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Review

Recent advances in high-throughput quantitative bioanalysis by LC–MS/MS

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Abstract

Liquid chromatography linked to tandem mass spectrometry (LC–MS/MS) has played an important role in pharmacokinetics and metabolism studies at various drug development stages since its introduction to the pharmaceutical industry. This article reviews the most recent advances in sample preparation, separation, and the mass spectrometric aspects of high-throughput quantitative bioanalysis of drug and metabolites in biological matrices. Newly introduced techniques such as ultra-performance liquid chromatography with small particles (sub-2 μm) and monolithic chromatography offer improvements in speed, resolution and sensitivity compared to conventional chromatographic techniques. Hydrophilic interaction chromatography (HILIC) on silica columns with low aqueous/high organic mobile phase is emerging as a valuable supplement to the reversed-phase LC–MS/MS. Sample preparation formatted to 96-well plates has allowed for semi-automation of off-line sample preparation techniques, significantly impacting throughput. On-line solid-phase extraction (SPE) utilizing column-switching techniques is rapidly gaining acceptance in bioanalytical applications to reduce both time and labor required to produce bioanalytical results. Extraction sorbents for on-line SPE extend to an array of media including large particles for turbulent flow chromatography, restricted access materials (RAM), monolithic materials, and disposable cartridges utilizing traditional packings such as those used in Spark Holland systems. In the end, this paper also discusses recent studies of matrix effect in LC–MS/MS analysis and how to reduce/eliminate matrix effect in method development and validation.

Keywords: LC–MS/MS bioanalysis; High-throughput; Sample preparation; On-line extraction; Ultra-performance liquid chromatography; Monolithic chromatography; Hydrophilic interaction chromatography; Matrix effect

Contents

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1. Introduction

Bioanalytical functions in the pharmaceutical industry are constantly under pressure to reduce development times. This is often accompanied with an increase in the number of biological samples requiring pharmacokinetic analysis and a decrease in the desired quantitation levels. Hyphenated techniques are examples of new tools that pharmaceutical industry adopted for developing fast and cost-effective analytical methods. One of the most prevalent hyphenated techniques, liquid chromatography-tandem mass spectrometry (LC–MS/MS), has led to major breakthroughs in the field of quantitative bioanalysis since the 1990s due to its inherent specificity, sensitivity, and speed. It is now generally accepted as the preferred technique for quantitating small molecule drugs, metabolites, and other xenobiotic biomolecules in biological matrices (plasma, blood, serum, urine, and tissue).

Samples from biological matrices are usually not directly compatible with LC–MS/MS analyses. Sample preparation has traditionally been done using protein precipitation (PPT), liquid–liquid extraction (LLE), or solid-phase extraction (SPE). Manual operations associated with these processes are very labor intensive and time-consuming. Parallel sample processing in 96-well format using robotic liquid handlers has significantly shortened the time analysts have to spend in the laboratory for sample preparation. An alternative sample extraction method that has generated a lot of interest in recent years is the direct injection of plasma using an on-line extraction method. A major advantage of on-line SPE over off-line extraction techniques is that the sample preparation step is embedded into the chromatographic separation and thus eliminates most of the sample preparation time traditionally performed at the bench.

Fast gradients and short columns were first utilized in early applications of high-throughput LC–MS/MS assays to reduce run times. Better understanding of how matrix effects can compromise the integrity of bioanalytical methods has re-emphasized the need for adequate chromatographic separation of analytes from endogenous biological components in quantitative bioanalysis using LC–MS/MS analysis. New developments from chromatographic techniques such as ultra-performance liquid chromatography with sub-2 mm particles and monolithic chromatography are showing promise in delivering higher speed, better resolution and sensitivity for high-throughput analysis while minimizing matrix effects.

2. Scope

The impressive growth in LC–MS/MS applications for quantitative bioanalysis has been documented in hundreds of articles in just the past 5 years, and a number of reviews dealing with one or more aspects of quantitative LC–MS/MS bioanalysis have been published [1–7]. In this paper, we focus on publications related to technology development for throughput improvement, associated applications, and discussions of key developments in quantitative analysis from 2002 to 2006.

3. Sample preparation

3.1. Automated off-line sample preparation

Adequate sample preparation is a key aspect of quantitative bioanalysis and can often be the cause of bottlenecks during high-throughput analysis. Sample preparation techniques in 96-well format have been well adopted in high-throughput quantitative bioanalysis. The techniques that can use the format include liquid–liquid extraction (LLE), solid-phase extraction (SPE), and protein precipitation (PPT). Typically, liquid transfer steps, including preparation of calibration standards and quality control samples as well as the addition of the internal standard (IS), were performed automatically using robotic liquid handling workstations for parallel sample processing.

The increasing demand for high-throughput causes a unique situation of balancing cost versus analysis speed as each sample preparation technique offers unique advantages. Dilute and shoot and protein precipitation (PPT) are popular techniques because of their simplicity. Sample preparation with PPT is widely used in bioanalysis of plasma samples. The method has been extended to quantitation of drug and metabolites from whole blood. Koseki et al. have developed a sensitive and specific LC–MS/MS method for the simultaneous determination of cyclosporine A (CsA) and its three main metabolites (AM1, AM4N, and AM9) in human blood [8]. Following protein precipitation, supernatant was directly injected into the LC/MS system. Overall, PPT offers a generic and fast sample preparation technique that can be easily automated. However, when analyzing supernatant from a plasma sample using PPT, salts and endogenous material are still present and can cause ion suppression or enhancement that will lead to higher variation from sample to sample.

Solid-phase extraction (SPE) has been automated with a broad range of sorbents (silica based or polymer based). Mixed-mode polymer-based sorbents (e.g., Waters Oasis MCX cartridge) were introduced in the late 1990's for the isolation of drugs with ionizable functional groups from biological fluids. The extraction procedures can be a generic protocol or can be optimized if better sample clean up is desired. The use of solid-phase extraction (SPE) often gives superior results to those by a PPT method but may not be as cost-effective as PPT due to the labor and material costs associated with the process. Mallet et al. [9] described a novel 96-well SPE plate that was designed to minimize the elution volume required for quantitative elution of analytes. The plate was packed with 2 mg of a high-capacity SPE sorbent that allows loading of up to 750 μL of plasma. The novel design permitted elution with as little as 25 μL solvent. Therefore, the plate can offer up to a 30-fold increase in sample concentration. The evaporation and reconstitution step that is typically required in SPE is avoided due to the concentrating ability of the sorbent. Yang et al. [10] developed a sensitive μElution solid-phase extraction (SPE) LC–MS/MS method for the determination of M+4 stable isotope labeled cortisone and cortisol in human plasma. In the method, analytes were extracted from 0.3 mL of human plasma samples using a Waters Oasis HLB 96-well

μElution SPE plate with 70 μL methanol as the elution solvent. The lower limit of quantitation was 0.1 ng/mL and the linear calibration range was from 0.1 to 100 ng/mL for both analytes.

