements were the most accurate in determining smoking status, with a sensitivity of 96% and a specificity of 99%. Other measures, such as saliva nicotine, breath carbon monoxide, and plasma thiocyanate, were found to be useful, but less reliable than saliva cotinine. The authors indicated that measurement of saliva cotinine provided a basis for categorization of the smoking status of an individual that was substantially more accurate than self-report.

Recently, Etzel[129] reviewed 43 reports concerning the use of saliva cotinine measures to discriminate between smokers and nonsmokers. Generally, nonsmokers and those exposed passively had cotinine concentrations below 5 ng/ml, but heavy passive exposure may result in concentrations equal or greater than 10 ng/ml; infrequent active smokers or regular active smokers with low nicotine intake had cotinine concentrations between 10 and 100 ng/ml; and regular active smokers had cotinine concentrations in excess of 100 ng/ml. The ability to categorize individuals into different risk groups by means of noninvasive cotinine saliva measurements provided objective criteria for use in future studies of the health effects of environmental smoke exposure.

XIV. CONCLUSIONS

Saliva testing for drugs can provide both qualitative and quantitative information on the drug status of an individual undergoing testing. Self-administration by the oral, intranasal, and smoked routes often produces "shallow" depots of drug that contaminate the oral cavity. This depot produces elevated drug concentrations that can be detected for several hours. Thereafter, saliva drug concentrations generally reflect the free fraction of drug in blood. Also, many drugs are weak bases, and saliva concentrations may be highly dependent on pH conditions. These factors lead to highly variable S/P ratios for many drugs. Table 18–3 provides a compilation of experimental and theoret-

TABLE 18–3
Experimental and Theoretical S/P (Total) Ratios, Physical Constants, and Assay Methodology for Drugs of Abuse

Drug	pK_a	% Protein bound	Calculated S/P (total)	Measured S/P (total)	Correlation with plasma	Assay method	Ref.
Alcohol	NA	0	1.08	1.08	0.98	Enz.	16
				1.08	0.96	Enz.	15
			1.17 (Pr)	1.04 (Pr)	0.99	GC-FID	17
				1.03	NA	Enz.	18
Amphetamines							
Amphetamine	10.0	16	2.62	2.76	NA	GC/MS	21
Methamphetamine	10.1	(0)	3.98	NA	—	—	131
Barbiturates							
Amobarbital	7.95	57.6	0.87	0.352	NA	GC-FID	27
Barbital	7.8	25	0.79	0.999	NA	GC-ECD	30
Hexobarbital	8.2	65.9	0.93	0.34	0.92	GC-NPD	31
Methaqualone	2.5	92	0.25	0.11	NA	GC-NPD	43
Pentobarbital	7.8	40	0.79	0.36	0.98	NA	33
Phenobarbital	7.2	55	0.54	0.32	0.98	GC	33
				0.294	NA	RIA	35
				0.285	0.97	EIA	38
				0.30	0.94	GC	39
				0.31	0.92	EIA	
				0.31–0.37	0.91	GC/MS	36
				0.32	NA	RIA	
				0.43	0.91	GC	132
				0.45	NA	GC	37
				0.41	0.96	GC	40
				0.33	0.87	UV	41
Secobarbital	7.9	50	0.82	0.30	NA	GC	43
Benzodiazepines							
Diazepam	3.3	91	1.0	0.017	0.96	GC-ECD	53
				0.013	0.90	GC-ECD	55
				0.035 (Pr)	0.97	GC-ECD	51
				0.029	0.99		
				0.016	NA	RIA	47
				0.016	0.89	GC-ECD	52
				0.012–0.026	NA	GC-NPD	50
Nordiazepam	3.5	86–90	1.0	0.018	0.97	GC-ECD	51
				0.029	0.81	GC-ECD	52
Chlordiazepoxide	4.6	89	1.0	0.03	0.96	RIA	58
Nitrazepam	3.2	55	1.0	0.03–0.08	0.47	GC-ECD	59
Caffeine	0.6	15	1.0	1.0	0.96	RIA	64
				0.70	NA	Enz.	67
				1.18 (F)	NA	HPLC	62
				0.55	NA	GC/MS	36
				1.07	0.84	HPLC	66
				0.90	0.96	RIA	65
				0.71	0.94	HPLC	133
				0.79	NA	GC	69
				0.72	0.95	Enz. HPLC	68
				0.79	0.98	HPLC	63
Cocaine	8.6	NA	3.80	0.5–3.0	0.89	GC-NPD	75
Inhalants	—	—	—	NA	—	—	77
LSD	6.6	90	1.41	NA	NA	—	88

(table continues)

Drug	pK_a	% Protein bound	Calculated S/P (total)	Measured S/P (total)	Correlation with plasma	Assay method	Ref.
Marijuana							
THC	9.5	90	1.0	>1	0.79	GC/MS RIA	106
Opiates							
Heroin	7.6	NA	2.81	Est.0.1	NA	GC/MS	110
Morphine	8.1	65	3.49	0.2	NA	RIA	111
Codeine	8.2	7	3.57	3.3	NA	GC-NPD	43
				1.0	NA	RIA	111
Phylocodeine	NA	NA	—	3.0	NA	HPLC	134
Hydromorphone	8.1	7	3.49	0.3–2.3	NA	RIA	113
Buprenorphine	8.3	96	3.65	0.1	NA	RIA	116
Meperidine	8.6	36	3.80	1.4–2.6	NA	GC	40
Methadone	8.3	84	3.65	0.51	NA	GC/MS	118
				2.0	NA	GC-FID	117
Phencyclidine	9.2	60	3.93	>2.0	0.92	LS	123
Tobacco							
Nicotine	6.2	5	1.18	NA	—	—	—
Cotinine	NA	3	NA	1.2–1.4	0.99	GC-TSD	126
				1.13	0.93	GC-TSD	135
				0.83 (U)	0.96	HPLC	136

Note: S/P ratios were calculated as S/P (total) ratios, assuming negligible drug binding to saliva protein, a plasma pH of 7.4, and a saliva pH of 6.8, unless reported otherwise. Abbreviations used are—NA, not available; Enz., enzyme; Pr, parotid saliva; GC-FID, GC with flame ionization detection; GC-ECD, GC with electron capture detection; GC-NPD, GC with nitrogen–phosphorus detection; RIA, radioimmunoassay; EIA, enzyme immunoassay; UV, ultraviolet spectrometry; F, Free Fraction; HPLC, high-performance liquid chromatography; Est., estimated; LS, liquid scintillation assay; GC-TSD, GC with thermionio-specific detection; U, ultrafiltrate. Analytical details are provided in the references.

ical S/P (total) ratios determined for several drugs. Estimation of theoretical S/P (total) ratios for acidic and basic drugs were based on the Henderson–Hasselbach equation.[130] Saliva pH was assumed to be 6.8, unless reported otherwise by the investigators. Generally, there was a high correlation of saliva drug concentrations with plasma, especially when contamination of the buccal cavity was eliminated. Assay methodology varied considerably, indicating that saliva assays could be readily developed from existing methodology.

