

In-Tip Solid Phase Microextraction for High Throughput Drug Analysis

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

This thesis describes the design of a convenient format of solid phase microextraction (SPME) for bioanalysis in pharmaceutical industry and the validation of the approach to the application. An automated in-tip SPME technique coupled with liquid chromatography (LC) and tandem mass spectrometry (MS/MS) for high throughput drug analysis has been developed and applied to the quantitative determination of various drug compounds in different biological fluids from drug discovery to clinical development.

The initial research in this thesis focused on a proof-of-concept study using manual multi-fiber approach to determine a drug compound in human plasma from a clinical trial. The proof-of-concept was achieved based on the validation data and a head-to-head comparison with conventional liquid-liquid extraction (LLE) method. An in-tip SPME technique was then proposed to explore the feasibility of SPME automation and two approaches of preparing in-tip SPME fibers were developed including fiber-packed and sorbent-packed fiber preparation. A simple and high throughput in-tip SPME fiber fabricating procedure based on polymer monoliths using photo-polymerization was introduced to prepare 96 fibers simultaneously. The biggest advantage of the in-tip SPME technique is that it is simple and easy to use for automation without introducing any additional devices and in the meantime, the simplicity of SPME is maintained.

Automated in-tip SPME was applied to routine drug analysis in drug discovery and development environment. One case study involved the determination of vitamin D₃ in human serum with derivatization and the in-tip SPME approach was compared with traditional LLE method using either tubes or 96-well plate extraction. Another study was to use hydrophilic interaction chromatography (HILIC) –MS/MS to determine three polar

compounds, imipenem (IMP), cliastatin (CIL) and β -lactamase inhibitor (BLI) simultaneously in different biological fluids including rat plasma and mouse blood. The results from both studies clearly demonstrated that in-tip SPME could be used as an alternative sample preparation method in bioanalytical analysis. Matrix effects in bioanalysis using automated in-tip SPME and LC-MS/MS were then thoroughly evaluated for the first time. Our study indicated that the assumption that SPME should provide sample clean up as effective as or better than solid phase extraction (SPE) with no or minimal matrix effects might not be always true, and matrix effects should be investigated in any SPME assays in bioanalysis.

Comparisons between in-tip SPME and other automated SPME approaches such as blade/thin film geometries were performed, and the advantages and limitations of using SPME versus conventional sample preparation methods including protein precipitation (PPT), LLE and SPE were summarized. Strategies for in-tip SPME method development and validation and the potential applications and future directions of in-tip SPME in bioanalysis were discussed.

Finally, kinetic models were established to describe SPME extraction and desorption processes in a complex matrix with both liquid and solid fiber coatings. The models were successfully applied to different scenarios to estimate the boundary layer (BL) thickness, extraction equilibrium time and total amount of analytes extracted at a given time. The excellent agreements between the model prediction results and experimental data indicated that the SPME modeling approach had great potentials to speed up SPME method development and fiber selection.

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List of Abbreviations

A	Surface area of a SPME fiber
ACN	Acetonitrile
ADME	Absorption, distribution, metabolism and elimination
AIBN	Azobisisobutyronitrile
AMS	Accelerator mass spectrometry
APCI	Atmospheric pressure chemical ionization
API	Atmospheric pressure ionization
APPI	Atmospheric pressure Photoionization
BL	Boundary layer
BLI	β -Lactamase inhibitor
BSA	Bovine serum albumin
CAR/PDMS	Carboxen/poly(dimethylsiloxane)
CARRS	Cassette accelerated rapid rat screen
C_b	Binding matrix concentration
C_b^0	Total concentration of binding matrix
C_{bs}	Bounded analyte concentration
CE	Collision energy
C_f	Analyte concentration on the surface of the fiber coating
CID	Collision induced dissociation
CIL	Cilastatin
C_s	Analyte concentration in the bulk of the sample matrix

C_s^0	Initial analyte concentration
C_s'	Analyte concentration in the boundary layer at the interface of the fiber coating and the boundary layer
CV	coefficient of variation
CW/TPR	Carbowax/templated resin
CXP	Collision cell exit potential
DBS	Dried blood spot
D_b	Diffusion coefficient of binding matrix in aqueous phase
D_{bs}	Diffusion coefficient of bounded analyte in aqueous phase
D_f	Diffusion coefficient in the fiber coating
DMPA	Dimethoxy- α -phenylacetophenone
DP	Declustering potential
D_s	Diffusion coefficient of analyte in the sample matrix
EDMA	Ethylene glycol dimethacrylate
EP	Entrance potential
ESI	Electrospray ionization
FDA	Food and Drug Administration
FT	Freeze thaw
GC-MS	Gas chromatography
GLP	Good laboratory practice
GPCho's	Glycerphosphocholines
HEPES	4-(2-Hydroxyethyl) piperazine-ethanesulfonic acid

h_f	Mass transfer coefficient in the fiber coating
HILIC	Hydrophilic interaction chromatography
HLB	Hydrophilic lipophilic balance sorbent
HN	Heated nebulizer
HQC	High quality control
h_s	Mass transfer coefficient in the boundary layer
HTS	High throughput screening
IMP	Imipenem
IPA	Isopropanol
ISTD	Internal standard
IV	Intravascular
J	Mass flux
K	Equilibrium constant of the analyte between the solid fiber coating surface and the sample matrix
K_a	Equilibrium binding constant between the analyte and the binding matrix
K_{fs}	Distribution coefficient of the analyte between the liquid fiber coating and the sample matrix
k_a	Rate constant for the association reaction
k_b	Rate constant for the dissociation reaction
LC	Liquid chromatography
LIMS	Laboratory information management system

