16 Direct Biofluid Analysis Systems

IAN A. BLAIR

Centers for Cancer Pharmacology and Excellence in Environmental Toxicology,
University of Pennsylvania, Philadelphia, PA, USA

16.1 Summary 1
16.2 Introduction 3
16.3 On-line SPE 3
16.4 RAM 8
16.5 Monolithic chromatography 11
16.6 Turbulent-flow chromatography 13
16.7 Conclusions and future directions 18
Acknowledgments 19
References 19

16.1 SUMMARY

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based methodology provides the most sensitive and specific methodology available for the analysis of small molecule drugs and endogenous metabolites in biofluids such as serum and plasma. However, the extraction and concentration of samples before the analysis represents a major hurdle for the implementation of routine assays and for assays when there are a large number of samples such as in drug disposition studies. This chapter provides a survey on the use of four different direct injection techniques that have been employed over the last decade for the analysis of biofluid samples. Specifically, background information, together with applications of solid-phase extraction (SPE), restricted access media (RAM), monolithic chromatography, and turbulent-flow chromatography have been reviewed. One of the major techniques for direct biofluid analysis utilizes on-line SPE. This is because a major drawback of off-line SPE procedures is that they often require many steps before reaching a concentrated extract suitable for the analysis, of which only a small portion is actually injected onto the LC column. In contrast, on-line SPE offers numerous advantages including the ability to readily automate the analyses. SPE sorbents include traditional materials, such as chemically bonded silica, ion-exchange and carbon-based materials, and some novel sorbents, such as RAM, molecularly imprinted polymers, immunosorbent, and monolithic material. Interfering substances present in the biofluids are flushed to waste while the analytes are retained

Encyclopedia of Drug Metabolism and Interactions, 6-Volume Set, First Edition.
Edited by Alexander V. Lyubimov.
© 2012 John Wiley & Sons, Inc. Published 2012 by John Wiley & Sons, Inc.

on the bonded phase of the SPE cartridge. Small molecule analytes are then eluted on-line, through a switching valve onto the LC analytical column. Simultaneously with the analytical separation, an exchange of the cartridge or reconditioning of the precolumn can take place.

The term *RAM* was introduced to describe a group of phases that allow direct and repetitive injection of untreated biological samples into reversed-phase LC systems. Macromolecules present in the biofluid samples cannot access the adsorption sites of porous supports because of their molecular size and so they elute in the void volume of the column. The RAM supports permit the separation of analytes through a combination of size-exclusion and conventional hydrophobic or ion-exchange interactions, which allows the passage of small molecules and restricts the access of macromolecules such as proteins. Two of the most popular RAM stationary phases are those employing internal surface reversed phase (ISRP) and alkyl-diol-silica (ADS) phase and there is an extensive literature describing the use of these columns. An interesting relatively recent development has involved the preparation of RAM using immobilized antibodies for the rapid and selective capture of small analytes by immunoextraction, giving rise to materials referred to as *immunoaffinity (IA)-RAM*. To make the required IA-RAM, an antibody for the desired target was first immobilized onto porous silica, with antibodies at or near the outer surface of the support then being treated with papain (or a related agent) to release and remove their binding domains. This resulted in a RAM support in which only antibodies deep within the pores remained intact and able to bind to the target analyte. This IA-RAM approach could potentially be used with many different antibodies to small molecule analytes and so could be useful for the rapid separation of drugs from drug–protein complexes.

Monolithic stationary phases comprise porous channels rather than beads found in conventional stationary phases employed for LC separations. They are typically prepared using a simple molding process carried out within the confines of a capillary. Monolithic stationary phases provide high rates of mass transfer at lower pressure drops, which enables much faster separations to be conducted when compared with conventional stationary phases. In addition, the nature of the pores in the monolithic phase allows easy permeability for large molecules. This means that monolithic columns can be used for direct biofluid analyses either as conventional LC columns or as sorbents in SPE columns for the extraction and concentration of small molecules. Turbulent-flow methodology was introduced specifically for the direct injection of biological samples without previous extraction or treatment onto an LC column packed with large particles. These large particles have an additional level of selectivity through different stationary phase chemistries (such as reversed phase or ion exchange) that can be added. Conventional LC methodologies employ parabolic flow profiles, while turbulent-flow LC (as its name implies) promotes turbulence in a packed column bed. The stream of mobile phase in the column moves so rapidly that it creates vacillating eddies within the stationary phase, which then cause flow equalization across the column. This results in a mobile phase front profile that is more like a plug than the parabola found in conventional LC separations. The eddies are also able to promote cross-channel mass transfer by convection within the interstitial spaces of the packed column bed. The use of fast mobile phase flow rates coupled with a plug flow profile results in an increase in the rate of change of the sample concentration gradient, thus increasing the effective diffusion rates within the pores. This turbulent-flow motion supports a more uniform velocity profile across the diameter of the column and controls effective

diffusion of the solutes. Therefore, analyte molecules can move quickly into and out of the pores and packing particles, which means that rapid chromatography can be conducted. The impressive sensitivity that has been attained with two-dimensional (2D) turbulent-flow LC-MS/MS suggests that it will be a powerful method for future routine applications for direct plasma and serum analyses of drugs and their metabolites, as well as endogenous metabolites.

16.2 INTRODUCTION

There is a compelling need for rapid, sensitive, and specific methods to analyze small molecule drugs, their metabolites, and endogenous metabolites for pharmaceutical development, as well as biomarker, therapeutic, and metabolomic applications [1–5]. The analysis of drugs in biofluids such as serum and plasma represents an important component in the determination of the physiological performance of a drug [6,7]. Therefore, a substantial amount of research has been conducted to develop rapid high throughput methods for the direct analysis of drugs and their metabolites in biofluids. Stable isotope dilution LC-MS/MS provides powerful bioanalytical methodology that combines high specificity with high sensitivity [8–10]. In spite of many advantages that have accrued due to recent advances and innovations in the area of instrumentation and dedicated software support, analysis of biofluid samples by LC-MS can be challenging and very time consuming [11]. One approach for improving sample throughput is to conduct direct on-line analysis of biofluid samples with no off-line extraction and purification. Such methods offer the advantage of reducing sample preparation steps and enabling effective preconcentration and cleanup of biological fluids. These procedures can also be readily automated, which reduces the requirements for handling potentially infectious biomaterial, improves reproducibility, minimizes sample manipulations, and limits potential contamination [12]. This chapter provides an overview on the use of four different direct injection techniques that have been employed over the last decade for the direct analysis of biofluid samples. Specifically, underlying principles involved in the application of SPE, RAM, monolithic chromatography, and turbulent-flow chromatography for direct analyses are addressed. A major issue that arises from direct analyses is the propensity for components of the biofluids to either suppress or enhance ionization in the analyzer region of the mass spectrometer [13]. Therefore, methodology for assessing such effects on different commonly used LC-MS ionization techniques is discussed [13,14]. A number of excellent review articles have appeared over the last decade describing the application of various methodologies for the direct analysis of biofluid samples [11,12,15–20]. Therefore, this chapter is focused primarily on recent advances that have been made in the application of the various direct on-line methods.

16.3 ON-LINE SPE

16.3.1 Background

Sample preparation is a major task in both regulated and unregulated bioanalytical laboratories. Sample preparation procedures significantly impact on assay throughput, data

quality, and analysis cost. Therefore, selecting and optimizing an appropriate sample preparation method is essential for successful method development using direct biofluid analysis methods [11]. Two of the major techniques that are employed utilize off-line SPE or on-line SPE. Significant drawbacks of off-line SPE procedures is that they can require many steps before reaching a concentrated extract suitable for the analysis, of which only a small portion is actually injected onto the LC column (Fig. 16.1) [21,22]. In contrast, on-line SPE offers numerous advantages including the ability to completely automate the analyses [22–24]. The on-line SPE approach involves the injection of a biofluid sample directly into an SPE cartridge, which retains the relevant analytes. SPE sorbents include traditional materials, such as chemically bonded silica, ion-exchange and carbon-based materials, as well as some novel sorbents, such as RAM, molecularly imprinted polymers, immunosorbents, and monolithic material [25]. Interfering substances present in the biofluid are flushed to waste, while the analytes are retained on the bonded phase of the SPE cartridge. They are then eluted on-line, through a switching valve onto the LC analytical column [23]. Simultaneously with the analytical separation, an exchange of the cartridge or reconditioning of the precolumn can take place.