There are many potential applications for saliva testing for drugs in the general areas of drug detection, treatment, and forensic investigations (Table 18–4). Saliva drug tests can reveal the presence of a pharmacologically active drug in an individual at the time of testing. Significant correla-

TABLE 18–4
Potential Applications of Saliva Testing for Drugs of Abuse

Detection
- Employment testing
- Health exams and insurance
- Epidemiology studies

Treatment
- Diagnosis
- Compliance
- Abstinence

Forensics
- "For cause" testing
- Driving under the influence of drugs (DUID)
- Evidence investigation

tions have been found between saliva concentrations of drugs and behavioral and physiological effects. It is anticipated that, over the next decade, saliva testing for drugs will develop into a mature science with new applications.

Future research is needed in pharmacokinetic modeling of drug and metabolite excretion in saliva. Also, to evaluate the potential of saliva for use as a matrix for workplace drug testing, data on detection times of illicit drugs and their metabolites in saliva are needed and saliva/urine ratios documented.

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Chapter 19

DRUG MONITORING: MODERN APPROACHES TO QUALITY ASSURANCE

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CONTENTS

I. INTRODUCTION

A large number of products used daily by people, such as foods, drugs, cosmetics, and medical devices, are regulated by the U.S. Food and Drug Administration (FDA). With that concept, the FDA may be considered as a consumer protection agency. The Medical Device Amendments to the Food, Drug, and Cosmetic Act (FD&C Act) were enacted in 1976. The Amendments directed the FDA to regulate medical devices to ensure their safety and effectiveness. In general, for medical devices, the agency looks at risks/benefits as they relate to the safety and effectiveness of the devices. In doing so, close attention is given to benefits as they relate to public health, and the risks that may occur from device malfunction, failure, or poor performance. Clinical laboratory devices in the U.S. can be marketed for research use, investigational use, and *in vitro* diagnostic (IVD), clinical use. Trying to bring these devices under proper and balanced regulatory process has been an

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ongoing challenge to the agency. FDA activities are not confined to product review only. Some resources and activities are directed toward compliance in manufacturing practices and postmarket surveillance of commercially distributed products.

The FDA's Center for Devices and Radiological Health (CDRH) looks at devices developed by new and/or old technology and evaluates them with regard to claimed intended uses and performance parameters. Before medical devices enter the market place, the FDA evaluates submissions from manufacturers to ensure that their devices are safe and effective. The FDA regulatory process as it relates to *in vitro* therapeutic drug monitoring devices is presented in this chapter. This includes an explanation of differences between the two types of pathways available to the manufacturers who desire to commercially distribute IVDs either through premarket notification (510 (k)) or through premarket approval (PMA) applications.

II. DEVELOPMENT AND EVALUATION OF NEW DRUGS

To market a new drug, a manufacturer must first obtain FDA approval of a new drug application (NDA). Before submitting an NDA, the manufacture must conduct preclinical (animal) tests and clinical (human) studies designed to demonstrate that the drug is safe and effective for its intended clinical use.

Under the FD&C Act of 1938, Congress passed the law prohibiting the shipment of any new drug for which the FDA has not approved an NDA. To enable sponsors of new drugs to conduct the necessary studies, Congress permitted the FDA to exempt a drug from this prohibition and certain other requirements of the Act for the limited purpose of conducting clinical studies. This is known as the investigational new drug (IND) process.

The research process begins with a pharmacotherapeutic concept. Generally, the investigator has a biological mechanism to regulate and/or a chemical lead to follow, either of which is related to the particular target disease. When a chemical lead is established, interesting compounds are prepared by chemists/biochemists with sophisticated techniques in a broad range of test systems, from subcellular levels to laboratory animals. The selected compounds with potential therapeutic applications are then subjected to toxicological testing that usually includes determination of lethal doses in at least three species of animals involving pathological studies for the detection of organ toxicity. At this point, an estimate is made of a therapeutic ratio by comparing an effective dose and toxic doses in the same species of animal models.

Compounds chosen for human study must first undergo additional toxicological evaluation and pharmacokinetic studies to determine how the drug is metabolized and excreted in animals. Compounds with useful potential are studied for clinical pharmacological applications. Selections are made among several analogs based on best estimates of therapeutic indices, absorption, and duration of desired effects. Finally, the drug must be prepared in a form that is stable and bioavailable.

Preclinical research data on the chemistry, pharmacology, and toxicology of the drug selected for human studies is then submitted to the FDA in a document called a "Claimed Exemption for an Investigational New Drug." The 1938 FD&C Act states that research on a new drug in human beings can be done only after a Notice of Claimed Exemption for an IND has been submitted to the FDA. The main purpose of the IND is to protect the safety of subjects in the investigational studies, while developing the data on safety and effectiveness necessary for marketing of the drug.

Once an IND is approved by the FDA, and the Institutional Review Board of the institution in which the drug will be studied approves the protocol, then clinical trials in humans can begin. This process is split into phases: Phases I to IV.

A. PHASE I

Phase I is initial safety studies on a new therapeutic drug, usually conducted in normal human volunteers. A thorough review of preclinical data, along with pharmacological and toxicological aspects of study, is recommended. After satisfactory completion of these studies, the drug is admin-

istered to a few volunteers, usually healthy subjects, but sometimes to patients also. This provides information on drug metabolism, excretion, and estimated potential of the drug for producing adverse effects. Generally, the drug administration begins at very low single doses, and the dosage is increased gradually. If single-dose studies show favorable effects, multiple-dose studies are initiated. In Phase I, emphasis is placed on developing the safety profile on the new drug. Throughout these studies, a variety of laboratory tests (e.g., clinical chemistry, hematology, pathology, immunology, and urinalysis, etc.) are conducted to detect any adverse effects. In cases of adverse effects (limiting the use of the drug), the drug study is stopped at this stage.