LLE	Liquid-liquid extraction
LLOQ	Lower limit of quantification
LPME	Liquid-phase microextraction
LQC	Low quality control
MeOH	Methanol
MEPS	Microextraction in packed syringe
MES	2-(N-morpholino)ethanesulfonic
MIP	Molecular imprinted polymer
MOPS	3-Morpholino-propanesulfonic acid
MQC	Middle quality control
MRM	Multiple reaction monitoring
MS/MS	Tandem mass spectrometry
MTBE	Methyl <i>tert</i> -butyl ether
<i>N</i>	Magnetic stirrer speed in revolutions per second
<i>n</i>	Amount of analyte absorbed onto the fiber
nd-SPME	Negligible depletion SPME
PA	Polyacrylate
PCC	Preclinical candidate compound
PDMS-DVB	Polydimethylsiloxane-divinylbenzene
PK	Pharmacokinetics
PO	Oral administration
PPB	Plasma protein binding
PPT	Protein precipitation

q	Amount of standard desorbed from the fiber after sampling time t
q_0	Initial amount of standard extracted onto the fiber
QC	Quality control
RAM	Restricted access media
R_e	Reynolds number
R_s	Radius of the stirring bar
r	Distance between the fiber and the center of the vial
SBSE	Stir bar sorptive extraction
S_c	Schmidt number
SEM	Scanning electron microscopy
S/N	Signal-to-noise ratio
SOP	Standard operation procedure
SPDE	Solid phase dynamic extraction
SPE	Solid phase extraction
SPME	Solid phase microextraction
t	Sampling time
TFC	Turbulent flow chromatography
THF	Tetrahydrofuran
TIC	Total ion chromatogram
TIS	Turbo ion spray
TOF	Time of flight
u	Flow velocity
u	Velocity vector

ULOQ	Upper limit of quantification
UPLC	Ultra-pressure liquid chromatography
ν	Kinematic viscosity
V_f	The volume of fiber coating
V_s	The volume of sample matrix
δ_f	The thickness of fiber coating
δ_s	The thickness of boundary layer

Chapter 1

Introduction

1.1 Sample Preparation in Bioanalysis

Bioanalysis is the quantitative determination of drugs and their metabolites in biological fluids. The technique is used throughout different drug development stages from drug discovery to clinical development, to help understand the metabolic fate and pharmacokinetics of chemicals in living cells, animals and human bodies. In addition, knowledge of drug levels in body fluids such as plasma and urine can optimize safety and efficacy of new drug therapies in humans. Although bioanalysis has been performed using various analytical instruments, most of these instruments cannot handle the sample matrices directly because of the multitude of substances present in the biological samples, such as proteins, salts, acids, bases, and numerous organic components with chemical properties similar to those of the analytes, these could potentially interfere with the analysis. Thus, sample preparation is usually necessary before analysis to clean up a sample and/or to concentrate a sample to improve its detection.

In bioanalytical sample preparation, there are two major goals: (i) remove unwanted matrix components that can cause interferences upon analysis, improving method specificity; and (ii) concentrate an analyte to improve its limit of detection. Ultrafiltration, dialysis and protein precipitation can be used to remove protein from biological samples. In contrast, liquid-liquid partition including LLE and liquid-phase microextraction (LPME),¹⁻¹¹ SPE^{1-4, 8, 12-15} and SPME,^{2-4, 11, 13-22} including fiber SPME, in-tube SPME, stir bar sorptive extraction (SBSE), solid phase dynamic extraction

(SPDE), and microextraction in a packed syringe (MEPS), are useful sample preparation techniques that can efficiently produce clean extracts for analysis. In addition, immunoaffinity extraction,²³⁻²⁴ molecularly imprinted polymer (MIP)-based extraction¹⁵ and membrane-based extraction²⁵⁻²⁶ are also specific and efficient sample preparation techniques. These techniques, which are based on the partition or adsorption of analytes, are able to remove the majority of the biological materials from the sample matrix prior to analysis. A reliable bioanalytical method is achieved with the successful combination of efficient sample preparation, adequate chromatographic separation and a sensitive detection technique. In the past decade, although LC-MS/MS has become a well established technology with regard to assay development in bioanalysis of small molecule drug candidates due to its high sensitivity, selectivity, and fast analysis with good precision and wide dynamic range, drug analysis in biological matrices using robust LC-MS/MS methods remains difficult, time-consuming, and sometimes quite challenging. Successful use of LC-MS/MS requires understanding the mechanism of various sample extraction processes and the underlying principles of both chromatography and MS.²⁷⁻³⁰

1.2 Bioanalysis in Pharmaceutical Drug Development

1.2.1 Roles of Bioanalysis

The drug discovery process is a series of stages through which compounds must pass in order to qualify for being a development compound. As shown in Figure 1, these stages represent various in vitro and in vivo tests that are performed on a series of compounds in order to select those few compounds that have the correct properties to

achieve the desired effect. Bioanalysis plays an integral role in the whole process and can be characterized as various levels based on different stages, as shown below.