Liquid–liquid extraction (LLE) gives excellent sample clean up but poses engineering difficulties for use in an automated high-throughput format. Several groups have developed different approaches to solve mixing and phase separation problems typically seen in a 96-well LLE method [11–18]. By using vigorous vortexing after well-controlled heat-sealing, or using repeated aspiration and dispensing by robotic liquid handler, common extraction solvents such as methyl *t*-butyl ether (MTBE) or ethyl acetate can be used in routine extraction of plasma, blood, or tissue samples. Wang et al. [12] have developed and validated a 96-well LLE assay, using LC/MS/MS in the atmospheric pressure chemical ionization (APCI) mode for simultaneous quantification of two human immunodeficiency virus (HIV) protease inhibitors, lopinavir and ritonavir, in human plasma. The sample preparation consisted of liquid–liquid extraction with a mixture of hexane and ethyl acetate using 100 μL of plasma. The method was validated over the concentration ranges of 19–5300 ng/mL for lopinavir and 11–3100 ng/mL for ritonavir. Zhang et al. [13] presented a 96-well LLE method for measuring zotarolimus drug concentrations from drug-eluting stents in swine artery samples. The authors used 100% swine blood as the homogenization solution to improve the consistency of the extraction recovery and stability of the zotarolimus in tissue homogenates. Xu et al. [17] described a 96-well liquid–liquid back extraction LC–MS/MS method for determination of a basic and polar drug candidate from human plasma samples. The analyte was extraction from plasma using MTBE first, followed by a back extraction from the organic phase into a small volume of acidified water. A linear range of 0.38-95.02 ng/mL was established for the method with good accuracy and precision. A similar approach was reported by Bolden et al. [18] in a liquid–liquid back extraction (LLE) procedure for sample preparation of dextromethorphan (DEX), an active ingredient in many over-the-counter cough formulations, and dextrorphan (DOR), an active metabolite of DEX, in human plasma. After back extraction, the acidified water isolated from the back extraction was analyzed directly by LC–MS/MS, eliminating the need for a dry-down step.

Combinations of sample preparation techniques have been developed to achieve desired sample extract purity with high-throughput. Xue et al. [19] investigated a simplified protein precipitation/mixed-mode cation-exchange solid-phase extraction (PPT/SPE) procedure. A mixture of acetonitrile and methanol along with formic acid was used to precipitate plasma proteins prior to selectively extracting the basic drug. After vortexing and centrifugation, the supernatants were directly loaded onto an unconditioned Oasis MCX μElution 96-well extraction plate, where the protonated drug was retained on the negatively charged sorbent while interfering neutral lipids, steroids or other endogenous materials were eliminated. Additional wash steps were deemed unnecessary and not performed prior to sample elution.

3.2. On-line solid-phase extraction

The on-line SPE technique offers speed, high sensitivity by the pre-concentration factor, and low extraction cost per sample, but typically requires the use of program controlled switch valves and column re-configurations. However, the on-line technique can be fully automated. Several generic approaches have recently been developed for on-line sample extraction coupled to LC–MS [7,24,28,32]. Different extraction supports allowing direct injection of biological fluids or extracts in various applications [20–42] are summarized in Table 1. These extraction supports or sorbents include restricted access media (RAM), large-size particle, monolithic material, and disposable cartridges. Most on-line SPE approaches use column-switching to couple with the analytical columns. Various column dimensions can be configured for the fast analysis of drug and their metabolites in biological matrix at the nanogram per mililiter level or lower.

One commercial automated on-line SPE system is the Symbiosis system manufactured by Spark Holland. It includes an autosampler (Reliance), two binary HPLC pumps, an on-line SPE unit with two high-pressure solvent delivery pumps (HDPs) and a combined valve systems to direct fluid for different steps of SPE. At the beginning of each run, an on-line SPE cartridge is loaded into the unit. After a conditioning step with high organic solvent and an equilibrium step with low organic aqueous solution, a sample is injected onto the cartridge and washed with aqueous solution. Proteins and other matrix materials from the sample are removed during the washing step. Analyte of interest is then eluted onto the analytical column and detected by mass spectrometry. During the sample elution step, a second sample is loaded to a new on-line SPE cartridge for the next analysis. In this parallel mode, the sample analysis cycle time approximates the LC run time without the time required for the SPE procedures. Since the on-line SPE cartridge is disposable and each sample uses a new cartridge, the carry-over problem from the extraction cartridge is eliminated. A generic method for the fast determination of a wide range of drugs in serum or plasma has been presented for the Spark Holland system [40]. The method comprises generic solid-phase extraction with HySphere particles, on-line coupled to gradient HPLC with tandem mass spectrometric detection. The optimized generic SPE-LC–MS/MS protocol was evaluated for 11 drugs with different physicochemical properties. Good quantification for 10 out of 11 of the pharmaceuticals in serum or plasma could be readily achieved. The quantitative assays gave recoveries better than 95%, lower quantification limits of 0.2–2.0 ng/mL, acceptable precision and accuracy and good linearity over 2–4 orders of magnitude. Carry-over was determined to be in the range of 0.02–0.10%, without optimization. An approach for on-line introduction of internal standard (IS) for quantitative analysis was developed on the Spark Holland system [41]. In this approach, analyte and IS were introduced into the sample injection loop in different steps. Analyte was introduced into the injection loop using a conventional autosampler (injector) needle pickup from a sample vial. IS was introduced into the sample injection loop on-line from a microreservoir con-

Table 1
A summary of commonly used extraction supports for on-line SPE and associated applications

Compound	Extraction support	Pre-treatment*	System	Analytical column	Isotope-labeled IS used?	Total run time	Low limit of quantitation	Ref
Rofecoxib (Vioxx) in rat plasma	Licrospher 60, RP-18 ADS, 0.76 mm × 50 mm, 40–63 μm	No	Home-built	Chromolith RP-18e 50 mm × 4.6 mm	No	5 min	40 ng/mL	[20]
Compound A (proprietary) in rat plasma	C18 RAM-ADS (Alkyl diol Silica), 25 μm 25 mm × 4 mm	No	Home-built	Not used	No	8 min	1 ng/mL	[21]
Cyclosporin A, Tacrolimus, and Sirolimus in human blood	Cohesive Cyclone 50 mm × 1 mm polymeric column, 50 μm	Protein precipitation, no dry-down step	Cohesive Technologies	Phenomenex Phenyl-Hexyl-RP, 50 mm × 2.1 mm, 5 μm	No	3 min	4.5 ng/mL for Cyclosporin A, 0.2 ng/mL for Tacrolimus, and 0.4 ng/mL for Sirolimus	[22]
Mycophenolic acid (MPA) and glucuronide metabolite (MPAG) in human plasma	Applied Biosystems Poros Perfusion column 30 mm × 1 mm	Protein precipitation, no dry-down step	Cohesive Technologies	Phenomenex Phenyl-Hexyl-RP, 50 mm × 2.1 mm, 5 μm	No	5 min	50 ng/mL for MPA and 100 ng/mL for MPAG	[23]
MK-0767, a dual PPAR alpha/gamma agonist in human plasma	Cohesive Turboflow C18 column 50 mm × 1.0 mm, 50 μm	No	Cohesive 2300 HTLC Turboflow system	ThermoHypersil Keystone ODS Hypersil C18, 5 μm, 30 mm × 4.6 mm	No	65 s	4 ng/mL	[24]
Dextrorphan and dextromethorphan in human plasma	Cohesive Cyclone C18 50 mm × 1 mm, 50 μm	No	Cohesive 2300 HTLC Turboflow system	Chromolith RP-18e 50 mm × 4.6 mm	No	1.5 min	5 ng/mL	[25]
Multiple compounds in human plasma	Cohesive Cyclone 50 mm × 0.5 mm column, 50 μm	No	Cohesive Aria TX-2 system	Zorbax C18, 50 mm × 4.6 mm	No	3.7 min	1 ng/mL	[26]
Ten compounds in Caco-2 cell based permeability study samples	Cohesive Cyclone trap column 50 mm × 0.5 mm column, 50 μm	No	Cohesive Aria TX-2 system	Waters Atlantis 50 mm × 2.1 mm	No	3.5 min	10–2500 nM	[27]
Eight analytes (Indiplon, Verapamil, et al.) in plasma	Phenomenex Strata-X SPE column 20 mm × 2.1 mm, 25 μm	No	Home-built	Chromolith RP-18e 50 mm × 4.6 mm	No	2.8 min	1.95 ng/mL	[28]
Piritramide in human urine	Oasis HLB extraction column 25 μm, 20 mm × 2.1 mm	No,	Home-built	Grom Sil 120 ODS-3 CP column, 5 μm, 150 mm × 2 mm	Yes	8.5 min	0.5 ng/mL	[29]
Terbutaline enantiomers in human plasma	Oasis HLB extraction columns 50 mm × 1.0 mm, 25 μm	No	Home-built	Chirobiotic T CSP, 5 μm, 100 mm × 4.6 mm	No	5.5 min	1 ng/mL	[30]
(*R*)- and (*S*)-propranolol in rat plasma	Oasis HLB extraction columns 0 mm × 1.0 mm, 25 μm	No	Home-built	Chirobiotic T CSP, 5 μm, 100 mm × 4.6 mm	No	10 min	2 ng/mL	[31]
Amprenavir and Atazanavir in human plasma	Chromolith C18 4.6 mm × 10 mm	Protein precipitation, no dry-down step	Home-built	Phenomenex Luna C18(2), 150 mm × 2.0 mm	No	4 min	2.77 ng/mL for Atazanavir, and 4.50 ng/mL for Amprenavir	[32]
Propranolol and Diclofenac in rat plasma	C18 HD 10 mm × 2 mm	No	Symbiosis, Spark Holland	C18 Luna column 50 mm × 2.1 mm, 5 μm, and C18 Chromolith column 50 mm × 2.1 mm,	No	2 min for Chromolith column and 4 min for Luna column	1 ng/mL	[33]