B. PHASE II

If Phase I study is successful, Phase II studies are conducted to evaluate safety and efficacy in selected populations of patients with the disease of condition the drug is claimed to treat. Generally, small numbers of patients are investigated intensively with specific studies designed for the type of drug and the disease to be treated. The dosage protocols begin with single-dose studies, followed by multiple-dose studies. This phase focuses on dose–response type of patient, frequency of dosing, and other clinical pharmacokinetic parameters. The main purpose of Phase II studies is to determine whether the drug has the expected therapeutic effect, the proper dose range producing the desired effect, and whether any adverse effects are observed that would limit the usefulness of the drug. Lack of efficacy in this phase can result in termination of the drug studies. Numerous factors are considered, such as the toxicological profile on other safety parameters and the expected results of the drug in patients.

C. PHASE III

Phase III studies are conducted after the drug efficacy is demonstrated. (These studies are conducted in patient populations for which the drug is eventually intended for clinical use.) In this phase, additional safety and efficacy data are generated in a relatively large number of patients in both controlled and uncontrolled trials. Most of the information generated in these studies are summarized in the labeling (package insert) of the drug. The Phase III studies are designed for a clinical site analogous to the environment in which the drug is intended to be used. Generally, masked comparative studies are conducted with placebo or a standard drug, and greater attention is given to observing effects and any potential interactions of the new drug with other medications of conditions. Satisfactory completion of well-controlled Phase III studies leads to collecting the clinical pharmacokinetic and toxicological data for an NDA and submitting them for approval to the FDA's Center for Drugs Evaluation and Research (CDER). In this review and approval process, the FDA determines whether the drug is safe and effective, can be manufactured consistently, and whether the benefits outweigh the risks. The FDA also approves the contents of the drug (package insert) that is distributed to the prescribing physician. Part of the NDA approval process is evaluation and approval of the labeling. The label typically provides information concerning dosages, directions of administration, conditions for which the drug is effective, contraindications (disease conditions in which the drug may be harmful), and warnings about known or suspected side effects and adverse reactions.

D. PHASE IV

Clinical studies are conducted for additional safety and efficacy after marketing. The operating term of this phase is "postmarketing surveillance" (e.g., a period after marketing). This may involve different formulations, dosages, durations of treatment, drug interactions, etc. In this phase, other types of patients (e.g., race and new age group) can be studied. The important aspects of these studies are to obtain detection and definition of previously unknown or inadequately identified adverse reactions and their related risk factors.

E. NEW DRUG APPLICATION

In the U.S., FDA approval is required for a new drug for the indications specified in the NDA. Usually, an NDA is submitted to the FDA at the completion of Phase III clinical studies to show that

safety and efficacy have been met. The NDA must include a collection of sufficient data to allow the agency to form the judgment that the drug is safe and effective. In special situations, wherein a drug is purported to be used to treat a rare disease or a very serious disease for which no satisfactory treatment is available, an NDA may be submitted at earlier stage of Phase III studies.

The drug efficacy is a relative concept that refers to the ability of a drug to bring out a beneficial clinical effect. Efficacy can be assessed at one or more levels of physiopharmacology (e.g., subcellular, cellular tissue, organ, and whole body). Safety, on the other hand, is the concept that refers to freedom from harm or damage resulting from adverse reactions that occur as a result of drug or nondrug use. Safety is usually measured in one or more combinations of physical characteristics (e.g., vital signs, neurological, ophthalmological, and general physical), laboratory diagnostic (e.g., clinical chemistry, hematology, and immunology), diagnostic medical tests and procedures (e.g., EKG and pulmonary), psychiatric evaluations, and observation of clinical signs and symptoms. Safety and efficacy are judged in relation to the specific clinical conditions for which the drug is intended to be used. A typical NDA contains all of the chemical, pharmacological, clinical, and manufacturing data that have been collected in Phases I to III (Table 19–1).

III. REGULATION OF MEDICAL DEVICES

The FDA has, among its responsibilities, the regulation of medical devices. A medical device is defined as "an instrument, apparatus, implement, machine, contrivance, implants, in vitro reagent or other similar or related article intended for use in the diagnosis of disease or other conditions, or in the cure, mitigation, treatment, or prevention of disease in man." The Medical Device Amendments required the FDA to classify all medical devices, including IVD devices. Although most drug monitoring *in vitro* devices are regulated by the FDA's CDRH, the ones used in the manufacture of *in vivo* drugs are regulated by the FDA's CDER. Before enactment of the 1976 Amendments, the FDA regulated medical devices under the definition of drug. The Medical Device Amendments, however, defined IVDs as, "In vitro diagnostic products are those reagents, instruments, and systems intended for use in the diagnosis of disease or other condition, including a determination of the state of health in order to cure, mitigate, treat, or prevent disease or its sequelae." Such products are intended for use in the collection, preparation, and examination of specimens taken from the human body. These products are devices as defined in Section 201(h) of the FD&C Act, and may also be biological product subject to Section 351 of the Public Health Service Act.[1]

The Medical Device Amendments of 1976 and the Safe Medical Devices Act of 1990 ([SMDA'90] with this Act being the major revisions of the 1976 Amendments) defined three classes of devices according to the level of regulation needed to ensure that a device is safe and effective.[2,3] Medical devices that existed before May 28, 1976 were classified into one of the three classes (Table 19–2). Medical devices, including IVD devices intended for clinical use, must be cleared or approved by the FDA. FDA authority for the regulation of devices is based on the two legislative

TABLE 19–1
Contents of the Overall Summary of an NDA

1. Proposed labeling
2. Drug's pharmacological class, scientific principles, intended use, and expected clinical benefits
3. Chemistry, manufacturing, and controls
4. Nonclinical pharmacology and toxicology
5. Human pharmacokinetics and bioavailability
6. Marketing history, if any
7. Clinical data, including statistical analysis
8. Conclusion, including benefit-to-risk considerations, proposed additional studies, and plans for postmarketing surveillance

TABLE 19–2
Classification of Devices

Categories	Level of controls
Class I	*General:* Devices require the lowest level of regulation. These devices are subject to "general control" requirements, which include manufacturer registration, device listing, "premarket notification," and GMP. Examples of Class I IVD devices are all of the preamendment and those after, postamendment, chromatographic and spectroscopic devices intended for general purpose use.
Class II	*Special:* Devices for which general control alone are insufficient to provide reasonable assurance of safety and effectiveness, and for which sufficient information exists to establish special controls to provide this assurance. These devices require in addition to general controls, special controls (e.g., guidelines, performance standards, postmarket surveillance, etc.). Until a special control is established by regulation, only general controls apply. Examples of Class II IVD devices are digoxin testing systems.
Class III	*PMA:* Devices in this class require the highest level of regulation, because the general and special controls are insufficient to provide a reasonable assurance of safety and effectiveness. This must be demonstrated by laboratory testing and clinical studies. Examples of Class III *in vitro* devices are cyclosporine testing systems.

acts stated previously. Two regulatory pathways are available to a manufacturer who desires to market a device commercially: the PMA and the premarket notification, also known as the 510 (k)—named after Section 510(k) of the Act.