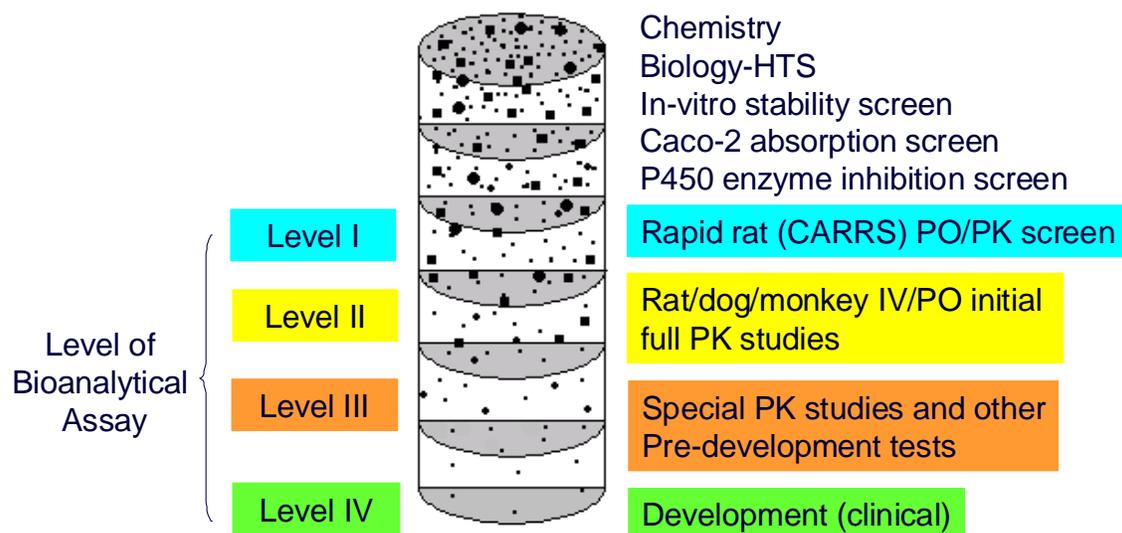


Figure 1-1 Stages in new drug discovery and levels of bioanalytical assay.

In the support of drug discovery and lead optimization, information about a molecule's absorption, distribution (including protein binding), metabolism, and elimination (ADME) is obtained through a list of typical experiments such as Caco-2 cells, PgP transport, in vivo pharmacokinetic profiling, in vitro protein binding, in vivo tissue distribution studies, metabolic stability screening, and P450 induction and inhibition studies. The rules for discovery screen assay (level I) and full PK assay (level II) are designed less strictly under non-good laboratory practice (GLP) and can be easily implemented in a high throughput manner for screening and lead optimization purposes. Level I assay only requires a two-point calibration curve with an appropriate internal standard. A one order of magnitude range is preferred to cover the samples, but two

orders of magnitude is acceptable. QC samples are not used and the assay is not validated. Since level II assays are used for lead optimization studies, assays need to be more rigorous than those of level I. This is because in lead optimization studies, the goal is to obtain enough data to calculate several PK parameters such as clearance, half-life, AUC and volume of distribution from about 30-60 samples for each compound. Normally, a multi-point standard curve ranging from 1 to 10000 ng/mL is used. The matrix of the calibration curve should be from the same animal species and matrix type as the samples. Again, QC samples are not required and the assay is not validated.

Once a drug compound reaches the preclinical development phase, its ADME characteristics are determined in many species of animals. At this point, a defined and validated assay is used repeatedly for the determination of drug concentrations from in vitro and in vivo samples. The major change in a level III assay is the use of QC samples. The addition of QC samples provides additional confidence in the results that are obtained with the assays. A minimum of six QCs at three concentrations (low, middle, high) are used and the QC standards should be frozen at the same freeze temperature as the samples to be analyzed. A major effort in the clinical phase of drug development is the determination of drug and metabolite concentrations in biological fluids after drug administration to humans. The pharmacokinetic data obtained is used to support drug development in assessing the therapeutic index, drug-drug interactions, dose regimes, etc. Thousands of samples are often obtained from a single clinical study and rapid sample turnaround is required to plan the next clinical study. Since drugs are often dosed at lower levels, very sensitive assays are required to detect the low levels of drugs circulating in biological fluids. In addition to sensitivity, selectivity and ruggedness are also very

important to the analytical method; therefore, the bioanalytical assays (level IV) should be fully validated under GLP rules and maintained during the clinical phase of drug development.

1.2.2 Current Practice and Future Trend

Drug discovery and development pharmacokinetic analysis include multiple steps, which need to be performed in sequence so that the PK results can be delivered to the discovery team. These steps are sample dosing and collection, sample transferring and tracking, sample preparation and analysis, and the final PK report preparation and distribution. It is very important to understand the potential bottlenecks of each step so that efforts can be made to streamline these steps so as to reduce the cycle time of the whole process.