Direct analysis of biofluid samples using SPE-based methodology has a long history because it can significantly increase sample throughput [12]. The overall methodology for LiChrograph OSP-2 (On-line Sample Preparation Unit, Merck, Darmstadt, Germany) Microlab SPE (Hamilton, Reno, NV, USA), and the widely used PROSPEKT (Programmable On-line Solid Phase Extraction Technique, Spark Holland BV, The Netherlands) systems have been discussed extensively in previous review articles [26–28]. Therefore, this chapter provides an overview of the symbiosis system developed by Spark Holland BV [29,30]. The system allows the use of

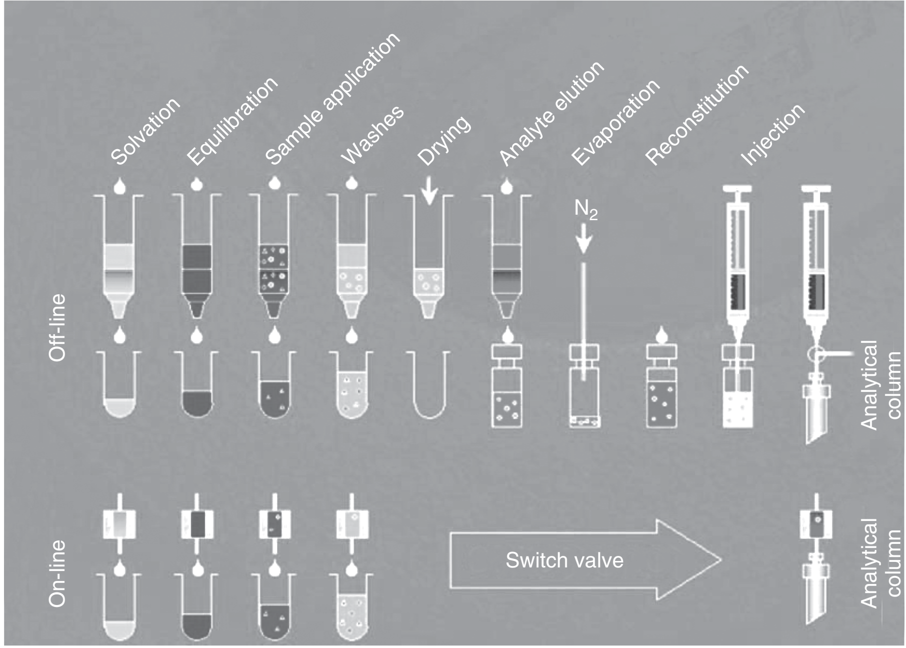


Figure 16.1 Comparison between on-line and off-line SPE methodology. *Source*: Reprinted with permission from Mitchell *et al*., 2010 [22]. (See color insert.)

two different extraction mechanisms on two separate disposable SPE cartridges in one sample run, which can significantly improve upon methods that employ single SPE cartridges. Disposable cartridges help to eliminate sample-to-sample carryover problems that can sometimes be observed when columns are reused, particularly for low level analytes. The Symbiosis system scan handle sample volumes from 10 μL to 10 mL and can add, mix, and dilute samples before the analysis. The principles of extraction are essentially the same as off-line SPE. However, the system design allows the SPE eluate to be directly injected onto the chromatography system, thus removing the need for any evaporation (Fig. 16.1). By avoiding evaporation steps, it is possible to significantly cut down on the amount of time required for the sample analysis. In addition, the ability to use two separate liquid flows for the extraction and chromatography steps significantly reduces the potential for carryover from sample to sample. This can be a particular problem when analyzing large concentration ranges of drugs and endogenous metabolites that are present in plasma and serum samples. Importantly, there is a large variety of stationary phases available for the SPE cartridges, which can help in the development of highly selective on-line extraction methodology.

The development of analytical methodology for sophisticated on-line SPE extractions requires a substantial amount of time to optimize all of the conditions. A particular advantage of the Symbiosis system is that the SPE cartridges can be washed with heated solvents. This can be useful when washing with organic solvents is precluded by the polarity of the analytes. Parameters such as elution time, elution flow rate, solvent polarity, and type of stationary phase have to be modified for different analytes. A recent study demonstrated that the performance of assays with on-line extraction was essentially identical with that obtained by off-line SPE extraction [22]. Therefore, the benefits generally only accrue for large sample batches. Consequently, off-line SPE extraction methodology generally remains the method of choice for small numbers of samples. However, for volatile or unstable samples, on-line methodology is preferable even for small sample numbers [29]. Both on-line and off-line SPE-LC-MS configurations can result in significant matrix effects, which depend on whether electrospray or atmospheric pressure chemical ionization (APCI) is being used and the particular extraction support that is utilized [31]. Therefore, it is necessary to assess the amount of suppression or enhancement of ionization that is occurring with different batches of plasma or serum samples [13].

16.3.2 Applications of On-Line SPE

A validated method for on-line SPE coupled with LC-tandem mass spectrometry (MS/MS) for analysis of the anxiolytic drug bromazepam in human plasma provides an illustration of how direct biofluid analyses can be conducted [32]. The method involved addition of carbamazepine internal standard, vortex-mixing, centrifugation, and injection of 100 μL of the supernatant. Analytes were ionized using positive electrospray mass spectrometry then detected by multiple reaction monitoring/MS. The calibration curve was linear from 1 ng/mL (limit of quantification) to 200 ng/mL with LC retention times for bromazepam and carbamazepine of 2.6 and 3.2 min, respectively. This automated method was successfully applied in a bioequivalence study of two tablet formulations. The comparison of different experimental conditions for establishing a dissolution profile *in vitro*, along with the bioavailability data that were generated,

made it possible to propose rationally based experimental conditions for a dissolution test of bromazepam tablets.

In the field of environmental toxicology, SPE-LC-MS/MS was employed for quantifying trace levels of 18 perfluorinated chemicals (three perfluorosulfonates, eight perfluorocarboxylates, and seven perfluorosulfonamides) in serum [29]. After dilution with 0.1*M* formic acid, an aliquot of 100 μL of serum was injected into a commercial column-switching system that allowed for concurrent SPE and LC-MS/MS analyses. Analytes were concentrated on a C18 SPE column and the eluates then automatically injected on a reversed-phase C8 analytical LC column. Excellent recovery was achieved for all analytes including the volatile sulfonamide derivatives, which could not be analyzed using traditional off-line SPE methods. The high throughput and low limits of detection (0.05–0.8 ng/mL) using a small sample volume and isotope dilution quantification make this method suitable for large-scale epidemiological studies.

A novel approach for on-line introduction of internal standard for quantitative analysis using LC-MS/MS was developed for use with on-line SPE methodology [33]. In this approach, the analyte and internal standard were introduced into the sample injection loop in different steps. The analyte was introduced into the injection loop using a conventional autosampler (injector) needle pickup from a sample vial, whereas the internal standard was introduced into the sample injection loop on-line from a microreservoir containing the internal standard solution. As a result, both analyte and internal standard were contained in the sample loop before the injection into the column. This new technique was applied for direct analysis of model compounds in rat plasma using on-line SPE-LC-MS/MS quantification. The assays yielded accuracy (85–119%), precision (2–16%), and analyte recovery comparable to those obtained using off-line internal standard introduction. Furthermore, on-line internal standard introduction allowed for nonvolumetric plasma collection and direct analysis without the need of measuring and aliquoting a fixed sample volume before the on-line SPE-LC-MS/MS analysis. Therefore, this methodology enabled direct plasma analyses to be conducted without any sample manipulation and preparation. On-line SPE coupled with capillary LC-ESI-MS was employed to improve the sensitivity for quantification of the antidepressant and anorectic drug fluoxetine (valium) in human plasma [34]. Before injection, the plasma was spiked with metronidazole (internal standard) and mixed with ammonium formate buffer for effective chloroform liquid–liquid extraction. The method was validated in the range 5–60 ng/mL fluoxetine. The method was then used to determine the amount of fluoxetine in a healthy male 14 h after an intake of one capsule of flutin, which contains 20 mg fluoxetine/capsule. The use of capillary LC increased the sensitivity by a factor of similar to 100 when compared with conventional LC systems.

The analysis of endogenous metabolites in plasma by direct SPE-LC-MS is exemplified by the studies conducted on the analysis of steroids in serum [35]. Following protein precipitation of 100 μL serum, on-line SPE and chromatographic separation were performed for 13 steroids in 1.8 min. Analyte identities were confirmed by the characteristic fragmentation patterns observed by MS/MS analyses in a quadrupole ion trap. The total run time of the method was 4 min. Limits of quantitation ranged between 50 pg/mL for testosterone and 48 ng/mL for dehydroepiandrosterone sulfate. The method was linear up to 7000 ng/mL for dehydroepiandrosterone sulfate, 500 ng/mL for cortisol, 125 ng/mL for 11-deoxycortisol, and 25 ng/mL for aldosterone, 17-hydroxyprogesterone, progesterone, testosterone, androstenedione, and β-estradiol, respectively. Accuracy ranged between 80% and 114%. Between-day variance at three

different concentration levels was $<15\%$. Excellent correlations with immunoassays were observed for testosterone, cortisol, and β-estradiol. Therefore, this novel on-line SPE-LC-MS/MS platform offers a very fast and reliable method for the quantification of steroid patterns for relatively routine laboratory applications. However, the limits of detection would need to be substantially lower for research applications such as the determination of breast and prostate cancer risk [3,36].