Table 1 (*Continued*)

Compound	Extraction support	Pre-treatment*	System	Analytical column	Isotope-labeled IS used?	Total run time	Low limit of quantitation	Ref
Multiple anti-retroviral drugs in human plasma	HySphere C18 HD	No	Symbiosis, Spark Holland	Synergy Max-RP, 150 mm × 2 mm, 4 μm,	No	3.3 min	6.5 ng/mL	[34]
Cortisol and 6β-hydroxycortisol in urine	HySphere C18 HD, 7 μm	No	Prospekt-2, Spark Holland	Symmetry Shield RP-18 column 100 mm × 2.1 mm, 3.5 μm,	No	9 min	1 ng/mL for 6β-hydroxycortisol and 0.2 ng/mL cortisol	[35]
Talinolol in human plasma	C8 End Capped (10 mm × 2 mm) cartridge	Protein precipitation, no dry-down step	Prospekt-2, Spark Holland	XTerra C18, 50 mm × 4.6 mm	No	4.8 min	2.5 ng/mL	[36]
Brostallicin in human plasma	HySphere Resin SH cartridges (10 mm × 2 mm)	No	Prospekt-2, Spark Holland	Platinum Cyano column (100 mm × 4.6 mm, 3.6 μm)	Yes	8 min	0.124 ng/mL	[37]
Clozapine and metabolites in human serum	HySphere C18 HD, 7 μm, 10 mm × 2 mm and HySphere-CN, 7 μm, 10 mm × 2 mm	No	Prospekt-2, Spark Holland	Zorbax Eclipse XDB-C18, 3.5 μm, two sizes tested	No	2.2 min	0.15–0.3 ng/mL	[38]
8-Oxo-7,8-dihydro-2′-deoxyguanosine in human urine	Inertsil ODS-3 column, 5 μm, 50 mm × 4.6 mm	No	Home-built	YMC Polyamine-II endcapped column, 5 μm, 150 mm × 4.6 mm	Yes	10 min	0.019 ng/mL	[39]

* A "No" in this column indicates pre-treatment was limited to sample transfer, internal standard addition, dilution, and centrifugation steps only.

taining the IS solution. As a result, both analyte and IS were contained in the sample loop prior to the injection into the column. The authors demonstrated comparable accuracy and precision to those obtained using off-line IS introduction (i.e., IS and analyte were pre-mixed before injection) while maintaining chromatographic parameters (i.e., analyte and IS elution time and peak width). This new technique was applied for direct analysis of model compounds in rat plasma using on-line solid-phase extraction (SPE) LC–MS/MS quantification. On-line IS introduction allows for non-volumetric sample (plasma) collection and direct analysis without the need of measuring and aliquoting a fixed sample volume prior to the on-line SPE-LC–MS/MS. The method enables direct sample (plasma) analysis without any sample manipulation and preparation. Koal et al. [34] developed a method for quantitation of 7 protease inhibitors and two non-nucleoside reverse transcriptase inhibitors in patient plasma samples. Only a sample dilution step was used to dilute samples and add internal standard before the analysis. Run time was only 3.3 min per sample and 6.6 min for the first sample. Alnouti et al. [33] reported another study with Symbiosis system connected to a Luna C18 analytical column or a Chromolith C18 monolithic column for analysis of two model compounds. Rat plasma spiked with the analytes was diluted with internal standard and injected directly into the system. Method development including on-line SPE cartridge selection and extraction condition optimization was performed by the Symbiosis system automatically. The total cycle time of 4 min with the Luna C18 column was reported. The run time was reduced to 2 min per sample for the monolithic column without compromising the quality and validation criteria of the method.

On-line SPE with high flow rate has been achieved by using extraction columns packed with large diameter particles. The extraction flow rate is typically set to 4–6 mL/min. Sample extraction occurs with very high solvent linear velocity without significant backpressure. Turbulent flow chromatography (TFC) columns marketed by Cohesive Technologies are widely used for this purpose. Turbulent flow in the extraction column results in rapid binding of small molecules to the absorbent while proteins being removed from the sample matrix. Minimum or no sample pre-treatment is required and significant sample preparation time is saved. Smalley et al. [27] reported a method using turbulent flow chromatography to analyze Caco-2 cell based permeability study samples. Ten compounds could be analyzed simultaneously in a cassette mode. The standard curve range for most compounds was 10–2500 nM with regression coefficients (r^2) greater than 0.99 for all compounds. The run time with individual sample was 6.5 min and was reduced to 3.5 min when Aria system equipped with a dual injection arm autosampler, dual injection ports, and multiplexed LCs was used. Chassaing et al. [26] demonstrated a parallel micro TFC method to analyze pharmaceutical compounds in plasma. Plasma samples were mixed with an equal volume of internal standard solution and injected onto a parallel Aria TX-2 system equipped with micro extraction columns. The narrow diameter of the TFC extraction column (0.5 mm i.d.) allowed the extraction flow rate to be reduced to only 1.25 mL/min. Special effort was made to lower the carry-over from both autosampler and extraction column.

The carry-over value was reduced to well below 0.2% for all six compounds used for method development.

Another commonly used on-line SPE sorbent material is restricted access material (RAM). With a small pore size, RAM works by eliminating the access of large molecules such as proteins to the inner surface of the particles. Small molecules can freely bind to the sorbent in the normal hydrophobic interaction mode. Proteins molecules quickly pass through the column and are washed out to waste. RAM columns have been used as the SPE and analytical column in the single column mode or coupled with another analytical column in column-switching mode. Vintiloiu et al. [20] demonstrated the work of combining RAM with turbulent flow chromatography for on-line extraction of rofecoxib (Vioxx) in plasma samples. The on-line SPE was performed on a column packed by the researchers (Licrospher 60, RP-18 ADS, 40–63 μm diameter) at a loading flow rate of 5 mL/min. After on-line SPE, the analyte was eluted and separated on a monolithic column. The total run time for the analysis was 5 min per sample. The lower limit of quantitation was 40 ng/mL. The extraction method showed good recovery and robustness after more than 200 plasma sample injections. Kawano et al. [42] developed an on-line SPE method with methylcellulose-immobilized cation-exchange RAM to analyze basic drugs in plasma. Samples were injected onto the RAM exchange column at a flow rate of 3 mL/min with and 0.1% acetic acid and then eluted onto a C18 analytical column by fast gradient with acetonitrile and ammonium acetate buffer at 0.5 mL/min. The total cycle time was 7 min per sample.