Traditionally, for preclinical studies, one animal species is dosed with one drug and blood samples are collected at a series of time points after the dose was administered in order to obtain the drug candidate's pharmacokinetics in a living system. This procedure is very time consuming and labor intensive because each individual sample must go through analysis in series. Currently, simultaneous multiple compound dosing, or "cassette dosing" (n-in-1), in which multiple compounds are dosed in one animal has greatly improved the throughput. This is achieved with the help of the selectivity of the mass spectrometers that can individually quantify the concentrations of each compound in the mixture³¹⁻³³. Animal samples are collected in a 96-well plate, which can be easily transferred using robotic systems for further sample preparation and analysis. Although only a small amount of sample is normally available, sensitivity is usually not an issue

and rapid sample turnaround is required. There is little time available for method development and, therefore, protein precipitation is the preferred sample preparation approach. However, in clinical analysis where drugs are more potent and are dosed at low levels, a large amount of sampling from the participating volunteers in clinical trials is often required with the utilization of individual collection tubes. Depending on the phase of the program and the type of drug under development, the number of blood samples collected during a clinical study could range from a few hundred to several thousand. The amount of work involved in sample collection, shipment, and tracking is quite substantial and, to date, this process is one of the major bottlenecks in PK analysis. Attempts have been made to automate the sample transfer process from tubes to plates using "de-capping/capping" robotic systems with barcode readers. Unfortunately, the approach has not been widely accepted due to many reasons, and, in most cases, clinical sample transfer is still a labor intensive, semi-automated procedure. Sample tracking for both preclinical and clinical is handled by Watson LIMS, a laboratory information management system, used by most pharmaceutical companies. With outsourcing activities increasing nowadays, it can be expected that developing a harmonic sample tracking system will be quite challenging.

The use of LC-MS/MS with triple-quadrupole mass spectrometers and an atmospheric pressure ionization (API) source, operated under selected reaction monitoring mode, has grown exponentially in the last decade. The principle of MS is to produce ions from analyzed compounds that are separated or filtered based on their mass-to-charge ratio (m/z). Tandem mass spectrometers make use of two mass analyzers: one for the precursor ion in the first quadrupole and the other for the product ion in the third

quadrupole after the collision-activated dissociation of the precursor ion in a collision cell. Innovative and successful research efforts on the design of an effective interface connection between LC (operated under atmospheric pressure) and MS (operated under a high-vacuum environment) have made the LC-MS/MS the most reliable technique for quantitative bioanalysis in drug discovery and development. Compared to clinical development, in which quality and robustness of the bioanalytical methods is more important; drug discovery is mainly focused on speed and high throughput. Thus, the applications of LC-MS/MS related approaches are quite different. LC-MS/MS using triple-quadrupole mass spectrometers operated under the SRM mode remains the workhorse for both environments. However, in drug discovery, new developments such as automated SRM MS/MS method optimization³⁴⁻³⁵ high-speed LC separation, including ultra-high pressure liquid chromatography (UPLC) system with sub-2 μm columns and multiplexed LC-MS/MS analysis, have been actively used.³⁶⁻⁴¹ In addition, other MS-based technologies, including online SPE-MS/MS, direct analysis using alternative ionization techniques, and LC-MS(MS) with other mass analyzer such as time-of-flight (TOF) analyzer have also emerged as promising techniques.⁴²⁻⁴⁹

In spite of the great success of LC-MS/MS technology that provided sensitive and specific detection of analytes of interest with adequate chromatographic separation, to obtain a clean sample extract is always the goal for bioanalytical chemists because a clean extract is a prerequisite for an accurate LC-MS/MS analysis. The importance of sample preparation cannot be over emphasized; and efforts to develop simple, fast, efficient, and high throughput sample preparation methods have never stopped. Table 1 summarizes some typical choices of sample preparation techniques in bioanalysis.

Table 1-1 Typical Choices of Sample Preparation Techniques in Bioanalytical Analysis

Typical sample preparation methods	Comments
<ul style="list-style-type: none">○ Dilution followed by injection (Dilute and shoot)○ PPT○ Filtration○ Protein removal by equilibrium dialysis or ultrafiltration○ LLE○ Solid support liquid-liquid extraction○ SPE (off line/online)○ Turbulent flow chromatography (TFC)○ Restricted access media (RAM)○ Monolithic columns○ Immunoaffinity extraction○ Combination of the above	<ul style="list-style-type: none">○ PPT, LLE and SPE are still the most routinely used sample preparation methods in drug discovery and development○ Direct injection and online extraction become more and more popular in bioanalysis○ Combination of different sample preparation approaches will be the solutions to achieve highly sensitivity assays in studies such as microdosing

It should be pointed out that each sample preparation method has its own advantages and limitations. For example, protein precipitation is often used as the initial sample preparation scheme in the analysis of new drug substance since it does not require any method development. Protein precipitation is simple, universal, inexpensive, and can be easily automated in microplates. However, matrix components are not efficiently removed and these will lead to significant matrix effects, which can directly affect the performance of the bioanalytical methods. On the other hand, the LLE method provides efficient sample cleanup as well as sample enrichment, and it is widely applicable for many drug compounds. However, a relatively larger amount of organic solvent is necessary and the whole sample preparation process is very labor intensive. Over the past decade, SPE has gradually replaced most LLE methods as the preferred technique to extract drug and metabolites from biological fluids prior to quantitative analysis.⁵⁰ Benefiting from the advances in the technology of sorbent chemistry, a wide range of

sorbents is now commercially available in various formats from cartridge to disk to microplate. These provide various applications not only for most classes of drugs and metabolites but also for ionic and highly polar compounds that are difficult to extract by other methods. High throughput SPE utilizes the 96-well plate format; although even a 384-well format has been reported.⁵¹⁻⁵² Regardless of all these great advantages, SPE suffers from the disadvantages of high cost compared with other techniques, long method development time, and the complexity and difficulty of mastering its usage.