In vitro devices in comparison to other types of medical devices are unique in the area of device regulation, because they have their own specific labeling regulation found under 21 CFR 809.10. These regulations delineate the format and content of the information that the manufacturer must provide to the user (information required to support device labeling and premarket submissions). The labeling regulation is divided into 15 major components (Table 19–3). The *in vitro* labeling regulations apply to both the PMA and 510(k) submissions. The clinical laboratory evaluation program requires product labeling that will provide adequate information about the product's use and expected performance. Of these various elements, the most important is the intended use. The intended use for a product determines the type of review and the data requirements that will likely be required in the course of review.

The manufacturer's first step in marketing a new device is to notify FDA at least 90 days in advance of its intent to market. Premarket notification is required of all devices marketed in the U.S. for the first time and of devices modified enough to affect their safety and effectiveness. In the last case, the manufacturer has the responsibility of determining if a change in a device is significant enough to require FDA review.

TABLE 19–3
Labeling for IVD Products

1. The proprietary name and established name
2. Intended use and indications for use
3. Summary and explanation of the test
4. Principles behind the operation of device procedures
5. Information on reagents
6. Information on instruments
7. Information on specimen collection and preparation
8. Procedures
9. Results
10. Limitations of the procedures
11. Expected values
12. Specific performance characteristics
13. A bibliography of pertinent references
14. Name and place of business
15. Date of the package insert

A. DRUG MONITORING DEVICES

Therapeutic drug monitoring IVD devices are classified as a total system (instrument and reagents) under a specified drug analyte. The analyte classification determines how the performance data are reviewed (Table 19–3). Most drug monitoring devices are Class II devices classified under a particular drug analyte category. For example, digoxin, lidocaine, procainamide, phenytoin, phenobarbital, amitriptyline, nortriptyline, vancomycin, methotrexate, etc. are classified as Class II analyte systems in conjunction with a specified instrument or immunoassay technology. Drug monitoring immunoassay system performance depends on several factors, including the quality of the antibody, the nature of the label, and the time of incubation. Therefore, the current IVD drug monitoring review criteria are based on the specific analyte application. Each analyte is specific in terms of its intended clinical use and risks associated with the interpretation of results under the laboratory certification programs, such as Clinical Laboratory Improvement Amendments and College of American Pathologists.

In the semantic framework of the FDA device evaluation process, a determination of whether a device is considered old or new is made based on whether it was in the commercial marketplace at the time of passage of the Medical Device Amendments of 1976. Therefore, most drug monitoring devices represent intended uses, for which it is likely that a manufacturer would be able to identify a predicate device (a device already in commercial distribution), a requirement for the premarket notification 510(k) process. Whether used as a total analyte system or in combinations as kits or systems, most such products currently submitted to the FDA for IVD use have in common clear intended uses, descriptions of analytes/metabolites, instructions for use, and other labeling that includes detailed information on product performance.[4]

Manufacturers must submit a premarket application to the FDA before commercially introducing the IVD device intended for use in a clinical laboratory. Successful completion of the regulatory review processes results in FDA clearance or approval, and ultimately commercial availability of quality IVD tests for use in the clinical laboratory. The 510(k) process is used to introduce new versions of products that are already in the marketplace (predicates) and is an administrative paper review. The PMA process is used to introduce brand-new products and is more than a paper review requiring clinical investigations, data, and clinical trials.

B. PMA APPLICATIONS

The FDA's Division of Clinical Laboratory Devices (DCLD) reviews fewer IVD PMA submissions, generally 1 to 2 dozen in various stages of review activity in the course of 1 year. (These PMAs are in various specialty areas: Clinical Chemistry, Toxicology, Hematology, Pathology, Immunology, and Microbiology.) PMA of a medical device must be obtained when there is no similar (predicate) device in commercial distribution, the device has a new intended use, or it has new high complexity technological features that raise new types of safety and effectiveness issues. The PMA process is intended to be a complete scientific and clinical evaluation of new or significant risk devices in terms of their safety and effectiveness. PMA submissions represent fundamentally new products with new concerns of safety and effectiveness. To support a conclusion of safe and effective use and performance, a sponsor must submit valid scientific and clinical data. Valid data may include appropriate clinical protocols, target population selection, control groups, appropriately recognized confirmatory tests, and statistical analysis, among other things (Table 19–4). After the PMA is reviewed by the FDA staff and expert advisory panel members, the sponsor makes a presentation before an advisory panel in an open public hearing. The advisory panel then gives its recommendations to the FDA. The PMA process has a regulatory period of 180 days. If, however, additional data are required or if there are major deficiencies, the clock stops until the requested information is provided by the sponsor of the PMA. An FDA-approved PMA is considered as an individual license that allows only the applicant to market that device. Examples of IVD drug monitoring devices that required individual PMAs are cyclosporine testing systems.[5]

TABLE 19–4
Contents of the Overall Summary of a PMA Application

1. Device generic/trade name
2. Intended use and indications for use
3. Device description—technology/methodology
4. Alternative practices and procedures
5. Potential adverse effects on health
6. Marketing history, if any
7. Summary of nonclinical studies (nonclinical studies conducted to determine the purity and specificity of the reagents, as well as assay performance characteristics)
8. Summary of clinical studies (clinical studies conducted in medical institutions and sponsor's facilities; this may involve retrospective and/or prospective clinical samples)
9. Results of statistical analysis
10. Conclusions drawn from studies

C. PREMARKET NOTIFICATION 510(k)

510(k) notification involves new versions of products already on the market before the Medical Device Amendments of 1976. DCLD currently handles approximately 1000 IVD 510(k)s per year. The focus of these reviews is analytical performance characteristics and/or other scientific data to demonstrate that the new/modified device is "substantially equivalent" to the predicate. Review of most 510(k) submissions is based on evaluation of an analysis of the fundamental analytical principles of a test, including accuracy, precision, sensitivity, specificity, interpretations, and impact of results on patients. 510(k) review is entirely a paper review; FDA does not regularly submit these products to direct (actual) laboratory evaluation, and the agency therefore has no hands-on experience with most devices under the 510(k) program.