A polar functionalized polymer (Strata-X, Phenomenex) has been explored as the extraction support in an on-line solid-phase extraction LC–MS/MS assay [28]. This newly developed SPE column allows direct analysis of plasma samples containing multiple analytes. A gradient LC condition was applied to separate eight analytes that cannot be distinctly differentiated by MS/MS. With a run time of 2.8 min per injection using a Chromolith column as the analytical column, 300 direct plasma injections were made on one on-line SPE column without noticeable changes in system performance.

Beside fast chromatographic separation, monolithic-phases have been investigated as extraction support for on-line SPE. Thanks to their high permeability, the extraction can be performed at a high flow rate without generating high backpressure. The flow remains laminar and is 5–10 times higher than the flow rates generally used with conventional supports. More details of using a monolith as the analytical column can be found in the separation section of this review article. Xu et al. [32] described an automated procedure using on-line extraction with monolithic sorbent for pharmaceutical component analysis in plasma by LC–MS/MS. A short monolithic C18 cartridge is used for high flow extraction at 4 mL/min. Plasma samples were subjected to protein precipitation first with acetonitrile, and the supernatant was diluted and loaded onto the monolithic cartridge. Sample elution was accomplished with narrow-bore LC–MS/MS system with a total analysis time of 4 min. Fig. 1 shows schematic diagrams of the instrumental setup for the on-line extraction with monolithic cartridge. A method for determination of Amprenavir (APV) and Atazanavir (AZV) in human plasma was

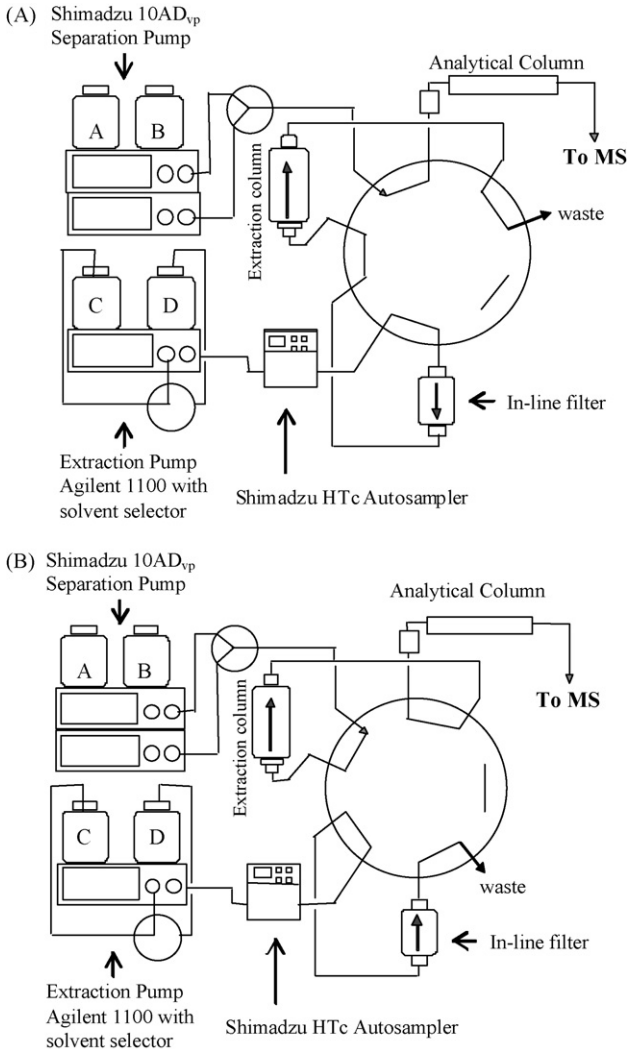

Fig. 1. Schematic diagrams of the instrumental setup for the on-line extraction with a Chromolith cartridge (4.6 mm × 10 mm) as extraction column: (A) sample loading and extraction mode; (B) elution and separation mode. Reproduced from ref. [32] with permission of Elsevier.

developed with this approach. Very low carry-over on the order of 0.006% was demonstrated using the monolithic-phase based method. The method has high recovery and good tolerance to matrix effect, which was demonstrated in 12 lots of plasma. The backpressure of the monolithic extraction cartridge remained unchanged after 450 samples injected. The performance of the monolithic-phase on-line extraction method was compared with that performed by an automated 96-well liquid–liquid extraction procedure, carried out using hexane and ethyl acetate as the extraction solvent. The results from both methods produced similar precision and accuracy.

Endogenous material from urine contains a great deal of amount of metabolic products that may present a significant challenge to assay developers and often require tedious sample preparation to remove the interfering small molecules. Method development for determining drug or metabolite concentrations from urine samples has been simplified with the implementation of on-line SPE. Because of its aqueous nature and lack of protein

content, urine samples can be easily loaded onto and cleaned by on-line SPE cartridges. Barrett et al. [35] developed a sensitive method for quantitation of urinary 6β-hydroxycortisol (6β-HC) and cortisol using on-line SPE and LC–MS/MS. Human urine samples were injected directly onto an on-line solid-phase extraction apparatus, Prospekt-2, followed by HPLC separation and LC–MS/MS detection. The lower limit of quantitation was 1 and 0.2 ng/mL for 6β-HC and cortisol, respectively.

4. Separation

4.1. Ultra-performance liquid chromatography with sub-2 μm particles

The use of smaller particles in packed-column LC is a well-known approach to shorten the diffusion path for a given analyte. As an approximation, the time required to achieve a given degree of resolution between two compounds decreases as the square of the particle diameter assuming everything else being constant [43]. Recent technology advances have made available reverse phase chromatography media with sub-2 μm particle size along with liquid handling systems that can operate such columns at much higher pressures. This technology termed ultra-performance liquid chromatography (UPLC), offers significant theoretical advantages in resolution, speed, and sensitivity for analytical applications, particularly when coupled with mass spectrometers capable of high-speed acquisitions. The principles of and recent developments in UPLC were reviewed by Mazzeo et al. [44].

In 2004, Waters commercialized the ACQUITY UPLC system which is able to work at pressures up to 1000 bar. Other manufacturers followed this approach, such as Jasco with the Xtrem LC capable of handling of pressures up to 1000 bar, and Agilent with 1200 Series Rapid Resolution LC system compatible with pressures up to 600 bar. Sub-2 μm particle columns have become available from almost all major column manufacturers. The strengths of UPLC technology promote the ability to separate and identify drug compounds with significant gains in resolution and sensitivity and marked reductions in the overall time of analysis. Since its introduction, ultra-performance liquid chromatography has served as a powerful analytical tool for high-throughput analysis. Al-Dirbashi et al. [45] reported a method for the determination of doxazosine in human plasma by UPLC–MS/MS. Plasma extracts after liquid–liquid extraction were separated on a C18 UPLC column packed with 1.7 μm particles. The total run time was 2 min. The calibration curve based on peak area ratio was linear up to at least 100 ng/mL, with a detection limit of 0.02 ng/mL. Wren and Tchelitcheff [46] investigated UPLC as an alternative to HPLC for the analysis of pharmaceutical development compounds. Data on three compounds were presented showing that significant reductions in separation time can be achieved without compromising the quality of separation. Apollonio et al. [47] assessed the separation of several commonly encountered amphetamine-type substances using the Acquity UPLC-Micromass Quattro Micro API MS system (Waters Corporation, USA). Using a poly-drug reference standard and whole blood extracts, the authors successfully separated and identified amphetamine, methamphetamine, ephedrine, pseudoephedrine, phentermine, MDA, MDMA, MDEA, and ketamine in less than 3 min. In addition to the significant reduction in overall run time, all peaks exhibited acceptable resolution, indicating the capability to separate 5–11 peaks in 1.75 min. Shen et al. [48] conducted validation of an bioanalytical method for determination of desloratadine and 3-hydroxydesloratadine using UPLC in conjunction with mix-mode solid-phase extraction. The dynamic range of the assay was from 0.025 to 10 ng/mL using 96-well solid-phase extraction. The total run time was slightly over 2 min per sample. The approach of orthogonal extraction/chromatography and UPLC significantly improves assay performance while also increasing sample throughput for drug development studies. Fig. 2 shows chromatograms from injection of an extracted LLOQ sample on a Shimadzu HPLC system employing a 5 μm, 2.1 mm × 50 mm, C18 column (A) and a Waters ACQUITY system with a 1.7 μm, 2.1 mm × 50 mm, C18 column (B).