Automation plays a pivotal role in allowing high-speed analysis to meet the ever-increasing demands in the current pharmaceutical environment. Automation results in greater performance consistency over time and more reliable methods of transfer from site to site. One of the current strategies for high throughput bioanalysis is to use well-established instrumentation; rigorous, standardized techniques, and automation wherever possible to replace manual tasks. The choices for automation differ in complexity according to the required task, and the size and the function of automated systems can vary from a small bench top to a large workstation. Commonly used commercially available liquid handling systems include: Hamilton STAR Line liquid handling workstations; TECAN Genesis liquid handling workstation; JANUS automated liquid handling system; and Tomtec Quadra liquid handling workstation. Most of these liquid handling systems have eight channels with fixed or disposable tips, although some have 96 probes that can transfer liquid to a 96-well plate simultaneously. In bioanalytical sample preparation, there are three major functions that involve liquid transfer: (1) standard curve and QC sample preparation; (2) sample transfer which includes the transfer of different types of samples such as standards, QCs, control blanks, internal

standards, study samples from different sources vials or containers to their destination vials, as well as organic solvent; and (3) extraction processes include protein precipitation, LLE and SPE. Performing a routine sample preparation procedure is one of the main uses for automation. However, developing some strategies on how to use automation effectively in bioanalytical assay development is also very important, as most of the automated instruments are not ideal for performing method development by using variable solvents and volumes.

Two new trends in drug development will definitely have great impact to bioanalysis in the near future. One is Microdosing⁵³⁻⁵⁵ and the other is Dried Blood Spot (DBS).⁵⁶⁻⁵⁸ Microdosing is defined as 1/100th or less than the pharmacological dose and should not exceed 100 µg. The purpose of microdosing is to reduce the resources spent on nonviable drug candidates and the amount of testing done on animals prior to first-in-man studies. Microdosing requires an extremely sensitive bioanalytical method to fully define the pharmacokinetic profile of a compound. Currently, accelerator mass spectrometry (AMS) is the most effective tool, which is particularly specialized, requiring trace amounts of a long-lived isotope in the molecule, such as ¹⁴C. A sophisticated AMS can measure a ratio of the trace isotope, ¹⁴C, to total carbon at parts per quadrillion levels. However, an AMS assay is quite expensive, as it involves a radiolabeled compound. In practice, this will be a complicating factor, which may cause delay and increase the cost of the microdosing studies. The development and validation of highly sensitive LC-MS/MS methods without using radiolabeled compounds will greatly prompt the wide application of microdosing in the early stage of drug development.

DBS involves the collection of blood on an absorbent storage medium prior to sample analysis. As the sampling technique dates back to the 1960s, DBS has very recently gained increasing interest in support of pharmacokinetic and toxicokinetic studies in small molecule drug discovery and development. The key features of DBS samples are the low blood volume requirement, potentially leading to decreased animal use, and the potential for simplified sample collection, storage, and shipment. The standard sample preparation approach for DBS analysis consists of punching out a disk from the card that contains the DBS followed by extraction of the analyte. This procedure is very time consuming and labor intensive. There is an emerging need for greater efficiency, either through automation of the sample preparation process, or alternative approaches that require minimal or no sample preparation.

1.2.3 Bioanalytical Assay Validation

There have been no clear guidelines to validate bioanalytical assays until a compound enters the development stage, where most of the assays are required to be fully validated under GLP regulations.⁵⁹⁻⁶⁰ Development and validation of a bioanalytical assay is divided into two major steps: establishing a method and routine drug analysis.

1.2.3.1 Establishing a Method

Typical method development and establishment for a bioanalytical method includes determination of (1) selectivity, (2) sensitivity, (3) calibration curve, (4) intra-day variation, (5) recovery, (6) stability, (7) matrix effects, (8) sample dilution, and (9) carry-over minimization.

Selectivity

The selectivity of the assay must be confirmed by processing control biological fluids from at least six different sources (or subjects) to demonstrate that no interfering compounds elute at the same retention times as the analyte(s) of interest and a suitable internal standard. For biological fluids with limited availability (e.g. spinal fluid or semen), the sensitivity of the assay may be assessed in a fluid originating from only one source.

Sensitivity

The sensitivity of the assay corresponds to the lower limit of quantification (LLOQ). The LLOQ is defined as the lowest concentration on the calibration curve that can be measured with acceptable precision and accuracy. In principle, the signal-to-noise (S/N) ratio of the analyte response should be at least 5 times the blank response.

Calibration (Standard) Curve

The concentration range over which the analyte will be determined must be defined in the method based on evaluation of stock standard solutions of analyte spiked into control biological fluids. At least six concentrations should define the calibration curve. A least-squares regression (weighted if appropriate) of response vs. concentration of the calibration standards should be determined. The curve fitting parameters should not be changed and should be used in all subsequent analysis of clinical samples. In addition to at least six non-zero samples, a calibration curve should also consist of a blank sample (matrix processed without internal standard) and a zero sample (matrix processed with internal standard).

Intra-day Variation

Determination of accuracy and precision should be accomplished by analysis of replicate sets of analyte samples of known concentration in a biological matrix.