The basic concept behind a 510(k) clearance is that the device submitted for review is very much like others already in commercial distribution in the U.S. Two important factors enter into arriving at such a conclusion: substantial equivalence and predicate device. The 510(k) application must show that the device is substantially equivalent to one or more predicate devices legally marketed in the U.S. A predicate device is often one that was legally marketed on or before passage of the Medical Device Amendments of 1976. However, a number of "me-too" predicate devices were first introduced in the marketplace after 1976. They have been declared by the agency to be substantially equivalent after a review and evaluation of a 510(k) application. Substantial equivalence for drug monitoring IVD devices is usually determined if the device being reviewed is found to have the same drug analyte, intended use, methodological outputs, and performance parameters, compared with the predicate device. Typically, in a 510(k), the sponsor describes the device analyte/metabolite, device methodology/technology, its intended use for monitoring in a hospital/physician/point-of-care setting, performance characteristics as shown in labeling and advertisements. The sponsor compares and contrasts the review device with similar legally marketed devices with supporting comparable data; or, in the case of modified devices (new matrices and new monitoring ranges), data to demonstrate that the sponsor has considered the effect of the change on safety and effectiveness, and has changed the labeling accordingly. In accordance with the device law of 1990 (SMDA'90), the 510(k) requires either a statement that the product is safe and effective or a statement that this information is available from the manufacturer upon request by the public.

The FDA evaluates the overall information in the 510(k) to determine if the device is equivalent to a predicate, in which case the device is "cleared" and the sponsor is free to market it. On the other hand, if it is new or quite different, then the device is considered to be not substantially equivalent (NSE) and is recommended for a PMA. Generally, in a typical modified drug monitoring IVD 510(k), the sponsor would provide the type of drug analyte, device technology features (e.g.,

radioimmunoassay, enzyme immunoassay, and fluorescence polarization immunoassay), reagent components (e.g., enzymes, receptors, antigens, and antibodies), detection systems (radiolabel and chemiluminescent), and overall technology assessment of the total system, compared with other cleared devices. The ability of a specified drug analyte-based technology system to perform equivalently to an already marketed device is considered to be adequate for 510(k) clearance. For example, the FDA has declared a number of drug monitoring devices using monoclonal antibodies substantially equivalent to those using polyclonal antibodies.

In situations where a brand-new drug analyte–technology system is declared as NSE, the sponsor has the right to petition for a reclassification into Class I or II. In evaluating new drug analytes or matrices, there is often lack of a "gold standard" against which to judge performance, and compromises are made in performance yardsticks. Data collection to establish safety and effectiveness is often complicated by either overt or latent bias; therefore, in new 510(k) matrix applications, determining acceptable limits for minimum performance can be difficult and challenging.

IV. DRUG MONITORING: CONCEPTS AND APPLICATIONS

Therapeutic drug monitoring is now a routine part of laboratory testing. Therapeutic drug monitoring is useful for those drugs exhibiting a pharmacological effect that is related to plasma concentration when the effects are not easily assessed by routine clinical observation. Serum levels of many of the therapeutic drugs administered to patients should be frequently determined, because of the possible toxic side effects caused by greater variability of utilization among the different individuals.[4]

Therapeutic drug monitoring becomes effective for drugs that have a narrow therapeutic index. Digoxin is an example of such a drug; it is very effective at enhancing the force of myocardial contraction of a failing heart, yet it can be very toxic when the blood level exceeds the narrow critical limit. Coupled with the narrow therapeutic range is a marked patient variability in response to the same dosage of drug. Although there is a direct relationship between toxic manifestation and circulating drug level, there may be an overlap of the therapeutic and toxic ranges. Intoxication symptoms are sometimes indistinguishable from the original heart condition for which the drug was prescribed. Digoxin levels are frequently monitored in critical patients and the dose adjusted based on the blood level to ensure safe and effective use. On the other hand, propranolol has a wider therapeutic index with a wider margin of therapeutic range of safety, and toxic manifestations are not clearly related to serum concentration. The correlation between symptoms of toxicity and plasma propranolol concentrations is variable.[6] Propranolol produces toxicity at larger doses; therefore, the standard dosing guidelines are more than adequate to provide safe dosage. Generally, for propranolol instead of frequent detection of serum levels, measurement of blood pressure is considered to be a simple, rapid, economical biological monitor for the effectiveness of the drug.

New IVD drug monitoring device technologies are available that are accurate, specific, and sensitive enough to detect small amounts of drug down to microgram and picogram levels. These technologies are able to measure the active drugs and pharmacologically important metabolites. This becomes important when the concentration of drug in the serum reflects the effective concentration of drug at the receptor site and when the development of a tolerance for the drug at the receptor site does not occur. In this situation, the therapeutic range needs to be well defined, with good correlation between serum concentration and drug efficacy.

Drug monitoring immunoassays of the 1990s incorporated features such as "walk-away" automation with batch and random-access capabilities. Major improvements were in multiple-drug analyte profiles (e.g., antiepileptics), laboratory information systems (LISs), and application of software for system interface and pharmacokinetics. Some of these improvements include better shelf-life with less disposables and lesser calibration. There were tremendous improvements in automated front-end hardware for sampling with positive sample identification interface capability with LISs, microsampling, profiles, and stat capability. It is expected that future drug monitoring immunoassay systems will incorporate instrument design features for minimum or self-maintenance with built-in

quality control (QC)/quality assurance (QA). There will be reagent stability check features for lot changes and on-board reagent storage. Future systems would be capable of minimizing the carry-over sampling problems and on-board dilution for out-of-range results automatically performed along with "hook effect" checks and the results automatically adjusted for dilutions thus checked and recorded.[7]

Current heterogeneous immunoassay systems offer versatility. Some of these systems can measure both small and large drug analytes. The physical separation steps applied in these systems eliminate interfering substances present in the patient's sample before measurement. Heterogeneous drug immunoassay systems—with less labor-intensive, less time-consuming, simple washing, and separation steps, yet capable of integrating into multichannel analyzers (e.g., clinical chemistry and hematology)—are considered desirable.

Homogeneous drug immunoassay systems are generally used for small drug analytes and for small sample size, with low reagent volume consumption. These systems are useful for size differences between unbound antigen (small) and antigen-bound antibody complex (large). Some assay systems require sample pretreatment to eliminate interferences in the patient's sample by minimizing high background signal or competition with the binding site (e.g., digoxin assay requiring acid precipitation or column pretreatment). Generally, small drug analytes present in relatively high concentration are measurable by heterogeneous immunoassay systems.