Other direct comparison experiments using UPLC–MS/MS and HPLC–MS/MS have shown that the UPLC–MS/MS improved cycle time by 50–100% with increased sensitivity. Churchwell et al. [49] explored the differences in LC–MS performance by conducting a side-by-side comparison of UPLC for several methods previously optimized for HPLC-based separation and quantification of multiple analytes with maximum throughput. Sensitivity increases with UPLC, which were found to be analyte-dependent, were as large as 10-fold and improvements in method speed were as large as 5-fold under conditions of comparable peak separations. Improvements in chromatographic resolution with UPLC were apparent from generally narrower peak widths and from a separation of diastereomers not possible using HPLC. Yu et al. [50] developed a quantitative UPLC–MS/MS protocol for a five-compound mixture in rat plasma. A similar high-performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS) quantification protocol was developed for comparison purposes. Both UPLC/MS/MS and HPLC/MS/MS analyses were performed in both positive and negative ion modes during a single injection. Peak widths for most standards were 4.8 s for the HPLC analysis and 2.4 s for the UPLC analysis. There were 17 to 20 data points obtained for each of the LC peaks. Compared with the HPLC/MS/MS method, the UPLC/MS/MS method offered 3-fold decrease in retention time, up to 10-fold increase in detected peak height, with 2-fold decrease in peak width. Limits of quantification (LOQs) for both HPLC and UPLC methods were evaluated. For UPLC/MS/MS analysis, a linear range up to four orders of magnitude was obtained with r^2 values ranging from 0.991 to 0.998. The LOQs for the five analytes ranged from 0.08 to 9.85 ng/mL. The carry-over of the UPLC/MS/MS protocol was negligible and the robustness of the UPLC/MS/MS system was evaluated with up to 963 QC injections.

4.2. Monolithic chromatography

There is considerable interest to improve throughput by using monolithic columns because they exhibit higher separation effi-

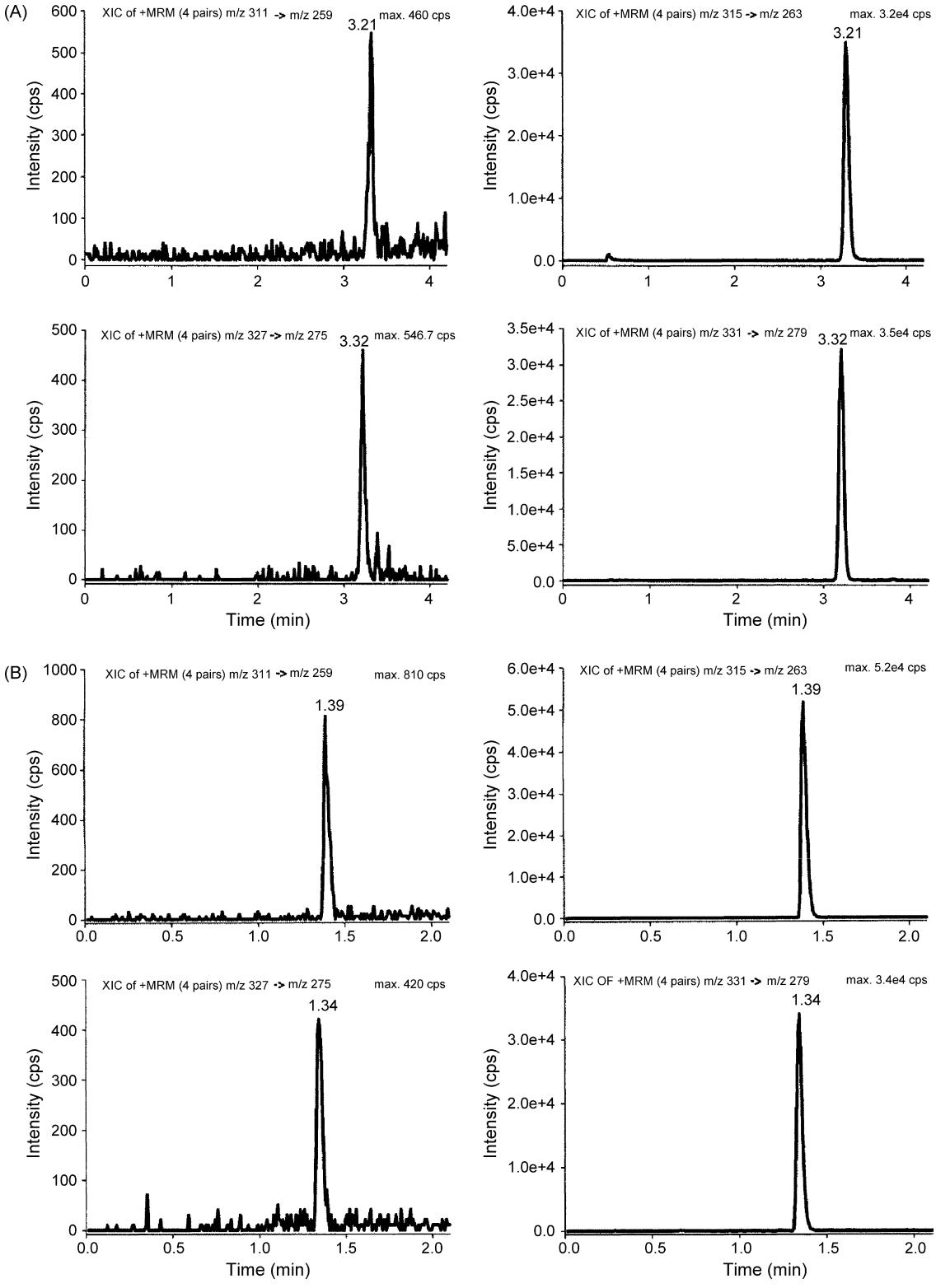

Fig. 2. (A) Multiple reaction monitoring chromatogram produced by injecting 30 μL of an reconstituted LLOQ sample from a human plasma assay on a Shimadzu HPLC system. From the top traces to the bottom traces are the MRM channels for monitoring desloratadine, ^{2}H4 desloratadine, 3-hydroxydesloratadine, and ^{2}H4 3-hydroxydesloratadine. (B) Multiple reaction monitoring chromatogram produced by injecting 15 μL of the reconstituted LLOQ sample on the ACQUITY UPLC system. From the top traces to the bottom traces are the MRM channels for monitoring desloratadine, ^{2}H4 desloratadine, 3-hydroxydesloratadine, and ^{2}H4 3-hydroxydesloratadine. Reproduced from ref. [48] with permission of Elsevier.