Recently, an on-line SPE-LC-MS/MS method was optimized for quantification of the anti-HIV (human immunodeficiency virus) peptide sifuvirtide in human plasma [37]. The SPE sorbents, loading buffer composition, and other aspects of the on-line SPE column were investigated in detail for efficiently extracting the interesting peptides and simultaneously discarding the large amount of proteins from the plasma. Method validation revealed a linear calibration curve that covered a wide range of 6.1–6250 ng/mL, with excellent correlation coefficients. The lower limit of detection with a signal-to-noise ratio >10 was 6.1 ng/mL. Impressively, more than 900 samples from a clinical trial were analyzed in a single week because of the rapid run time of 6.5 min for individual samples.

16.3.3 Background and Applications of Miniaturized SPE (μ-SPE) Cartridges

μ-SPE (micro solid-phase extraction) cartridges are available as a component of the RapidFire System (BIOCIUS Life Sciences, Wakefield, MA), which also uses innovative microfluidics technology. This system is emerging as a useful approach to direct biofluid analysis. Its utility was demonstrated by comparing a high throughput RapidFire MS System with a scintillation proximity assay [38]. The RapidFire System allows continuous SPE extractions to be performed in 5–10 s time intervals [39]. Thus, this technology performs rapid SPE purification of biofluids in 96 or 384 well plates followed by MS-based analysis [40,41]. Extracted analytes are rapidly eluted to a mass spectrometer after every SPE step, and analytes that are present in each well are quantified sequentially. Initial high throughput applications have focused on the analysis of enzyme incubation mixtures [38,41–43]. For example, a series of 120 compounds was evaluated for potential drug–drug interaction by incubating over a range of eight test concentrations and against a panel of six cytochrome P450 (CYP) enzymes, 1A2, 2C8, 2C9, 2C19, 2D6, and 3A4 [43]. The on-line SPE-MS/MS system reduced analysis time to <15 min per 96-well plate, translating to a 15-fold time savings compared to the 2-min LC/MS/MS method. There was an excellent correlation between this new rapid high throughput method and previous methods for the individual CYP enzymes.

The success of direct analyses of biofluids from enzyme assays using μ-SPE cartridges has encouraged investigators to test more challenging biofluids such as plasma. A presentation at the recent Applied Pharmaceutical Analysis meeting in Baltimore, MD explored the use of μ-SPE-based methodology for the high throughput direct analysis of drugs in plasma samples [44]. Encouragingly, the assay results from RapidFire-MS/MS analysis correlated well with the results from conventional LC-MS/MS analyses. Use of stable isotope labeled and structural analog internal standards effectively compensated for matrix effects. When the ion source conditions were optimized to minimize interference from an acyl-glucuronide metabolite, parent drug concentrations were comparable to those obtained from LC-MS/MS system. Therefore, co-eluting metabolites had a negligible impact on quantitation. Even though there were variable amounts of sample-to-sample carryover, no significant

impact on quantitation results was observed. Therefore, the μ-SPE-based RapidFire System might be a useful tool for high throughput MS quantitation of *in vivo*-derived biofluid samples in situations where large sets of samples justify the up-front burden of method development [44].

16.4 RAM

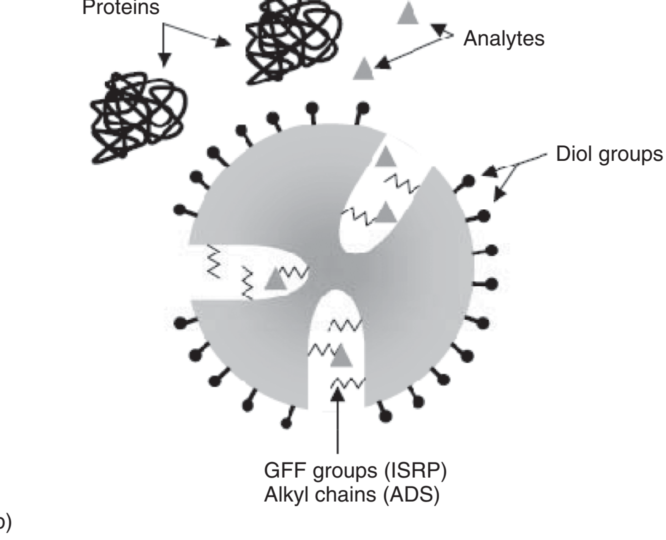
16.4.1 Background

The term *RAM* was introduced by the Desilets *et al*. to describe a group of phases that allows direct and repetitive injection of untreated biological samples into a reversed-phase LC systems [45]. Macromolecules present in the samples cannot access the adsorption sites of porous supports because of their molecular size and so they elute in the void volume of the column [46]. RAM supports permit the separation of analytes through a combination of size-exclusion and conventional hydrophobic or ion-exchange interactions, which allows the passage of small molecules and restricts the access of macromolecules such as proteins [47]. Macromolecular exclusion can be performed using a physical diffusion barrier based on the size of the pore or by a chemical diffusion barrier created by a network covering the surface of the support, which consists of covalently or adsorptive-bonded synthetic polymers, natural polymers, or proteins at the outer surface of the silica. It has been suggested that RAM sorbents can be divided into four groups: physical diffusion barriers with either unimodal, bimodal phases, chemical diffusion barriers with either unimodal, or bimodal phases (Fig. 16.2a) [28,47]. RAM stationary phases such as ISRP (Fig. 16.2b), semipermeable surface, ADS (Fig. 16.2b), shielded hydrophobic phase, mixed-function phase, and protein-coated silica have all been employed for LC-MS-based direct plasma analyses [46]. Commercial columns are available for each of these applications. The two most popular RAM stationary phases are those employing ISRP and ADS. A large number of applications, together with the commercially available columns that were used, have been described in the excellent extensive review by Souverain *et al*. [48].

An interesting relatively recent development has involved the preparation of RAM using immobilized antibodies for the rapid and selective capture of small analytes by immunoextraction, giving rise to materials referred to as *IA-RAM* [49]. To make the required IA-RAM, an antibody for the desired target was first immobilized onto porous silica, with antibodies at or near the outer surface of the support then being treated with papain (or a related agent) to release and remove their binding domains. This resulted in a RAM support in which only antibodies deep within the pores remained intact and able to bind to the target analyte. This IM-RAM approach could potentially be used with many different antibodies to small molecule analytes and so could be useful for the rapid separation of drugs from drug–protein complexes. It could also be particularly valuable for separating endogenous DNA bases such as 2′-deoxy-guansoine (dGuo) from 7,8-dihydro-8-oxo-dGuo (8-oxo-dGuo) in plasma samples. This would help prevent the artifactual formation of 8-oxo-dGuo when there is an excess of dGuo present [50]. The need for RAM capillary columns that could be used for low flow applications that enhance detection sensitivity was recognized by Jarmalaviciene *et al*. [51]. They have developed different strategies for the preparation of novel shielded polymeric reversed-phase monolithic material in the presence of different numbers

RAM phase	Diffusion barrier	Surface topochemistry	Type
	Physical	Homogeneous	A
	Physical	Heterogeneous	B
	Chemical	Homogeneous	C
	Chemical	Heterogeneous	D

(a)



(b)

Figure 16.2 (a) Classification of RAM supports. *Source*: Reprinted with permission from Cassiano *et al*. [47]. (b) Schematic representation of internal surface reversed-phase (ISRP) with GFF groups and alkyl-diol-silica (ADS) with alkyl chains. *Source*: Reprinted with permission from Souverain *et al*., 2004 [48].

of reactive groups and concentrations of the coating polymer. Inverse size-exclusion chromatography was used for investigation of the pore structure properties of the beds. The resulting RAM capillary columns were then applied for nanochromatography of biological fluids containing a mixture of the sedative benzodiazepine drugs nitrazepam and medazepam.

16.4.2 Applications of RAM

As might be predicted, RAM can be used as the solid support for both off-line and on-line SPE of small molecules in plasma as well as the stationary phase for LC separations [28,48]. There are many applications appearing in the current literature that use the RAM for this purpose rather than conventional LC procedures. A recent application described the use of an LC system that was equipped with an on-line dilution system for on-line SPE analysis [52]. Use of a six- to eightfold on-line dilution ratio for plasma samples resulted in almost quantitative recovery of both acidic and basic drugs from plasma. It was found that the relationship between the on-line dilution times and drug recovery efficiencies from plasma could be explained in terms of the binding constant between the drug and albumin. The applicability of this column-switching LC with an on-line dilution system and the effectiveness of the extraction procedure were confirmed by a simultaneous determination of the basic compound, ER-118585, and its metabolites in canine plasma.