- Prepare and analyze replicates of six standard curves
- Calculate the mean, standard deviation, and coefficient of variation (CV) for each analyte used to construct the standard curve
- Intraday precision should not exceed 15% for each concentration on the calibration curve, except for LLOQ not exceed 20%
- Assay accuracy mean values should not deviate by more than 15% of the nominal value at each concentration, except LLOQ where a deviation of 20% is acceptable
- LLOQ is the lowest point on the calibration curve. The precision should not exceed 20% and accuracy should within $\pm 20\%$ of the nominal value.

Recovery

"Recovery" is the extraction efficiency of an analytical process reported as a percentage of the known amount of an analyte carried through the sample extraction and processing steps of the method. Recovery should be determined by comparing the detector response obtained from an analyte added to and extracted from the biological matrix to the detector response of an analyte added to a biological fluid extract obtained from the same volume of the biological fluid as used for analyte extraction.

Recovery experiments should be performed at three concentrations (low, middle, and high), and results reported as a mean concentration at each level obtained from six

different lots of matrix. Recovery of the internal standard(s) should also be determined at the concentration used in the construction of the calibration curve.

Stability

Storage stability of the analyte in each biological matrix and the influence of freeze-thaw cycles should be examined. A set of QC samples should be prepared by spiking biological fluids with analyte to yield low QC (within 3x the concentration of the LLOQ), middle QC (near the center) and high QC (near the upper boundary). The QC standard solutions in biological fluids are subdivided into suitable aliquots and stored at -20°C or other temperatures adequate for long-term storage of clinical specimens.

The standard used for preparation of QCs must be weighed separately from that used for calibration standards. Thaw replicate (n=5) standards and assay along with the calibration curve. The calculated mean values of QC samples should not deviate by greater than 15% of the nominal value. CV for QC replicates should not exceed 15% as well. LQC, MQC and HQC samples should be examined for their freeze-thaw (F-T) stability. At least three aliquots at each concentration should be stored at the intended storage temperature for at least 24 h and thawed unassisted at room temperature. When completely thawed, the sample should be refrozen for at least 12 h under the same conditions and thawed a second time. The F-T cycle should be repeated and then analyzed on the third cycle. The mean values should not deviate by greater than 15% of the control values (one F-T cycle). CV of QC replicates should not exceed 15%. Long-term stability is determined and confirmed by the analysis of frozen QC samples during the course of clinical studies, which could last from several months to years. Room temperature and auto-sampler stability can be also performed using frozen QC samples.

Matrix effects

In the case of HPLC-MS/MS based methods, matrix effects should be investigated to ensure that the precision, selectivity and sensitivity of the method is not compromised when biofluid samples from different subjects participating in different clinical studies are analyzed. One of the simplest ways to demonstrate the absence of the matrix effect in biofluids from different sources is to perform precision and accuracy determination in biofluids from six different sources (lots) if available rather than six replicates from a single source (subject). If intraday precision and accuracy values obtained from six different sources meet the criteria described above, matrix effect may be considered as not having a significant impact on assay performance. The absence of matrix effect needs to be demonstrated when major changes in the assay are made including changes in the extraction procedure, chromatography, and in the MS interface etc.

Sample Dilution

In order to demonstrate the stability to dilute samples above the upper limit of the standard curve, experiments should be performed using biofluid samples at a concentration 10x higher than the upper limit of quantitation (ULOQ) and diluting them after an F-T cycle, with a control biofluid to a concentration within the standard curve range. The mean values should not deviate by greater than 15% of the nominal value and CV for QC replicates should not exceed 15%.

Carry-over Minimization

Carry-over should be assessed by injecting an extracted double blank immediately after an extracted high standard. If the analyte response in the double blank does not exceed 20% of the LLOQ, the extent of carry-over is deemed to not have a negative impact on assay performance; otherwise, an attempt should be made to minimize the carry-over.

1.2.3.2 Routine Drug Analysis

Calibration Curve and Integration

A standard curve, utilizing the model established during prestudy assay validation, should be generated daily and used to calculate the concentration of analyte in the unknown and QC samples assay in that analytical run. Seventy-five percent, or a minimum of six standards whichever is greater should fall within $\pm 15\%$, except for LLOQ, when it should be $\pm 20\%$ of the nominal value. All samples in an analytical run, including standard curve and QC samples, should be integrated in a consistent manner.

QC Samples

Duplicate sets (or a minimum of 5% of the unknowns) of frozen QC samples for at least three concentrations (low, middle, and high) should be processed along with the unknown clinical samples during each analytical run. The results of the analysis of QC samples provide the basis of accepting or rejecting the run. At least 67% of the total QC samples and at least 50% of QC samples at each concentration should be within $\pm 15\%$ of their respective nominal values.

1.3 SPME

SPME was first introduced by Pawliszyn and co-workers in the early 1990s.⁶¹ SPME is a solvent-free sample preparation technique that integrates sampling, isolation, and concentration into one step, and has been developed to address the need for rapid sampling and sample preparation in both laboratory and on-site. Its simplicity of use, relatively short sampling processing time and fiber reusability has made SPME an attractive choice for many analytical applications. Since its invention, SPME has been widely applied to environmental, food, metallic, forensic, and pharmaceutical analysis.^{16, 19, 62-63}

1.3.1 Introduction to SPME

SPME involves the use of a fiber coated with an extracting phase, that can be a liquid (polymer) or a solid (sorbent), which extracts different kinds of analytes including both volatile and non-volatile from different kinds of media. The quantity of analyte extracted by the fiber is proportional to its concentration in the sample as long as equilibrium is reached. In case of short pre-equilibrium times, convection or agitation is required to accelerate the approach to equilibrium. After extraction, the SPME fiber is transferred to the injection port of separating instruments, such as a gas chromatography or liquid chromatography where desorption of the analyte takes place and analysis is carried out. Figure 1-2 illustrates the first commercial SPME device made by Supelco Inc., where a small diameter fused-silica fiber coated with a small volume of extraction phase is contained in a specially designed syringe, moving the plunger allow for exposure of the fiber during extraction and desorption and for its protection inside the needle during storage and penetration of the septum.