ciency at high flow velocities when compared to conventional LC columns [51–58,25]. The structural characteristics of the monoliths used in chromatography and those of the conventional beds of particulate packing materials are very different. An important characteristic of monoliths is their high external porosity resulting from the structure of the network of through-macropores. Another is the structure of the stationary phase skeleton that consists in a network of small, thin threads of porous silica. The networks of the two phases twist around each other and provide the intricate structure of the monolithic medium. These two structural characteristics allow the combination of a low hydraulic resistance of the column to the stream of mobile phase and an enhancement of the rate of the mass transfers of the sample molecules through the beds of these continuous porous stationary phases. Consequently, the chromatographic behavior of monolithic columns differs markedly from that of the conventional columns packed with spherical particles. Two types of monolithic supports are currently available, namely organic polymers such as polymethacrylates, polystyrenes, or polyacrylamide and inorganic polymers based on silica and more recently on carbon and zirconia. In LC, monoliths made of silica produce better chromatographic performances than organic polymers. Monolithic silica columns developed from a sol–gel process [51,52] have been commercialized by Merck (Darmstadt, Germany) and Phenomenex (Torance, CA, USA) under the brand names Chromolith^{TM} and Onyx^{TM}, respectively. They possess macropores of 2 μm and mesopores of 13 nm. The main feature of silica rod columns is a higher total porosity, approximately 15% higher than of conventional particulate HPLC columns. The resulting column pressure drop is therefore much lower, allowing operation at higher flow rates including flow gradients. Consequently, HPLC analysis can be performed much faster, as it is demonstrated by various applications. Because of the high permeability of monoliths, several columns can be coupled in series (L >1 m) to generate high efficiency (N >100,000 theoretical plates). However, the large internal column diameter (e.g., 4.6 mm in Chromolith and Onyx) of currently available monolithic silica columns is not fully compatible with MS and requires a high consumption of organic solvent. When coupled to an ESI interface, the use of splitter is required to keep the flow rate entering the source within optimum conditions.

A high-throughput LC/MS/MS method [55] using a Chromolith RP-18 monolithic column was developed for the determination of bupropion (BUP), an antidepressant drug, and its metabolites, hydroxybupropion and threo-hydrobupropion (TB), in human, mouse, and rat plasma. After semi-automated 96-well liquid–liquid extraction, analytes were separated with a mobile phase delivered isocratically at 5 mL/min, the eluate was split post-column to 2 mL/min and directed into a turbo-ionspray source. Chromatographic separation of bupropion and its metabolites was achieved within 23 s. The method was linear over a concentration range of 0.25–200 ng/mL for bupropion and threo-hydrobupropion, and 1.25–1000 ng/mL for hydroxybupropion. The monolithic column performance as a function of column backpressure, peak asymmetry, and retention time reproducibility was adequately maintained over 864 extracted plasma injections. Barbarin et al. [56] presented a high-throughput LC–MS/MS method for the determination of methylphenidate (MPH), a central nervous system stimulant, and its de-esterified metabolite, ritalinic acid (RA) in rat plasma samples. A separation of these two compounds was achieved in 15 s by employing a 3.5-mL/min flow rate, a porous monolithic column and a turbo-ionspray source compatible with relatively high flow rates. Overall 768 protein-precipitated rat plasma samples (eight 96-well plates) containing both MPH and RA were analyzed within 3 h and 45 min. The calibration curves ranged from 0.1 to 50 ng/mL for MPH and from 0.5 to 50 ng/mL for RA. Baseline resolution of MPH and RA was consistent throughout analysis.

A monolithic column was directly compared with a conventional C18 column as the analytical column in method validation of a drug and its epimer metabolite [57]. Because the chosen drug and its epimer metabolite have the same selected reaction monitoring (SRM) transitions, chromatographic baseline separation of these two compounds was required. Sample preparation, mobile phases and MS conditions were kept the same in the column comparison experiment. The eluting flow rate for the monolithic column system was 3.2 mL/min (with 4:1 splitting) and for the conventional C18 column system was 1.2 mL/min (with 3:1 splitting). The monolithic column system had a run time of 5 min and the conventional C18 column system had a run time of 10 min. The methods on the two systems were found to be equivalent in terms of accuracy, precision, sensitivity and chromatographic separation, but the monolithic column method increased the sample throughput by a factor of two.

The significantly improved separation speed by monolithic columns demanded higher throughput on sample extraction. An attractive approach using monolithic separation is to combine it with high-flow on-line extraction, which allowed for the fast extraction and separation of samples. Zeng et al. [58] used such an approach for multiple-component quantitative LC–MS/MS assays of drug candidates in biological fluids. An evaluation of the approach was performed using a mixture of fenfluramine, temazepam, oxazepam, and tamoxifen in plasma. A considerably reduced run time was achieved while maintaining good chromatographic separations. A total cycle time of 1.2 min was achieved which included both sample extraction and subsequent monolithic column separation via column-switching. A total of over 400 plasma samples were analyzed in <10 h in routine support of drug discovery programs. Zhou et al. [25] developed a high-throughput LC–MS/MS method that combined on-line sample extraction using turbulent flow chromatography with a monolithic column separation, for direct injection analysis of drugs and metabolites in human plasma samples. By coupling a monolithic column into the system as the analytical column, the method enables running "dual-column" extraction and chromatography at higher flow rates, thus significantly reducing the time required for the transfer and mixing of extracted fraction onto the separation column as well as the time for gradient separation. It was demonstrated that the total run time for this assay with a baseline separation of two analytes is less than 1.5 min.

4.3. Hydrophilic interaction chromatography (HILIC)

Hydrophilic interaction liquid chromatography (HILIC) coupling with mass spectrometry has been gaining recognition as a valuable technique for analyzing polar molecules in biological matrix in recent years [59–62]. Polar compounds typically have very limited retention on reversed-phase (RP) columns. In order to separate the analyte from the matrix interference, reverse phase HPLC mobile phase with a very low organic content must be used. Sometimes trifluoroacetic acid or ion pair reagents have to be added into the mobile phase. When using ESI-MS, the very high aqueous mobile phase can cause low ionization efficiency. HILIC is a useful technique for the retention of polar analytes that offers a difference in selectivity compared to traditional reversed-phase chromatography. The highly volatile organic mobile phases used in HILIC provide increased electrospray ionization-mass spectrometry (ESI-MS) sensitivity. Although some column companies are marketing column specific for HILIC, most columns used with normal phase HPLC such as pure silica columns or cyano columns can operate in HILIC conditions. The retention of an analyte on a HILIC column is determined by its polarity. Elution is driven by the water content in the mobile phase. HILIC often yields narrower peak, which further improves the signal noise ratio.

Eerkes et al. [59] developed a bioanalytical method using automated sample transferring, automated liquid–liquid extraction (LLE) and hydrophilic interaction liquid chromatography-tandem mass spectrometry for the determination of fluconazole in human plasma. After liquid–liquid extraction, the extract was evaporated to dryness, reconstituted, and injected onto a silica column using an aqueous-organic mobile phase. The chromatographic run time was 2.0 min per injection.