Another interesting example of on-line SPE analysis involved the extraction of the proton-pump inhibitor, lansoprazole from plasma using an octyl RAM bovine serum albumin column (C-8 RAM BSA) [53]. Enantioselective LC separations were performed on an amylose tris(3,5-dimethoxyphenylcarbamate) chiral column. The method was applied to the analysis of the plasma samples obtained from nine volunteers who received a 30-mg oral dose of racemic lansoprazole and was able to quantify the enantiomers of lansoprazole in all the samples that were analyzed. RAM-based SPE extraction methodology has proved to be particularly useful for the analysis of the radioactive components in plasma taken during positron emission tomography (PET) measurements using [^{11}C]-labeled radiopharmaceuticals [54]. Plasma samples were analyzed directly, following a simple filtration, by the use of a small RAM SPE column, combined with a monolithic LC analysis in a column-switching system. Up to 4 mL of plasma could be analyzed by this method within 4.5–7 min in a fully automated process. A large number of samples could be analyzed during a 90-min PET scan. This method made it possible to conduct accurate determinations of the radioactive components in plasma even at 90 min after injection of a [^{11}C]-labeled radiopharmaceutical.

An important aspect of on-line RAM SPE for routine analyses is the ability to concentrate the sample into a small volume. Santos-Neto *et al*. found that the peak-focusing efficiency of the RAM SPE column was more effective in back-flush compared to fore-flush mode [18]. Their system was able to concentrate polar drugs and their metabolites reaching quantifiable results as low as 1 ng/mL utilizing a sample volume of only 333 nL of plasma. New column hardware was developed to circumvent clogging problems that were experienced with plasma injections. The glass fiber filter frit, which is commonly used, was replaced with a short piece of 20-μm internal diameter fused silica capillary tubing. The RAM SPE columns were then able to handle up to 60 injections without any deterioration in separation efficiency. The columns also had a

high loading capacity, making the saturation of the MS detector, the limiting factor on the linear dynamic range. Using this method, it was possible to simultaneously separate and detect 10 drugs and metabolites within 8 min.

A simple and rapid RAM-based LC-MS method was used for direct analysis of the antibiotic rifaximin in rat serum [55]. Separation of rifaximin from serum was achieved by the injection of rat serum onto a Supelco LC-Hisep LC column, which contains a shielded hydrophobic RAM stationary phase. The linear range of the assay was a 0.10–20 ng/mL of serum. The validated RAM-based LC method was successfully applied to pharmacokinetic studies of rifaximin in serum after oral administration to rats. RAM LC has also been employed for coupled-column separations [56]. A RAM bovine serum albumin RAM LC column was used in the first dimension in order to exclude macromolecules and retain small molecules. An amylose tris[(*S*)-1-phenylethylcarbamate chiral column was used in the second dimension. This fully validated method has provided the first example of the rigorous analysis of both enantiomers of the analeptic drug modafinil and its two major metabolites in plasma. The coupled RAM-chiral column method showed good linearity, precision, accuracy, sensitivity, and selectivity, allowing it to be used for pharmacokinetic studies. Importantly, the quality of the performance of both columns was maintained with over 280 plasma injections.

16.5 MONOLITHIC CHROMATOGRAPHY

16.5.1 Background

Monolithic stationary phases comprise porous channels rather than the beads found in conventional stationary phases employed for LC separations [12,16]. They are typically prepared using a simple molding process carried out within the confines of the capillary [57]. Monolithic stationary phases provide high rates of mass transfer at lower pressure drops, enable much faster separations and the nature of the pores allows easy permeability for large molecules (Fig. 16.3) [58]. This means they can be used for direct plasma analyses either as conventional LC columns [59,60] or as sorbents in SPE columns for the extraction and concentration of small molecules [20]. Silica-based monolithic stationary phases are prepared from tetraalkoxysilane by

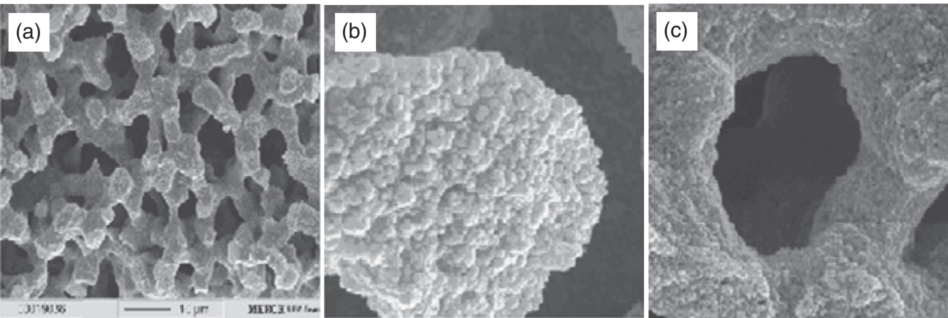


Figure 16.3 (a) SEM-picture of the typical porous structure of monolithic silica columns. (b) The mesoporous structure of the silica skeleton. (c) The macropores or through-pores. *Source*: Reprinted with permission from Hayes *et al*., 2000 [58].

a sol-gel method. This can provide either micrometer-size through-pores or high specific surface areas that are well suited for small molecules in LC modes. In contrast, organic polymer-based monolithic stationary phases including monolithic molecularly imprinted polymers have been applied to LC analysis of macromolecules and they have also been employed as sorbents for both on-line and off-line SPE extraction of plasma. The most commonly reported organic polymers are based on polystyrenes, polymethacrylates, and polyacrylamides. Monolithic phases have a number of potential advantages over more conventional silica-based particulate materials, as outlined in the excellent review by Saunders *et al*. [61]. Polymer monoliths can be adapted to many bioanalytical situations such as sample preparation and concentrations as well and capillary LC separations, which explains their increasing use for direct plasma analyses. These materials have some interesting properties when compared to more traditional particulate materials, such as their high permeability for liquids and biological samples, making them ideal for SPE applications.

16.5.2 Applications of Monolithic Chromatography

Many new applications on the monolithic SPE in sample extraction and concentration (as a sample extraction and concentration tool before more conventional LC separations) have appeared over the last decade [10,25]. A comprehensive review of the use of monolithic chromatography has appeared recently [20]. Although many of the

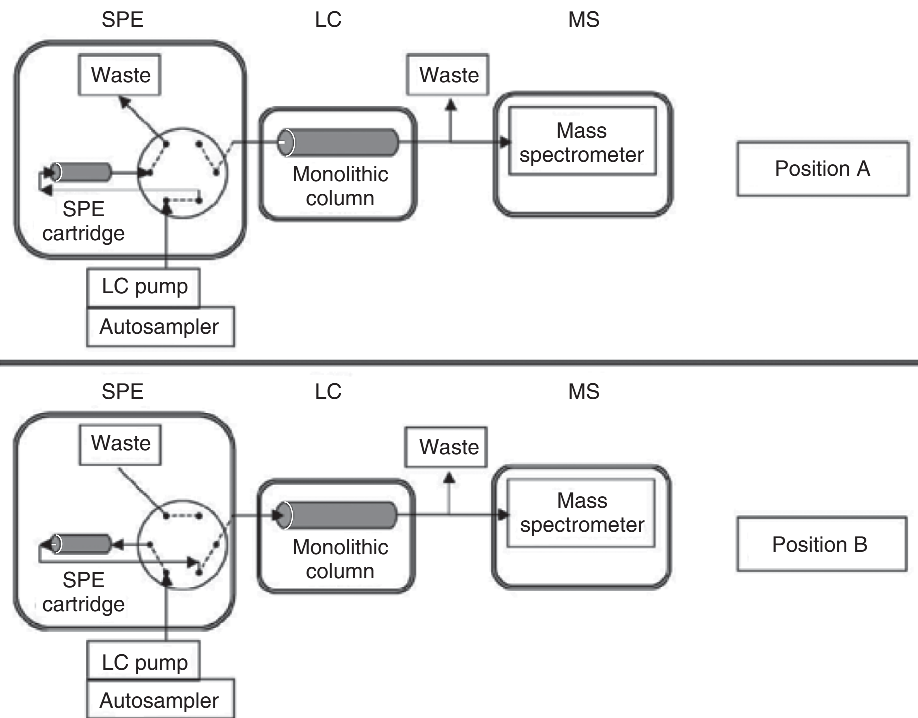


Figure 16.4 Schematic diagram of the on-line SPE monolithic LC/MS/MS configuration. *Source*: Reprinted with permission from Zang *et al*., 2005 [62].

applications do not relate specifically to direct plasma analyses on a monolithic column itself, the examples that have been provided can be used as the basis for embarking on the use of this type of methodology. In an interesting example, Zang *et al*. developed on-line SPE LC/MS/MS assay using a monolithic LC column for the direct analysis of plasma samples containing multiple analytes [62]. A simple column-switching configuration that required only one six-port valve and one LC pumping system was employed for on-line plasma sample preparation and subsequent gradient LC separation using a monolithic Chromolith Speed ROD RP-18e (4.6 × 50 mm i.d., Merck) (Fig. 16.4). The method was found to perform satisfactorily for direct plasma analysis with respect to assay linearity, specificity, sensitivity, precision, accuracy, carryover, and short-term stability of an eight-analyte mixture in plasma. Gradient LC conditions were applied in order to separate the eight analytes that could not be readily differentiated by MS/MS analysis. With a run time for every injection of 2.8 min, a minimum of 300 direct plasma injections were made on one on-line SPE column without noticeable changes in system performance. Owing to the ruggedness and simplicity of this system, the authors suggested that generic methods could be easily developed for the analysis of a wide variety of small molecules in plasma in a high throughput manner without laborious off-line sample preparation.