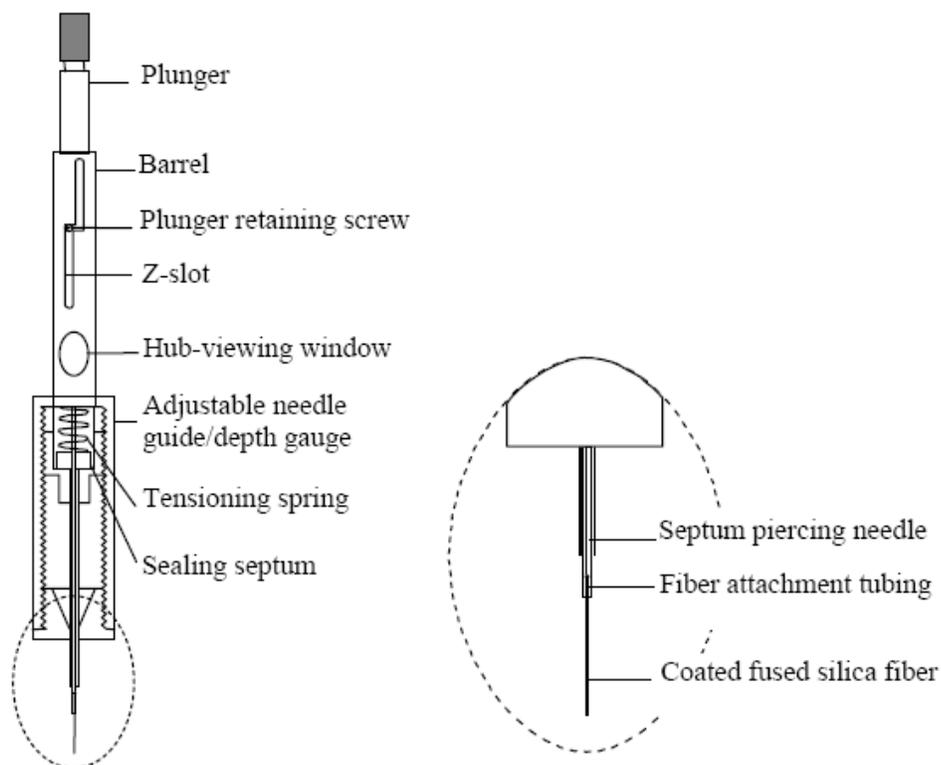


Figure 1-2 Commercial design of SPME device

Fibers and capillary tubes coated with an appropriate extraction phase are often used for SPME. However, other configurations such as stir bar sorptive extraction (SBSE), and membrane or thin-film SPME have also been developed (Figure 1-3). Two types of fiber SPME techniques can be used for analyte extraction: (i) headspace and (ii) direct extraction. Headspace SPME is suitable for the extraction of volatile and semivolatile analytes from the vapor phase above gaseous, liquid or solid samples; while direct SPME is used for the extraction of nonvolatile analytes or those with very low volatility, by immersing a fiber directly into liquid samples. In direct SPME, the amount of analyte extracted by the fiber at equilibrium can be expressed as:

$$n = \frac{K_{fs} V_f V_s C_s^0}{K_{fs} V_f + V_s} \quad \text{Equation 1.1}$$

where C_s^0 is the initial concentration of a given analyte in the sample, V_s is the sample volume, V_f is the fiber coating volume, and K_{fs} is the fiber/sample matrix distribution coefficient.

When the sample volume is large (i.e. $V_s \gg K_{fs} V_f$), the amount of analyte extracted becomes independent of sample volume and can be described as:

$$n = K_{fs} V_f C_s^0 \quad \text{Equation 1.2}$$

In this equation, the amount of analyte extracted is proportional to the volume of extraction phase; therefore, SPME assay sensitivity could be increased by increasing extraction volume. Furthermore, increasing the surface area-to-volume ratio will result in enhanced sensitivity and fast extraction rate.

Recently, great efforts have been focused on developing new SPME devices to increase extraction recovery.⁶⁴⁻⁶⁶ However, the simplicity of SPME technique such as easy to handle, little equipment, and solvent-less characteristics should not be abandoned.