A. 510(k) EVALUATIONS

Most drug monitoring 510(k)s as presented to the FDA include clinical as well as analytical sample data that is reviewed to evaluate the test's analytical performance within a clinical framework. The FDA review requires meticulous attention to details of analytical performance parameters and significant information for the proposed labeling. For a quantitative drug monitoring test, minimum requirements for review of a 510(k) generally include: (1) information on bias comparing the new method by linear regression to a reference and/or a predicate method; (2) information on precision using an analysis of variance analysis to allow comprehensive assessment of components of variation; and (3) testing designed to evaluate analytical sensitivity and analytical specificity.

Accuracy is the extent to which measurement is close to true value. The reference method determines the true value. Usually, sponsors identify in their premarket submissions a reference or predicate method applicable to their product methodology. For accuracy, comparison with the reference method is reported with systematic bias at each selected clinical decision point with confidence intervals. For those assays wherein a reference or definitive method is not available, alternative method comparison data are acceptable.[4] There are two approaches to an evaluation of the accuracy of the drug monitoring test. The first approach is to analyze a reference drug material with a known concentration level by the method under evaluation. The reference material provides a specific target value for the method it is to be compared with. Repeat analysis of the material will indicate the level of bias. The second approach is to analyze known patient samples in parallel by two methods: (1) the method under evaluation and (2) a reference or predicate method.

In the absence of reference or definitive methods for certain drug analytes, generally, indirect approaches for accuracy are commonly used. These include recovery, linearity, parallelism, interference, carryover, and calibration stability, In premarket submissions, accuracy is also assessed by comparative studies to other legally marketed predicate device technologies that have well-characterized documented parameters published in refereed journals.

For precision, QC materials covering two to three different levels are selected within, below, and above suggested reference range of the assay. Generally, these QC materials are analyzed in replicate of 20. The results are expressed as the mean, standard deviation, and coefficient of variation. The within-run precision (±5 to 10% range) is considered acceptable for the clinical usefulness of the device. In addition, it is recommended to compare patient results on different lots because QC materials may not always show the same variation as patient sample because of the matrix effect of lyophilization of QC material.

The design of the drug testing system and selection of reagent components determine the extent of imprecision. Both the antibody concentration and the affinity constant used in the type of reagent system have some bearing on the precision. For a system using antibody coated for the solid phase, the amount of antibody coating will determine the extent of antigen binding. The coating process for the competitive assay (limited amount of antibody coated) may contribute to imprecision; thus, uncoated or partially coated tubes will contribute to falsely low values. Sometimes, the antibody conjugate contributes to the amount of signal generated, which in turn affects the sensitivity and precision of the assay. The type of substrate used may also contribute to the enzymatic reaction and the final color development or fluorescence. The actual assignment of the calibrator's value and stability is another dimension that may contribute to imprecision. Other testing components—such as type of separation technique, incubation temperature, diluents, wash solutions, quench solutions, QC materials, pH, and ionic strength—may affect the precision. The detecting device component may also introduce imprecision (e.g., spectrophotometry and fluorometry). The curve-fitting models for data management systems could affect the total precision. Sometimes, the use of the logit–log model to linearize the calibration curve could have imprecision at both ends of the curve. Sample-related, nonspecific interference factors, as well as specific interferences such as heterophilic antibodies, may affect both precision and accuracy. Other factors are sample carryover and high-dose hook effect.

The test of specificity is an integral part of the development of a new drug monitoring assay. There is always a potential for interference from both endogenous physiological conditions and exogenous substances. Drug immunoassays identify known interfering substances and determine the level of specificity of the total test system. This includes endogenous substances (such as hemoglobin, bilirubin, and lipids) and exogenous substances (such as commonly administered drugs or drugs most likely to be used by the particular patient population). Other nonspecific sample-related interference substances may be present that cross-react with the antigen for the antibody. For example, in the digoxin assay, digoxin-like immunoreactive substances (DLIS) have been reported to cause false-positives in pregnant women, neonates, and patients with liver or renal diseases.[6] Some immunoassay systems recommend use of a column separation pretreatment step to remove DLIS.

To test for specificity, specimens containing no drugs are tested first to verify that no endogenous plasma constituents cause interference. The "blank" specimens can be from patients, as well as healthy individuals. Generally, known drugs commonly prescribed with the target drug are tested and are shown either not to interfere or the percentage extent of interference.[5]

The main objective of the drug testing system design is to maximize the accuracy (e.g., to minimize the error in), the measurement of analyte concentrations in a specified range. In the near future, the increasing use of nonisotopic immunoassay systems is likely to have the greatest impact on standardization and total quality assurance of the system. Good manufacturing QA programs play a significant role in the development and validation of drug monitoring test systems, not only from a bias and precision point of view, but also evaluation aspects of performance, such as robustness toward sensitivity, specificity, etc. that can impact on the reliability of these systems once placed in laboratory practice. The sponsors of drug monitoring premarket submissions can play a key role in quality planning design stage of the device by using existing, well-accepted protocols, such as the National Committee for Clinical Laboratory Standards (NCCLS) guidelines (e.g., EP5-T2—Precision Performance, EP7-P—Interference Testing, EP9-T—Method Comparison, EP10-T2—Evaluation of Clinical Laboratory Methods, and C28-A—Reference Intervals).

V. GLOBAL HARMONIZATION OF QUALITY SYSTEM STANDARDS AND RELATED REGULATIONS

The Global Harmonization Task Force has membership from Europe, Canada, the U.S., Japan, industry, and government. The objective of this task force is to facilitate the move toward harmonization of international standards and to facilitate worldwide marketing of new products through

less duplicative testing to meet the standards of different countries. The Harmonization Task Force and the International Standards Organization (ISO) formed Technical Committee 210, which is working on the application of quality system standards for medical devices. The Japanese Ministry of Health (MHW) has issued a new Medical Device Good Manufacturing Practice (GMP) proposal, which is very close to ISO 9001 plus European Standard (EN) 46001. This contributes to potential harmonization of medical device GMPs. Other countries are expected to proceed in the same general direction. These activities will lead to the U.S. and other countries' "harmonizing" and mutually recognizing quality system standards (e.g., the European Unions "EN 46001" standard and the FDA's proposed new GMPs). The future work of ISO/TC 210 with respect to quality systems will focus on the refinement of the basic documents and related guidances. In the course of its deliberations, TC 210 is expected to take into account the practices of other countries, including the U.S. FDA's GMP regulations.