A particularly good example of direct plasma analysis using monolithic LC-MS/MS is provided by the method developed by Hsieh *et al*. [15]. The method was employed for high-speed direct simultaneous determination of a drug discovery compound and its major circulating metabolite (M-72) in rat plasma. This methodology made use of flow programming and an alkyl-bonded silica rod column for fast macromolecule removal and chromatographic separation without the need for significant sample preparation. After 200 plasma injections on the monolithic silica column (50 × 4.6 mm i.d.), consistent column efficiency of approximately 39,000 theoretical plates per meter could be obtained together with reproducible retention times. The apparent on-column recoveries of 12 test compounds in rat plasma samples were $>90\%$. The direct plasma injection method was tested over a three-day period, where inter-day coefficients of variation of $<15\%$ were observed for both analytes.

16.6 TURBULENT-FLOW CHROMATOGRAPHY

16.6.1 Background

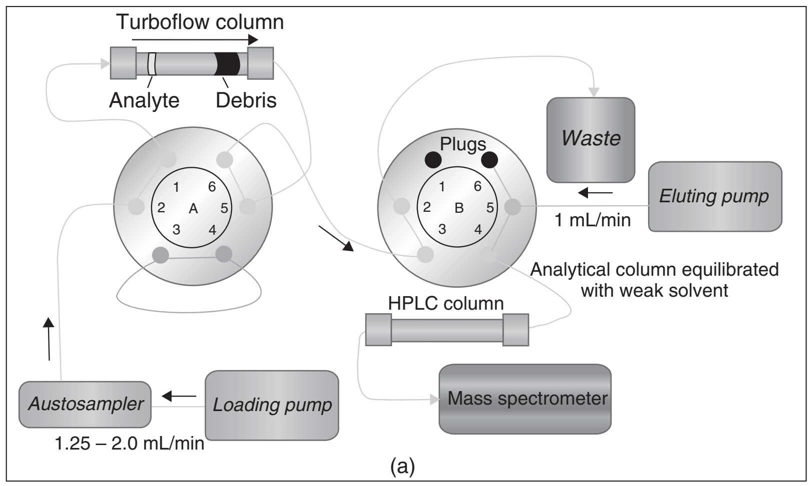
Turbulent-flow methodology was developed by Quinn and Takarewski specifically for the direct injection of biofluids without previous extraction or treatment onto an LC column packed with large particles [19]. These large particles have an additional level of selectivity through different stationary phase chemistries (such as reversed phase or ion exchange) that can be added. Conventional LC methodologies employ parabolic flow profiles, whereas turbulent-flow LC (as its name implies) promotes turbulence in a packed column bed. The stream of mobile phase in the column moves so rapidly that it creates vacillating eddies within the stationary phase, which then cause flow equalization across the column. This results in a mobile phase front profile that is more like a plug than the parabola found in conventional LC [63]. The eddies also promote cross-channel mass transfer by convection within the interstitial spaces of the packed column bed [63]. The use of fast mobile phase flow rates coupled with a plug flow

profile results in an increase in the rate of change of the sample concentration gradient, thus increasing the effective diffusion rates within the pores. This turbulent-flow motion supports a more uniform velocity profile across the diameter of the column and controls effective diffusion of the solutes. Therefore, analyte molecules can move quickly into and out of the pores and packing particles, which means that rapid chromatography can be conducted.

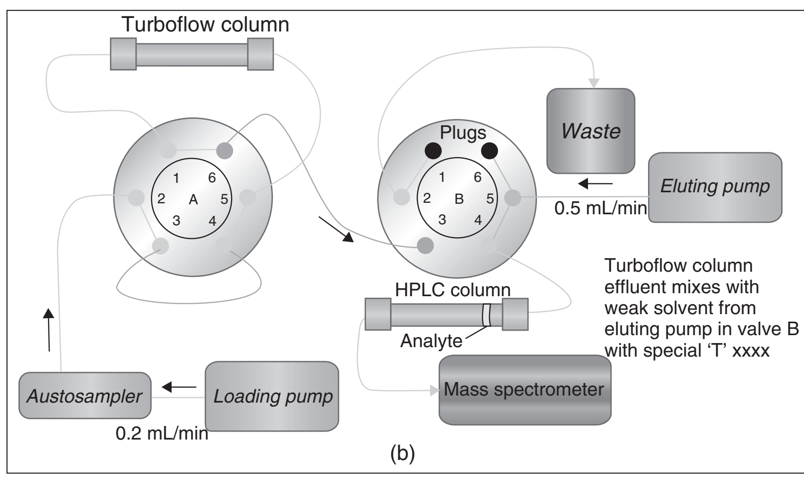
Typically, a biofluid sample is injected onto a turbulent-flow LC column at flow rates of between 1.5 and 5.0 mL/min. Initially, aqueous buffers are used as the mobile phase, so that small molecules are retained on the column through diffusion into the particle pores. The proteins that are present in the sample rapidly elute from the column and are washed to waste. Once the small molecules have been extracted from the biofluid into the stationary phase, they are then either eluted from the turbulent-flow column to a conventional analytical LC column in a 2D approach as described by Jemal [64], Wu *et al*. [65], and Chassaing *et al*. [66], or they are analyzed directly on the same column as described by Ayrton *et al*. [63]. Not surprisingly, the 2D approach has become the method of choice because it allows for much more selectivity as well as improved sensitivity through the use of low flow rates on the analytical column. For use of the turbulent-flow column as the initial extraction method, it has been recommended that the small molecule analytes should be eluted onto the analytical column using solvent that has been stored in a holding loop [19]. The holding loop should have a volume at least 10 times that of the turbulent-flow column and is typically filled with organic mobile phase (for reversed stationary phase) or pH buffered solutions (for ion-exchange phases) [19]. The use of switching valves to obtain optimal system performance is shown in Fig. 16.5.

A major issue with all biofluid assays is the degree of suppression or enhancement of ionization that can occur with LC-MS-based methodology [67]. This issue is extremely important when nonidentical or deuterium labeled standards are employed because there can be significant separation between the analyte and its relevant internal standard [68]. Suppression effects can potentially be problematic even with [^{13}C] and [^{15}N]-labeled internal standards because they can significantly affect the lower limit of quantitation. The current regulatory requirements include the need for the assessment and elimination of the matrix effect in bioanalytical methods that are submitted to the Food and Drug Administration [13]. These effects can be determined by infusing the analyte and observing the suppression and ionization caused by the LC effluent [14]. A robust procedure has also been described in which five different plasma lots are analyzed [13]. This makes it possible to determine the absolute effects for particular plasma samples or the relative effects with different plasma samples. The magnitude of matrix suppression and enchantment effects on ionization efficiency appears to be dependent on the actual mechanism of ionization. Under otherwise identical sample

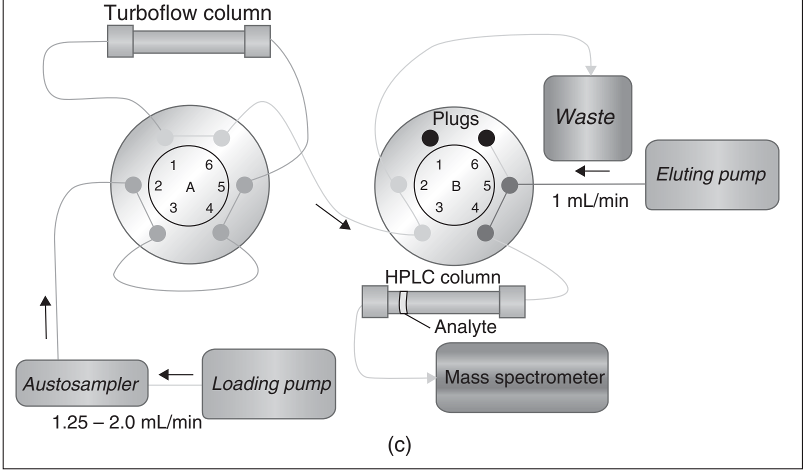
Figure 16.5 (a) Loading step: turbulent-flow removed proteins from the plasma through the turbulent-flow (Turboflow) column while small molecules are retained. (b) Transfer step: flow from both pumps is combined through the T-rotor seal, which allows the loop contents to transfer the analytes retained on the turbulent-flow column into a stacked band on the analytical column. (c) Elution step: the analytes are eluted from the analytical LC column using an isocratic or gradient mobile phase. The turbulent-flow column is washed and the loop is filled and closed in preparation for the next injection. *Source*: Reprinted with permission from Chassaing and Robinson, 2009 [19].