In a typical off-line sample preparation procedure using liquid–liquid extraction (LLE), solid-phase extraction (SPE), or protein precipition (PPT), the organic extracts need to be evaporated and reconstituted. The evaporation step could be very time-consuming if the water content of the organic extracts is high. The use of HILIC could eliminate the evaporation and reconstitution steps that hamper improvement of throughput and automation. With the high organic mobile phase, samples can be dissolved in organic solvent and injected without the problem of mismatching with mobile phase and peak shape deterioration. Thus it is possible to have samples injected onto columns after protein precipitation, liquid/liquid extraction, or solid-phase extraction without the steps of dry-down and reconstitution. Xue et al. [60] developed and validated a single-pot liquid–liquid extraction (LLE) with HILIC-MS/MS method for the determination of Muraglitazar, a hydrophobic diabetes drug, in human plasma. After extraction with acetonitrile and toluene, the organic layer was then directly injected into an LC/MS/MS system. Chromatographic separation was achieved isocratically on a Hypersil silica column with a mobile phase containing 85% of methyl *t*-butyl ether and 15% of 90/10 (v/v) acetonitrile/water and 0.3% trifluoroacetic acid. Post-column mobile phase of 50/50 (v/v) acetonitrile/water containing 0.1% formic acid was added. The standard curve, ranged from 1 to 1000 ng/mL, was fitted to a $1/x$ weighted quadratic regression model. The modified mobile phase was more compatible with the direct injection of the commonly used extraction solvents in LLE. Furthermore, the modified mobile phase improved the retention of Muraglitazar, a hydrophobic compound, on the normal phase silica column. In comparison with a reversed-phase LC/MS/MS method, this single-pot LLE, HILIC/MS/MS method improved the detection sensitivity by greater than 4-fold based upon the LLOQ signal to noise ratio. Song and Naidong [61] demonstrated a similar approach of eliminating evaporation and reconstitution steps in 96-well LLE by using HILIC-MS/MS on silica column with high organic/low aqueous mobile phase. Omeprazole, its metabolite 5-OH omeprazole, and internal standard, desoxyomeprazole, were extracted from 0.05 mL of human plasma using 0.5 mL of ethyl acetate in 96-well plate format. A portion (0.1 mL) of the ethyl acetate extract was diluted with 0.4 mL of acetonitrile and 10 μl was injected onto a Betasil silica column. Mobile phase with linear gradient elution consists of acetonitrile, water, and formic acid. The flow rate was 1.5 mL/min with total run time of 2.75 min. The method was validated for a low limit of quantitation at 2.5 ng/mL for both analytes.

Deng et al. [62] coupled high-flow on-line reversed-phase extraction with normal phase on silica columns with aqueous-organic mobile phase LC–MS/MS to quantify drug candidates in biological fluids. The orthogonal separation effect obtained from this configuration considerably reduced matrix effects and increased sensitivity for highly polar compounds as detected by selected reaction monitoring. This approach also significantly improved the robustness and limit of detection of the assays. An evaluation of this system was performed using a mixture of albuterol and bamethan in rat plasma. The system has been used for the quantitation of polar ionic compounds in biological fluids in support of drug discovery programs.

5. Mass spectrometric detection and system multiplexing

Multiplexing, or parallel LC–MS/MS, is widely accepted as a way to increase bioanalysis throughput [63–66]. The concept of multiplexing originated from taking advantage of the time difference between the chromatography run time and the mass spectrometer data acquisition time. The mass spectrometer data acquisition time often occupies only a small portion of the total chromatography run time. Most of time the mass spectrometer is idle in waiting for the next sample to come. In multiplexing setups, multiple LC systems or columns are connected in parallel to a single mass spectrometer. Samples are introduced to the LC systems in a staggered fashion so the analyte reaches the mass spectrometer from each LC system in serial order without overlapping. Multiple samples can be analyzed within the same time period required for one sample to be analyzed with a single LC system. Hsieh et al. [63] reported their validation of an LC–MS/MS method with multiplexing HPLC. A Leap HTS Twin-PAL with two injection syringes was used. With high-speed on-line extraction using turbulent flow columns from Cohesive Technologies, the method has a sam-

ple to sample cycle time as low as 0.4 min. Lindqvist et al. [64] made a system with three parallel HPLC columns and one mass spectrometer. Samples were injected by an autosampler and directed to three columns and the mass spectrometer by two six-port valves and one multiposition valve. The timing of the valves' action was controlled by the autosampler time program. More than 2.5 times throughput increase was achieved, as the "per sample" analysis time was decreased from 8 to 3 min.

Sample extraction, separation, and detection performed in a four-channel parallel format that resulted in an overall throughput of about 30 s per sample from plasma have been reported by Deng et al. [66]. After automated solid-phase extraction, the extracted plasma samples were injected onto four parallel monolithic columns for separation via a four-injector autosampler. The use of monolithic columns allowed for fast and well-resolved separations at a considerably higher flow rate without generating significant column backpressure. This resulted in a total chromatographic run cycle time of 2 min on each 4.6 mm × 100 mm column using gradient elution. The effluent from the four columns was directed to a triple quadrupole mass spectrometer equipped with an indexed four-probe electrospray ionization source (Micromass MUX interface). The performance of this system was evaluated by extracting and by analyzing twelve 96-well plates (1152) of human plasma samples spiked with oxazepam at different concentrations. The good separation efficiency provided by this system allowed for rapid method development of an assay quantifying the drug candidate and its close structural analog metabolite. The method was cross-validated with a conventional LC–MS/MS assay.

High-field asymmetric waveform ion mobility spectrometry (FAIMS) is another technology used by the bioanalytical practitioner to improve selectivity, sensitivity, and throughput. FAIMS separates ions at atmosphere pressure by transmitting a subset of ions and filtering out chemical background and isobaric endogenous interferences. It is installed between the atmospheric pressure ionization sprayer and mass spectrometer orifice. Ions formed from the ion source are carried by a stream of gas through a pair of closely spaced electrodes. A high frequency asymmetric waveform voltage (dispersion voltage, DV) and a dc voltage (compensation voltage, CV) are applied across FAIMS electrodes. The transmission of ions are based on the mobility of the ions in the electric field which can be adjusted by changing the compensation voltage. Ions from different compounds with same mass may have different mobility values. Thus, FAIMS can separate background and interference that is not distinguishable by quadruple mass spectrometer. As an additional filter between HPLC and mass spectrometer, FAIMS technology can improve method selectivity, reduce noise, simplify HPLC condition and shorten method development time. Kapron et al. [65] reported a study of analyzing an amine compound using FAIMS to eliminate its N-oxide metabolite interference. When analyzed using traditional LC–MS/MS method, this co-eluting metabolite caused interference that made the analysis results unreliable. The interference was due to conversion of the metabolite to the drug molecule in the ion source. After applying FAIMS, this metabolite interference was successfully removed.

6. Matrix effect

Often described in the literature as other terms such as matrix ionization effect or ion suppression effect, matrix effect is a phenomenon observed when the signal of analyte can be either suppressed or enhanced due to the co-eluting components that originated from the sample matrix. When a rather long isocratic or gradient chromatographic program is used in the quantitative assay, matrix effect may be not present at the retention time for an analyte. However, in the case of high-throughput LC–MS/MS analysis, matrix effect is one of the major issues to be addressed in method development and validation, especially when analyte is not well separated from the LC-front.

One problem brought by matrix suppression effect is reduced sensitivity when analyte signal is suppressed. Detailed studies on matrix effects revealed that the ion suppression or enhancement is frequently accompanied by significant deterioration of the precision of the analytical method as demonstrated by Matuszewski et al. [72]. The authors studied the precision (%R.S.D.) upon repetitive injection of post-extraction spiked plasma samples as a function of the analyte concentration for a single lot and for five different lots of plasma. While for the single plasma lot the precision is acceptable, it may not be when different plasma lots are taken into account as shown in Fig. 3. Generally, matrix effect impacts more on the low end of calibration curve than the mid range or highly end.

When discussing matrix effects, it is useful to discriminate between ion suppression (or enhancement) by the matrix at one hand, and different matrix effects exerted by different sample lots at the other hand. A useful nomenclature was suggested by Matuszewski et al. [72] and is adopted in this article. The difference in response between a neat solution sample and the post-extraction spiked sample is called the absolute matrix effect, while the difference in response between various lots of post-extraction spiked samples is called the relative matrix effect. If no counteraction is taken, an absolute matrix effect will primarily affect the accuracy of the method, while a relative matrix effect will primarily affect the precision of the method.