On November 23, 1993, the FDA published a proposed revision to its current GMP regulation for medical devices. This proposed regulation is compatible with the ISO 9001 quality standard and EN 46001—specific requirements for the application of EN 29001 to medical devices. The intent of this revision is to structure the proposed revisions as closely as possible to the international standard and to use similar terminology where appropriate. The FDA refers to the proposed new GMP regulations as quality system regulations.

Harmonization of the FDA's GMP device regulations with the good manufacturing rules of the European Community (EC), and with comparable rules under developments in Japan and Canada, will contribute to the likelihood that manufacturers need to develop one quality system for their primary markets. Furthermore, the devices manufactured in conformance to an internationally accepted standard will improve the quality of medical products wherever they are produced. The major change in the FDA's revised GMP is from a focus on production process to a focus on the QA of the finished devices. GMP regulations are one of those common elements, with the underlying standards and guidance toward harmonization as European legislation is implemented and standards are being harmonized at a global level. The role of standards can be somewhat active when a manufacturer is designing and manufacturing to meet certain standards. When designing the drug monitoring *in vitro* medical devices, standards are an active way of ensuring that devices reach a uniform quality level (Figure 19–1).

The introduction of the FDA's revised GMP states that it is based on the principles of QA. The first principle states that safety and effectiveness must be addressed at the initial design stage and built into the finished product. Compliance with GMP will help ensure that quality is built in the device, because this relates to the design and validation of the process. The major change to the GMP is addition of design controls to help ensure that safety and effectiveness criteria are met. The second principle of the GMP is based on process validation and process control, because it is difficult and costly to inspect and test quality periodically in the finished product. The third principle is the adoption of rigid manufacturing controls to increase the probability that the finished product will meet specifications. By revising GMP and adopting the quality system approach, one can increase the scope to include design and produce a quality system that, if manufacturer's conform to it, will result in a safe and effective device.

A. THE IVD DIRECTIVE

The IVD Directives specify the essential requirements that must be met by manufacturers wishing to affix the CE mark to their products. The CE mark will allow these products to be marketed throughout the European Union. According to the proposal, manufacturers of most IVD products would demonstrate conformity with the requirements of the Directive through the mechanism of self-certification (i.e., by drawing up an EC declaration of conformity described in Annex 3 of the directive. It is expected that this procedure would require IVD manufacturers to establish a production QA system. The IVD Directive is predicated on a quality management scheme largely grounded in a GMP/quality assessment system.

Figure 1
Drug Monitoring Device Quality Control

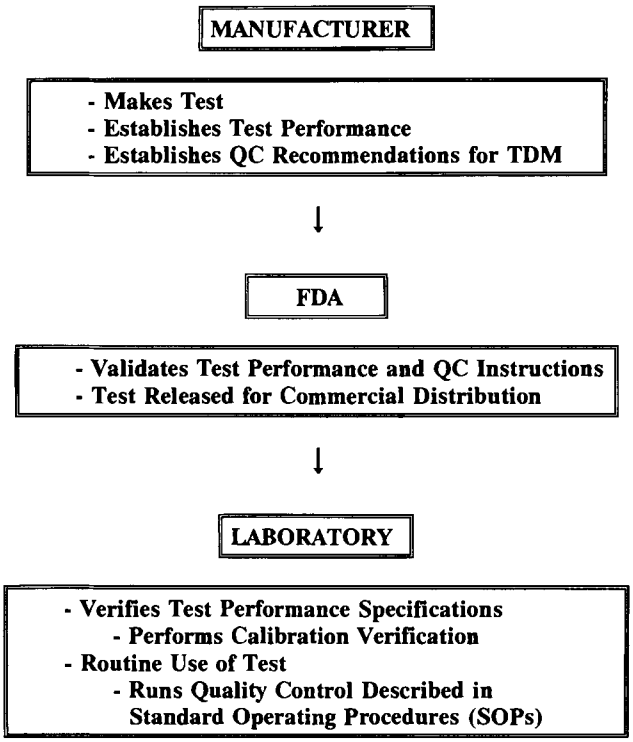

FIGURE 19–1. Drug monitoring device QC. TDM, therapeutic drug monitoring.

B. IVD HARMONIZATION

The NCCLS (Pennsylvania), in its official designation as the Secretariat of the ISO (Geneva, Switzerland), has established a new IVD Technical Advisory Group ISO/TC 212—clinical laboratory testing and IVD test systems. Formation of ISO/TC 212 provides a forum for coordination of international standardization and harmonization in the clinical laboratory medicine services and IVD medical devices. NCCLS proposed and offered to manage this new Technical Committee as part of its growing involvement in international standardization and in response to encouragement by a number of U.S. and international organizations. As manager of ISO's standards development process, NCCLS's role is a global one to be conducted on behalf of the laboratory medicine community throughout the world. The main goal of ISO/TC 212 is to establish and recommend standardization and guidance in the field of clinical laboratory testing and IVD test systems. This includes, for example, quality management, pre- and postanalytical procedures, analytical performance, laboratory safety, reference systems, external QA, accreditation, and ethics.

The NCCLS proposal to the ISO emphasized the role of international standards in supporting the worldwide interchangeability of test methods and IVD testing systems. International standards can play significant roles in promoting uniform practices, harmonizing national and regional requirements, facilitating data and information exchange, ensuring the safety of both patients and the users of clinical laboratory instruments and test systems, and facilitating economic factors of international commerce.

The standards development work of ISO/TC 212 will complement the work of the European Committee for Standardization (CEN; Brussels, Belgium)—TC 140 on Clinical Laboratory Medicine. CEN/TC 140, administered by the Deutsches Institut für Normung, is primarily concerned with production requirements and specifications for IVD products and test systems. The

ISO/TC 212's scope of work is directed toward overall practice of laboratory medicine. However, it seems that there is considerable interest in standardization and harmonization of *in vitro* medical devices that at least one *ad-hoc* working group will be directed toward international harmonization of this activity.

C. TOTAL QA

Clinical laboratories must define their service goals, objectives, and clinical and analytical requirements for testing processes to determine whether acceptable quality is being achieved. This involves control of analytical variables, including technology, standardization and calibration, protocols and procedures, and maintenance and monitoring of essential equipment and materials. This also involves actions of people and quality performance management in a periodic systematic consistency of outputs conforming to established laboratory performance standards (Table 19–5).