(a)



(b)



(c)

extraction and chromatographic conditions, the matrix effect was absent when APCI was employed but very significant with electrospray ionization [13]. Therefore, a thorough consideration of the ionization technique that is employed can be an important component of the analytical development procedure. A fully automated turbulent-flow method for the analysis of antidepressants in serum investigated the suppression of plasma constituents [69]. This study revealed that all of the suppression occurred before the relevant analytes eluted from the column and illustrated the power of turbulent-flow extraction methodology (Fig. 16.6).

16.6.2 Applications of Turbulent-Flow Chromatography

The quantification of Vitamin D in serum and plasma is an important diagnostic test of nutritional status that is often performed by radioimmunoassay procedures. Stable isotope dilution LC-MS/MS is in principle more accurate and specific and can be employed for multiple vitamin D metabolites [2]. However, time-consuming methods are required for extraction and purification of vitamin D and its analogs, which have restricted the use of LC-MS approaches. The analysis of plasma and serum vitamin D is very challenging because the concentrations are often in the low ng/mL (low nM) range [2]. A turbulent-flow on-line extraction-based 2D method was reported recently for the analysis of 25-hydroxy vitamin D3 (25OHD3), and 25-hydroxy vitamin D2 (25OHD2), the two most important vitamin D metabolites [70]. The second dimension was conducted using a Hypersil gold aQ LC column (Thermo Fisher) coupled with APCI-MS. Assays were linear from 3.0 to 283 nmol/L for 25OHD3 and from 4.6 to

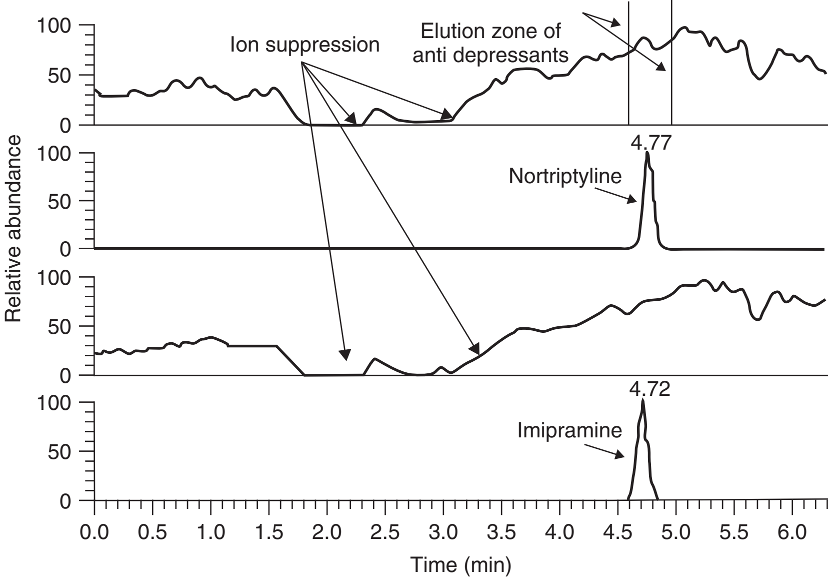


Figure 16.6 Study of the ion suppression phenomenon by means of continuous infusion of 20 antidepressants at 100 ng/mL and parallel injection of extracts of blank serum samples: the example of the nortriptyline and imipramine extracted-ion chromatograms. *Source*: Reprinted with permission from Sauvage *et al*., 2006 [69].

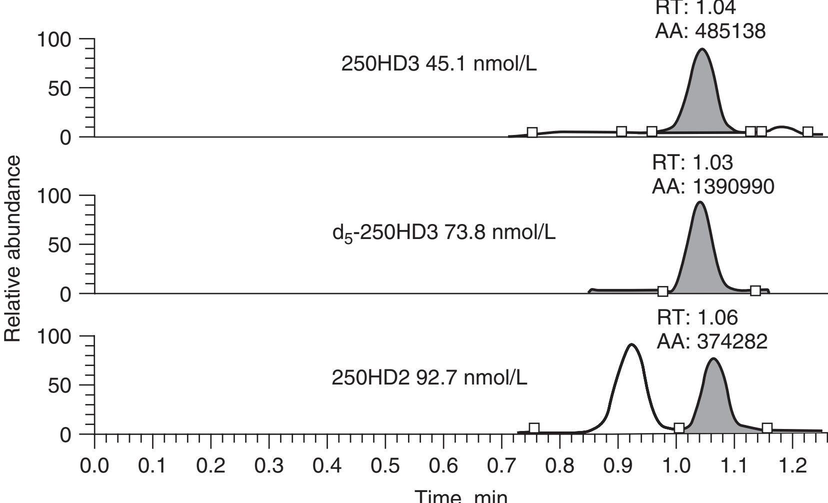


Figure 16.7 Chromatogram from 2D turbulent-flow LC-MS analysis of a patient's serum sample with mid-levels of 25OHD2 and 25OHD3. *Source*: Reprinted with permission from Bunch *et al*., 2009 [70].

277.9 nmol/L for 25OHD3 with chromatographic run times of only 1 min (Fig. 16.7). Importantly, no carryover or ion suppression was observed, lending further credence to the concept that APCI is less susceptible to matrix effects than other ionization techniques [13].

An area that offers significant future applications is in the field of metabolomic analyses because it is very difficult to analyze the polar metabolites without causing significant suppression of ionization as well as contamination of the ion source from plasma constituents [8]. A recent study has compared the use of protein precipitation with a 2D turbulent-flow LC-MS/MS approach [5]. This comparison has shown that turbulent-flow chromatography could be effectively used with a substantial reduction of the time required for sample handling. Furthermore, there were substantial differences in the overall metabolite profiles for the two methods. This was ascribed to greatly reduced amounts of phospholipids (ca. 10-fold reduction) for the turbulent-flow methodology compared with protein-precipitated samples. Interestingly, the majority of ions were detected in the mass range of m/z 200–400 compared with m/z 400–600 for the protein-precipitated samples. It was suggested that this could have been due in part to the loss of small molecules bound to proteins in the turbulent-flow method and suggest that a combination of both methods could maximize differences in metabolomic characterizations.

An impressive example of the sensitivity that can be attained with 2D turbulent-flow LC-MS/MS methodology was reported recently for the analysis of plasma testosterone [71]. Total analysis time was 1.15 min per sample when using the multiplexing system that was developed. Testosterone concentrations were measured directly from 150 μL of serum or plasma without derivatization or liquid–liquid extraction. The lower limit of quantification was 300 fg/mL and the assay was linear up to 200 ng/mL. Furthermore, the method compared very well with an established radioimmunoassay. Therefore,

this high throughput method appears to be suitable for quantifying the expected low-testosterone concentrations seen in women, children, and hypogonadal males and for monitoring testosterone suppressive therapy in prostate cancer patients.

In the field of drug disposition, a recent article described a fully automated turbulent-flow 2D LC-MS/MS method for the detection of tricyclic antidepressant drugs (amitriptyline, desipramine, imipramine, and nortriptyline) in serum [72]. Human serum and an internal standard were injected directly onto a turbulent-flow Cyclone-P on-line SPE column (0.5 × 50 mm, Thermo Fisher). Following removal of serum proteins and other components the analytes were transferred to a Hypersil Gold C-18 (50 × 3 mm, Thermo Fisher) analytical column. Gradient elution was performed with water and acetonitrile each containing 0.1% formic acid. The antidepressant drugs were ionized and detected over a 3.5 min analysis time using electrospray-ionization/MS. Matrix effects were characterized and carryover, precision, linearity, recovery, and limits of detection and quantitation were evaluated. The limits of detection and quantitation for all drugs were <3 ng/mL and <20 ng/mL, respectively. Recoveries were between 97% and 114%. On the basis of the validation data, this specific, sensitive fully automated method can be employed for rapid quantitation of tricyclic antidepressants in serum.

16.7 CONCLUSIONS AND FUTURE DIRECTIONS

Over the last decade, the direct analysis of biofluid samples has become a realistic alternative to time-consuming off-line extraction and purification procedures [11,12,15–20, 65]. This evolution has resulted in essentially two different approaches (SPE and turbulent-flow chromatography) for use in combination with MS instrumentation. On-line SPE coupled with conventional LC-MS has become a very mature method with the ability to conduct efficient on-line purification coupled with disposable columns [11,29,30]. The solid-phase sorbents can be of the conventional modified silica supports as well as RAM and monolithic sorbents. Intriguing future directions of this methodology will be the application of IM-RAM sorbents for the highly selective analysis of small molecules [49] and the use of μ-SPE-columns [38,40,41].