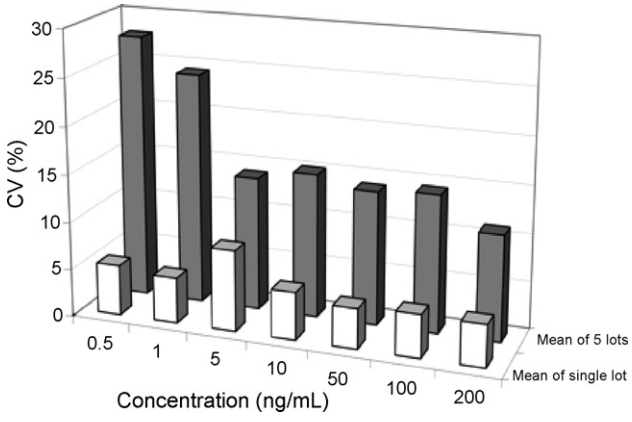

Fig. 3. Precision ($n = 5$, %CV) of a bioanalytical method at various analyte concentrations, determined in either a single plasma lot or in five different plasma lots. While for the single plasma lot the precision is acceptable, it is not when five different plasma lots are taken into account. Reproduced from ref. [73] with permission of American Chemical Society.

The matrix effects are generally due to the influence of co-eluting compounds on the actual analyte ionization process, that is, they happen well before the analyte ions enter the high vacuum of the mass analyzer. Matrix effects are known to be both compound and matrix dependent. It was demonstrated that matrix-induced ion suppression is especially important for early eluting compounds, while later eluting compounds are not affected as often. Suppression or enhancement effects may be exerted by any co-eluting component entering the atmospheric pressure ionization source via the liquid stream. Some mobile-phase additives are also known to suppress or enhance analyte response. Although such effects are sometimes called matrix effects, it appears useful to discriminate between effects due to the analytical system, for example, mobile-phase composition, source parameters, and effects due to the actual analyte matrix.

Matrix effect could be introduced from a formulation agent used in toxicological studies. Larger et al. [69] observed strong ion suppression in a preliminary pharmacokinetic study from a polysorbate co-solvent, which, if undetected, would have given highly erroneous pharmacokinetic results and possibly could have led to the inappropriate elimination of a promising drug candidate. Some excipients commonly used in formulations are polydispersed polymers, for which very limited pharmacokinetic information is available. Further investigation is needed to better understand the mechanisms of ion suppression and the kinetics of the suppressing species to allow the development of new LC–MS/MS based analytical strategies, which will not be subject to such ionization interferences.

Absolute matrix effect can be easily detected by comparing the response obtained from a neat solution and that from a post-extraction spiked sample. Difference in response indicates ion suppression or ion enhancement. To pinpoint the location of matrix peaks or affected region in the chromatogram by matrix effect, the analyte solution is usually post-column infused into the ion source while a blank matrix extract is injected through a column. For testing of relative matrix effect, samples from different sources or lots must be analyzed. Often plasma samples from different lots are spiked with analyte at the low end of calibration curve (i.e., samples at low quality control or lower level quantitation limit) and tested. Matuszewski [73] described a simple experimental approach for studying and identifying the relative matrix effect in quantitative analyses by LC–MS/MS. It was shown that the variability of standard line slopes in different lots of a biofluid [precision of standard line slopes expressed as coefficient of variation, CV (%)] may serve as a good indicator of a relative matrix effect and, it is suggested, this precision value should not exceed 3–4% for the method to be considered reliable and free from the relative matrix effect liability.

Endogenous phospholipids cause ion suppression in both positive ESI and negative ESI modes and must be removed or resolved chromatographically [71,74]. It is suspected that one major contributor to matrix effects are Glycerophosphocholines (GPCho's) because of their surfactant-like properties. A method was developed for detecting GPCho's during LC–MS/MS method development [74]. The approach uses high energy in-source collisionally induced dissociation (CID) to yield trimethylammonium-ethyl phosphate ions (m/z 184), which are formed from mono- and disubstituted GPCho's. The resulting ion is selected by the first quadrupole (Q1), and monitored without further fragmentation.

Due to their unpredictable character, matrix effects in quantitative analysis using LC-ESI-MS or LC-APCI-MS are a serious concern. It is clear that the use of real sample extracts is necessary already at an early stage of method development, as the matrix effect may have serious impact on the choice of the most appropriate sample pre-treatment method, ionization method and mode, and even the most adequate mobile-phase composition. A study was performed where high-flow on-line reversed-phase extraction was coupled with normal phase chromatography on silica columns. Matrix effects were reduced considerably by this orthogonal separation configuration [66].

The best way to eliminate the influence of matrix effects on the accuracy and precision of a quantitative method is through the use of stable isotope labeled internal standards [68,73]. It is important to add stable isotope labeled internal standards prior to sample pre-treatment. In that way, they can correct for analyte losses during sample pre-treatment as well as matrix-related suppression or enhancement during analyte ionization. Although it is generally believed that the use of an isotopically labeled internal standard corrects for almost all matrix effects, data reported for the bioanalysis of mevalonic acid indicate that this assumption needs to be demonstrated during method development and validation [67]. Wang et al. [76] reported that retention time difference between analyte and isotopically labeled internal standard could lead to the variability of a method's precision. In addition, mutual suppression or enhancement of responses of an analyte and its co-eluting isotopically labeled internal standard has occasionally been reported [80]. However, calibration curves were linear if an appropriate IS concentration was selected for a desired calibration range to keep the response factors constant.

Obviously, limited availability and high costs have hampered the wide application of isotopically labeled internal standards. Advanced sample pre-treatment methods can help in reducing or eliminating matrix effects [70,75], and together with efficient chromatographic separation, one may eliminate the sample constituents responsible for the matrix effects. Alternatively, one may reduce or eliminate the influence that matrix effects have on the accuracy and/or precision of the method by one or a number of the following measures: change to a different MRM channel, change to another ionization methods, and/or change the mobile-phase composition [77,78]. It has been shown that the precision of a method in which an analog internal standard is used, can be significantly improved by modifying the mobile-phase conditions in such a way that analyte and analog internal standard co-elute [79].

7. Conclusion and future perspectives

In this article, we reviewed recent progress made in several areas including sample preparation, separation and detection. Although much of the emphasis is put on the first two areas, it should be noted that the progress in mass spectrometer designs over the years provided the basis for sensitive detection of ever more potent drug candidates from biological matrices. Without

sensitivity gains, many of the commonly used approaches such as the "dilute and shoot" would not be practical.

The results from many applications cited in this article have demonstrated that innovative chromatography technologies are re-shaping the ways that separations are performed in high-throughput laboratories. Together with advancements made in laboratory automations like parallel sample processing, column-switching, and usage of more efficient extraction supports for SPE, they drive the trend towards less sample clean-up time in today's quantitative bioanalysis field. Importantly, the efficiencies are accomplished without compromising the quality of assay such as precision, accuracy, selectivity, and robustness. On the other hand, we recognize that some of these techniques such as Spark Holland or UPLC systems need specialized equipments. Some of the materials like certain types of extract sorbents or specialized columns are not cost-effective yet to many users. Most of the techniques described in the article continue to be developing. For example, the achievement of small particle UPLC has not been fully extended from reversed-phase to other types of stationary phase. Monoliths made of silica possess a limited pH range over which they are applicable (2 to 8). There is a need of more dimensions and different type of monolithic column, especially microbore monolithic columns so that highly efficient separation can be performed using less HPLC solvent. The separation efficiency of such columns can be optimized with improved fabrication. Nevertheless, further expansion or advancement of these techniques will be beneficial to bioanalytical scientists in either developing strategies for a new method or modernizing a high-throughput laboratory.

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