QA represents those practices and procedures that are generally recommended for ensuring the desired quality goals. QA is defined in the revised GMP as all activities necessary to provide confidence in the manufacturing process. QA defined this way relates mostly to production. A quality system is defined by ISO 8402 as all things necessary to manage quality. ISO 8402 is entitled "Quality-Vocabulary." Therefore, the big change is a change in scope, moving from a production GMP to one that covers essentially the entire life cycle of the device. The manufacturer must ensure the application of quality principles to the design control, manufacturing process, and continuous quality output of the product. Manufacturers and the FDA have a process for validating the performance and QC requirements of IVD devices through product review and the evaluation program described earlier in this chapter. As part of the PMA and 510(k) evaluation process, a manufacturer has the responsibility to present data verifying that the validity checks and other controls have been effective in preventing the reporting of erroneous results when test analysis is done by the intended users. For therapeutic drug monitoring instruments, kits, or test systems cleared or approved by the FDA for IVD use, minimal QC requirements are described in the labeling. When using these test systems, laboratories must at minimum: (1) follow the manufacturer's instructions, (2) have a procedure manual, and (3) perform calibration procedures and QC levels according to standard operating procedures (Figure 19–1). Drug testing device QC is dependent on those techniques and procedures that require periodic monitoring of quality performance indicators. Generally, these are quantitative techniques that monitor particular sources of errors, estimate the magnitude of the errors, and alert laboratory personnel when to look out for quality shift indicators (Table 19–6).

The goal of a therapeutic drug monitoring should be to provide test results of the quality needed for sound medical decision-making. Monitoring of therapy requires relatively "rigid" analytical performance. For example, an antiepileptic drug with a tight therapeutic range of 10 to 30 μg/ml is dif-

TABLE 19–5
Total QA

QA is the comprehensive set of written policies, procedures, and practices necessary to ensure the overall quality goals of laboratory tests:

1. Follow manufacturer's instructions
2. Have procedure manual describing processes for testing and for reporting patient test results
3. Perform and document calibration procedures described in the labeling
4. Perform and document controls applicable to drug monitoring analyte system described in the labeling
5. Correct problems and errors and document remedial actions
6. Conduct proficiency testing to assess test accuracy and identify training needs
7. Participate in QA networks of other laboratories using similar technologies

Note: QC is a subset of the total QA process.

TABLE 19–6
QA Procedures

QA procedures are a total set of laboratory materials and testing systems used to:

1. Monitor the performance of laboratory systems (reagents, instruments/kits, and/or operators)
2. Monitor the accuracy and precision of a test
3. Ensure that proper testing conditions and instructions have been met

Note: A QA check needs to be an integral part of the analysis of each patient's test sample, and this includes monitoring for system error, as well as for operator error.

ficult to monitor by a testing device that has a coefficient of variation of 15 to 20%. Such a device can only be useful for detecting compliance or gross toxicity. In selecting devices for therapeutic drug monitoring, the laboratory personnel should consider expected analytical performance (e.g., accuracy, precision, sensitivity, and specificity) and operational characteristics (e.g., calibration, applicable QC, normal maintenance features, and reliability of reagents and material supplies). Linkages and participation in QA networks are important in establishing and maintaining quality output of drug monitoring laboratory (Table 19–7). Thus, an effective QA program is important in achieving reliable test results.

D. THE FDA'S NEW MANAGEMENT INITIATIVES FOR IVD MEDICAL DEVICES

The FDA's CDRH is working on procedures to raise the quality and improve the backlog of premarket submissions. CDRH's DCLD is working to improve its review process and create a balance between workload and workforce to minimize future backlogs in premarket submissions (510(k)s and PMAs). To address these concerns, CDRH introduced a comprehensive management action plan in June 1993 for improving the efficiency of its administrative work process.

TABLE 19–7
QC Categories (QC of IVD Devices May Be Composed of Two Different Categories)

External QC	Device QC
External samples run in parallel with patient samples to assess the analytical reliability of the total test system. In actual laboratory practice, these are handled in exactly the same manner as the patient samples and are in an identical matrix. Results generated are used to evaluate all components of the total analytical system from specimen preparation to output of test results.	Device components to complement or augment external QC samples. QC components include a wide variety of mechanisms internal to a device that can be used to evaluate parts of the system with the following examples: 1. *System check*—the operating integrity of the testing system (internal electronic calibration) 2. *Validity check*—proper procedures (internal design components to monitor that proper reagents have been added in the proper order and the proper sequences are followed) 3. *QC check*—the integrity of reagents and sample (internal components used to check for volume flow, integrity of antigen/antibody, or the activity of important reaction ingredients)

Note: Appropriate control limits can be established for both external QC samples and device QC components, and these mechanisms can be used together to monitor or assess devices and components involved in the testing process.

One key item in this plan was a tier/triage program for premarket applications. The tier/triage program was designed to allow levels of review to be adjusted according to device risk. DCLD is interested in redirecting its workforce to deal with newer and more complex premarket submissions. Other initiatives currently underway at DCLD include the availability of a proposal for handling replacement reagents of preexisting or expanded instruments, a downclassification of immunohistochemical stains, a reclassification of serum tumor markers for monitoring, and development of a regulatory scheme for handling analyte-specific reagents used as components or materials for in-house ("home-brew") assays.

A draft proposal for 510(k) modifications entitled, "Deciding When to Submit a 510(k) for a Change to an Existing Device," was made available. This draft document is intended to provide guidance to manufacturers for submitting a new 510(k). DCLD is also involved in a proposed pilot program for third-party review of 510(k)s. In this pilot, CDRH will allow outside parties to conduct 510(k) reviews before final FDA sign off. Finally, DCLD is actively participating in the U.S. Technical Advisory Group for ISO/TC 212 on clinical laboratory testing and IVD test systems.

The FDA now has nearly 20 years of regulatory experience in the area of IVDs. The agency recognizes dual goals in the regulation: to prevent bad products from being marketed, while fostering rapid introduction of innovative and good new ones into the diagnostic arena. Application of the initiatives described herein should enhance the FDA's ability for success in meeting these goals.

VI. CONCLUSIONS

IVD drug monitoring devices are reviewed primarily for safety and effectiveness with regard to their claims for intended use and performance. The FDA's mission is to facilitate the development of those devices that would clearly benefit or advance the public health. The DCLD is responsible for the premarket review and evaluation of all drug monitoring *in vitro* medical devices. A central theme over the past few years has been the development of a standardized model for scientific review and evaluation. The FDA contributes to the quality of IVD devices by providing oversight and objective review, by setting thresholds for product safety and effectiveness, and by ensuring that organized data and appropriate labeling are present in support of a device's intended use and indications for use. Drug monitoring test systems provide unique regulatory and scientific challenges to the FDA in its efforts to conduct its review objectives in conjunction with global harmonization.

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