Turbulent-flow chromatography has become a flexible high sensitivity system, which employs a 2D LC-MS/MS approach [19]. The turbulent-flow component is employed to concentrate the sample and to remove proteins and phospholipids [5]. The resulting extract is then separated on an analytical column, which provides enormous flexibility in terms of flow rate and column type. The analytical column is then linked to a suitable ionization technique for high sensitivity MS/MS analysis. A series of novel applications has appeared recently for the analysis of both drugs and endogenous metabolites, suggesting that this methodology could hold the key for future complex metabolomic applications [5]. A particularly attractive feature of the turbulent-flow 2D LC-MS/MS system is the ability to remove constituents from the biofluid that cause suppression or enhancement of ionization [69]. This makes it a realistic proposition to conduct analyses in the subpicogram range [70], which raises the possibility that turbulent-flow methodology can be employed in the future for the routine direct analysis of plasma and serum for low abundance endogenous metabolites such as vitamins and steroids [70,71]. Similarly, the recent application of 2D turbulent-flow LC-MS/MS to the analysis of tricyclic antidepressants in serum [72] suggests that this could be a very

useful technique in the future for the direct analysis of drugs and their metabolites in plasma and serum.

Recently, a novel system incorporating an automated direct analysis in real time (DART) ionization source coupled with a triple-quadrupole mass spectrometer was developed and evaluated for direct analysis of drugs in biofluids [73]. This system can potentially eliminate the need for sample cleanup and chromatographic separations. Additional pumping of the mass spectrometer ion source was employed to compensate for the increased vacuum load from the high flow helium introduced by the DART. This resulted in an improvement of detection sensitivity by a factor of 10- to 100-fold and minimized matrix effects on a diversified group of analytes. The system was employed for the direct analysis of biofluid samples with similar results to those obtained with conventional LC-MS/MS methods. Therefore, this new automated DART-triple quadrupole mass spectrometer system has significant potential future for high throughput direct biofluid analyses [73]. Finally, the μ-SPE-based RapidFire System might be a useful tool for high throughput MS quantitation of *in vivo* biofluid samples in situations where large sets of samples justify the up-front burden of method development [44]. Clearly, direct analysis of biofluid samples has come of age and is now a realistic alternative to more time-consuming off-line purification procedures particularly when large numbers of sampled are involved.

ACKNOWLEDGMENTS

I acknowledge the support of NIH grants U01ES016004 and P30ES013508 and PA Department of Health grant PA4100038714.

REFERENCES

1. Prakash C, Shaffer CL, Nedderman A. Analytical strategies for identifying drug metabolites. Mass Spectrom Rev 2007;26:340–369.
2. Vogeser M, Parhofer KG. Liquid chromatography tandem-mass spectrometry (LC-MS/MS)–technique and applications in endocrinology. Exp Clin Endocrinol Diabetes 2007;115:559–570.
3. Blair IA. Analysis of estrogens in serum and plasma from postmenopausal women: past, present, and future. Steroids 2010;75:297–306.
4. Ciccimaro E, Blair IA. Stable-isotope dilution LC–MS for quantitative biomarker analysis. Bioanalysis 2010;2:311–341.
5. Michopoulos F, Edge AM, Theodoridis G, *et al*. Application of turbulent flow chromatography to the metabonomic analysis of human plasma: comparison with protein precipitation. J Sep Sci 2010;33:1472–1479.
6. Mullett WM. Determination of drugs in biological fluids by direct injection of samples for liquid-chromatographic analysis. J Biochem Biophys Methods 2007;70:263–273.
7. Xu RN, Fan L, Rieser MJ, *et al*. Recent advances in high-throughput quantitative bioanalysis by LC-MS/MS. J Pharm Biomed Anal 2007;44:342–355.
8. Medvedovici A, Albu F, David V. Handling drawbacks of mass spectrometric detection coupled to liquid chromatography in bioanalysis. J Liq Chrom Relat Tech 2010;33:1255–1286.
9. Shou WZ, Zhang J. Recent development in high-throughput bioanalytical support for *in vitro* ADMET profiling. Expert Opin Drug Metab Toxicol 2010;6:321–336.
10. Hsieh Y. HPLC-MS/MS in drug metabolism and pharmacokinetic screening. Expert Opin Drug Metab Toxicol 2008;4:93–101.

11. Chang MS, Ji Q, Zhang J, *et al*. Historical review of sample preparation for chromatographic bioanalysis: pros and cons. Drug Dev Res 2007;68:107–133.
12. Hsieh Y. Direct plasma analysis systems. In: Korfmacher WA, editor. Using mass spectrometry for drug metabolism studies. Boca Raton (FL): CRC Press; 2005. 151–174.
13. Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. Anal Chem 2003;75:3019–3030.
14. King R, Bonfiglio R, Fernandez-Metzler C, *et al*. Mechanistic investigation of ionization suppression in electrospray ionization. J Am Soc Mass Spectrom 2000;11:942–950.
15. Hsieh Y, Wang G, Wang Y, *et al*. Direct plasma analysis of drug compounds using monolithic column liquid chromatography and tandem mass spectrometry. Anal Chem 2003;75:1812–1818.
16. Souverain S, Rudaz S, Veuthey JL. Use of monolithic supports for fast analysis of drugs and metabolites in plasma by direct injection. Chromatographia 2003;57:569–575.
17. Sadilek P, Satinsky D, Solich P. Using restricted-access materials and column switching in high-performance liquid chromatography for direct analysis of biologically-active compounds in complex matrices. Trac, Trends Anal Chem 2007;26:375–384.
18. Santos-Neto AJ, Markides KE, Sjoberg PJR, *et al*. Capillary column switching restricted-access media-liquid chromatography-electrospray ionization-tandem mass Spectrometry system for simultaneous and direct analysis of drugs in biofluids. Anal Chem 2007;79:6359–6367.
19. Chassaing C, Robinson S. Turbulent flow chromatography: an evolving solution for bioanalysis. Chromatogr Today 2009;2:20–24.
20. Yang GL, Liu HY. Application of monolithic stationary phases in solid-phase extraction and pharmaceutical analysis. Curr Pharm Anal 2010;6:213–224.
21. Deng Y, Wu JT, Lloyd TL, *et al*. High-speed gradient parallel liquid chromatography/tandem mass spectrometry with fully automated sample preparation for bioanalysis: 30 seconds per sample from plasma. Rapid Commun Mass Spectrom 2002;16:1116–1123.
22. Mitchell RJ, Christian R, Hughes H, *et al*. The application of fully automated on-line solid phase extraction in bioanalysis. J Pharm Biomed Anal 2010;52:86–92.
23. Rodriguez-Mozaz S, Lopez de Alda MJ, Barcelo D. Advantages and limitations of on-line solid phase extraction coupled to liquid chromatography-mass spectrometry technologies versus biosensors for monitoring of emerging contaminants in water. J Chromatogr A 2007;1152:97–115.
24. Deng Y, Zhang H, Wu JT, *et al*. Tandem mass spectrometry with online high-flow reversed-phase extraction and normal-phase chromatography on silica columns with aqueous-organic mobile phase for quantitation of polar compounds in biological fluids. Rapid Commun Mass Spectrom 2005;19:2929–2934.
25. Chen LG, Wang H, Zeng QL, *et al*. On-line coupling of solid-phase extraction to liquid chromatography - a review. J Chromatogr Sci 2009;47:614–623.
26. Hennion MC. Solid-phase extraction: Method development, sorbents, and coupling with liquid chromatography. J Chromatogr A 1999;856:3–54.
27. Rossi DT, Zhang N. Automating solid-phase extraction: current aspects and future prospects. J Chromatogr A 2000;885:97–113.
28. Boos KS, Grimm CH. High-performance liquid chromatography integrated solid-phase extraction in bioanalysis using restricted access precolumn packings. Trac, Trends Anal Chem 1999;18:175–180.
29. Kuklenyik Z, Needham LL, Calafat AM. Measurement of 18 perfluorinated organic acids and amides in human serum using on-line solid-phase extraction. Anal Chem 2005; 77:6085–6091.
30. Koal T, Sibum M, Koster E, *et al*. Direct and fast determination of antiretroviral drugs by automated online solid-phase extraction-liquid chromatography-tandem mass spectrometry in human plasma. Clin Chem Lab Med 2006;44:299–305.

31. Souverain S, Rudaz S, Veuthey JL. Matrix effect in LC-ESI-MS and LC-APCI-MS with off-line and on-line extraction procedures. J Chromatogr A 2004;1058:61–66.
32. Goncalves JCS, Monteiro TM, Neves CSD, *et al*. On-line solid-phase extraction coupled with high-performance liquid chromatography and tandem mass spectrometry (SPE-HPLC-MS-MS) for quantification of bromazepam in human plasma - an automated method for bioequivalence studies. Ther Drug Monit 2005;27:601–607.
33. Alnouti Y, Li M, Kavetskaia O, *et al*. Method for internal standard introduction for quantitative analysis using on-line solid-phase extraction LC-MS/MS. Anal Chem 2006; 78:1331–1336.
34. Saber AL. On-line solid phase extraction coupled to capillary LC-ESI-MS for determination of fluoxetine in human blood plasma. Talanta 2009;78:295–299.
35. Ceglarek U, Kortz L, Leichtle A, *et al*. Rapid quantification of steroid patterns in human serum by on-line solid phase extraction combined with liquid chromatography-triple quadrupole linear ion trap mass spectrometry. Clin Chim Acta 2009;401:114–118.
36. Penning TM, Lee SH, Jin Y, *et al*. Liquid-chromatography mass spectrometry (LC-MS) of steroid hormone metabolites and its applications. J Steroid Biochem Mol Biol 2010; 121:546–555.
37. Wang QQ, Xiang SS, Jia YB, *et al*. An improved on-line solid phase extraction coupled HPLC-MS/MS system for quantification of Sifuvirtide in human plasma. J Chromatogr B 2010;878:1893–1898.
38. Quercia AK, LaMarr WA, Myung J, *et al*. High-throughput screening by mass spectrometry: comparison with the scintillation proximity assay with a focused-file screen of AKT1/PKB alpha. J Biomol Screen 2007;12:473–480.
39. Kool J, Lingeman H, Niessen W, *et al*. High throughput screening methodologies classified for major drug target classes according to target signaling pathways. Comb Chem High Throughput Screen 2010;13:548–561.
40. Forbes CD, Toth JG, Ozbal CC, *et al*. High-throughput mass spectrometry screening for inhibitors of phosphatidylserine decarboxylase. J Biomol Screen 2007;12:628–634.
41. Holt TG, Choi BK, Geoghagen NS, *et al*. Label-free high-throughput screening via mass spectrometry: a single cystathionine quantitative method for multiple applications. Assay Drug Dev Technol 2009;7:495–506.
42. Jonas M, LaMarr WA, Ozbal C. Mass spectrometry in high-throughput screening: a case study on acetyl-coenzyme a carboxylase using RapidFire–mass spectrometry (RF-MS). Comb Chem High Throughput Screen 2009;12:752–759.
43. Lim KB, Ozbal CC, Kassel DB. Development of a high-throughput online solid-phase extraction/tandem mass spectrometry method for cytochrome P450 inhibition screening. J Biomol Screen 2010;15:447–452.
44. Jian W, Romm MV, Edom RW, *et al*. Evaluation of RapidFire ultra-fast online SPE-MS/MS system for analysis of *in vivo* bioanalytical samples. Appl Pharm Anal (Baltimore, MD) 2010.
45. Desilets CP, Rounds MA, Regnier FE. Semipermeable-surface reversed-phase media for high-performance liquid chromatography. J Chromatogr 1991;544:25–39.
46. Cassiano NM, Barreiro JC, Moraes MC, *et al*. Restricted-access media supports for direct high-throughput analysis of biological fluid samples: review of recent applications. Bioanalysis 2009;1:577–594.
47. Cassiano NM, Lima VV, Oliveira RV, *et al*. Development of restricted-access media supports and their application to the direct analysis of biological fluid samples via high-performance liquid chromatography. Anal Bioanal Chem 2006;384:1462–1469.
48. Souverain S, Rudaz S, Veuthey JL. Restricted access materials and large particle supports for on-line sample preparation: an attractive approach for biological fluids analysis. J Chromatogr B 2004;801:141–156.

49. Wa CL, Mallik R, Hage DS. Development of immunoaffinity restricted access media for rapid extractions of low-mass analytes. Anal Chem 2008;80:8751–8762.
50. Mangal D, Vudathala DK, Park JH, *et al*. Analysis of 7,8-Dihydro-8-oxo-2′-deoxyguanosine in cellular DNA during oxidative stress. Chem Res Toxicol 2009;22:788–797.
51. Jarmalaviciene R, Kornysova O, Bendokas V, *et al*. Restricted-access media development for direct analysis of drugs in biofluids using capillary liquid chromatography. Anal Bioanal Chem 2008;391:2323–2328.
52. Yamamoto E, Kato T, Mano N, *et al*. Effective on-line extraction of drugs from plasma using a restricted-access media column in column-switching HPLC equipped with a dilution system: application to the simultaneous determination of ER-118585 and its metabolites in canine plasma. J Pharm Biomed Anal 2009;49:1250–1255.
53. Gomes RF, Cassiano NM, Pedrazzoli J, *et al*. Two-dimensional chromatography method applied to the enantiomeric determination of lansoprazole in human plasma by direct sample injection. Chirality 2010;22:35–41.
54. Gillings N. A restricted access material for rapid analysis of [C-11]-labeled radiopharmaceuticals and their metabolites in plasma. Nucl Med Biol 2009;36:961–965.
55. Rao RN, Shinde DD, Agawane SB. Rapid determination of rifaximin in rat serum and urine by direct injection on to a shielded hydrophobic stationary phase by HPLC. Biomed Chromatogr 2009;23:563–567.
56. Cass QB, Ferreira GT. A method for determination of the plasma levels of modafinil enantiomers, (+/−)-modafinic acid and modafinil sulphone by direct human plasma injection and bidimensional achiral-chiral chromatography. J Pharm Biomed Anal 2008;46:937–944.
57. Klodzinska E, Moravcova D, Jandera P, *et al*. Monolithic continuous beds as a new generation of stationary phase for chromatographic and electro-driven separations. J Chromatogr A 2006;1109:51–59.
58. Hayes JD, Malik A. Sol-gel monolithic columns with reversed electroosmotic flow for capillary electrochromatography. Anal Chem 2000;72:4090–4099.
59. Zeng H, Deng Y, Wu JT. Fast analysis using monolithic columns coupled with high-flow on-line extraction and electrospray mass spectrometric detection for the direct and simultaneous quantitation of multiple components in plasma. J Chromatogr B 2003;788:331–337.
60. Wu JT, Zeng H, Deng Y, *et al*. High-speed liquid chromatography/tandem mass spectrometry using a monolithic column for high-throughput bioanalysis. Rapid Commun Mass Spectrom 2001;15:1113–1119.
61. Saunders KC, Ghanem A, Hon WB, *et al*. Separation and sample pre-treatment in bioanalysis using monolithic phases: A review. Anal Chim Acta 2009;652:22–31.
62. Zang X, Luo R, Song N, *et al*. A novel on-line solid-phase extraction approach integrated with a monolithic column and tandem mass spectrometry for direct plasma analysis of multiple drugs and metabolites. Rapid Commun Mass Spectrom 2005;19:3259–3268.
63. Ayrton J, Dear GJ, Leavens WJ, *et al*. The use of turbulent flow chromatography mass spectrometry for the rapid, direct analysis of a novel pharmaceutical compound in plasma. Rapid Commun Mass Spectrom 1997;11:1953–1958.
64. Jemal M. High-throughput quantitative bioanalysis by LC/MS/MS. Biomed Chromatogr 2000;14:422–429.
65. Wu JT, Zeng H, Qian M, *et al*. Direct plasma sample injection in multiple-component LC-MS-MS assays for high-throughput pharmacokinetic screening. Anal Chem 2000;72:61–67.
66. Chassaing C, Luckwell J, Macrae P, *et al*. Direct analysis of crude plasma samples by turbulent flow chromatography/tandem mass spectrometry. Chromatographia 2001;53:122–130.
67. Remane D, Wissenbach DK, Meyer MR, *et al*. Systematic investigation of ion suppression and enhancement effects of fourteen stable-isotope-labeled internal standards by their native analogues using atmospheric-pressure chemical ionization and electrospray ionization and the relevance for multi-analyte liquid chromatographic/mass spectrometric procedures. Rapid Commun Mass Spectrom 2010;24:859–867.

68. Wang S, Cyronak M, Yang E. Does a stable isotopically labeled internal standard always correct analyte response? A matrix effect study on a LC/MS/MS method for the determination of carvedilol enantiomers in human plasma. J Pharm Biomed Anal 2007;43:701–707.
69. Sauvage FL, Gaulier JM, Lachatre G, *et al*. A fully automated turbulent-flow liquid chromatography-tandem mass spectrometry technique for monitoring antidepressants in human serum. Ther Drug Monit 2006;28:123–130.
70. Bunch DR, Miller AY, Wang SH. Development and validation of a liquid chromatography-tandem mass spectrometry assay for serum 25-hydroxyvitamin D2/D3 using a turbulent flow online extraction technology. Clin Chem Lab Med 2009;47:1565–1572.
71. Salameh WA, Redor-Goldman MM, Clarke NJ, *et al*. Validation of a total testosterone assay using high-turbulence liquid chromatography tandem mass spectrometry: Total and free testosterone reference ranges. Steroids 2010;75:169–175.
72. Breaud AR, Harlan R, Di Bussolo JM, *et al*. A rapid and fully-automated method for the quantitation of tricyclic antidepressants in serum using turbulent-flow liquid chromatography-tandem mass spectrometry. Clin Chim Acta 2010;411:825–832.
73. Yu S, Crawford E, Tice J, *et al*. Bioanalysis without sample cleanup or chromatography: the evaluation and initial implementation of direct analysis in real time ionization mass spectrometry for the quantification of drugs in biological matrixes. Anal Chem 2009;81:193–202.