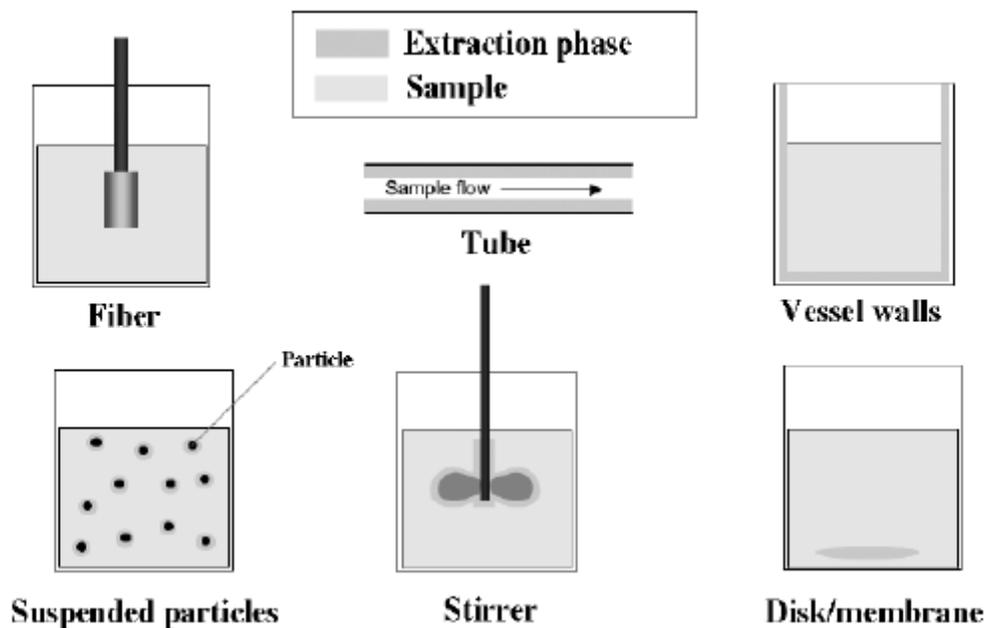


Figure 1-3 SPME and related microextraction techniques

1.3.2 Calibration in SPME

Base on the understanding of fundamental principles governing the mass transfer of analytes in multiphase systems, several calibration approaches have been developed in SPME. These can be classified as traditional calibration methods; equilibrium extraction, exhaustive extraction, pre-equilibrium extraction, and diffusion-based calibration.⁶⁷ Each calibration method has its own advantages and limitations. Calibration methods that are suitable for SPME bioanalysis are summarized and the characteristics of these methods are discussed.

Calibration Curve with Internal Standard

This traditional calibration approach involves the preparation of several standard solutions in a sample matrix with the addition of a compound that is different from the analyte. A calibration plot is developed by determining the ratio of the peak area of the

analyte to the internal standard for calibration solutions that contain different concentrations of the analyte with a fixed concentration of the internal standard. This ratio is subsequently used to calibrate the unknown samples. This calibration method does not require extensive sample preparation, but the sampling procedure and chromatographic conditions must remain constant for both sample and the standard solutions. The internal standard is used to compensate for the matrix effect, losses of analytes during sample preparation and irreproducibility in parameters. Analog compounds are often used, but stable isotope-labeled standards are highly recommended to achieve a satisfactory result although the compounds may not always be available. Calibration curve with internal standard is the simplest and the most widely used calibration approach in SPME high throughput bioanalysis.

Equilibrium Extraction

In equilibrium extraction, a small amount of extraction phase is exposed to a sample matrix until equilibrium is reached. If only two phases are considered, the equilibrium conditions can be described by Equation 1.1. Equation 1.1 indicates that the amount of analyte extracted onto the coating is linearly proportional to the analyte concentration in the sample, which is the analytical basis for quantification using SPME. When the sample volume is very large, Equation 1.1 can be simplified to Equation 1.2 which illustrates the advantage of equilibrium extraction where the amount of the extracted analyte is independent of the sample volume. This calibration approach is useful for *in vivo* SPME because at equilibrium, the amount of analyte extracted is independent of the agitation conditions so that the exact blood flow rate does not need to be known. The only requirements that must be met are (i) that equilibrium is established

for both in vivo sampling situation and calibration samples, and (ii) that the calibration matrix closely matches the system under study. The biggest limitation is that the approach requires very thin coatings so that equilibrium can be reached in a reasonable amount of time.

Diffusion-based Calibration

A theoretical model based on a diffusion-controlled mass transfer process to describe the entire kinetic process of SPME yields following equation: ⁶⁸⁻⁶⁹

$$n = [1 - \exp(-at)] \frac{K_{fs} V_f V_s}{K_{fs} V_f + V_s} C_0 \quad \text{Equation 1.3}$$

where a is a rate constant that is dependent on the extraction phase and sample volumes, the mass-transfer coefficients, the distribution coefficients, and the surface area of the extraction phase. This dynamic model suggests that a linear relationship exists between the absorbed analyte and its initial concentration in the sample matrix.

Based on the dynamic model of SPME, Chen et al. ⁷⁰⁻⁷¹ demonstrated the isotropy of absorption and desorption in the SPME liquid coating fiber and developed a new calibration method so called standard-in-fiber technique. Briefly, an appropriate standard, either an isotopically-labeled analogue of the analyte or any compound with mass transfer kinetics similar to that of the analyte, is preloaded on the fiber coating. During extraction, this preloaded standard is desorbed from the coating to the system under study. This desorption process can be used to calibrate the process of extracting the analyte of interest into the coating. The amounts of analyte extracted and the desorbed standard depend on the degree and uniformity of agitation in the system, the composition of sample matrix, and the exact length of sampling time. The whole procedure

compensates well for any potential sources of variability and, therefore, permits accurate quantitative analysis. The concentration of analyte in the sample can be determined by:

$$C_0 = \frac{nq_0}{q_0 - Q} \cdot \frac{1}{K_{fs}V_f} \quad \text{Equation 1.4}$$

where C_0 is the initial concentration of analyte, Q is the amount of standard remaining in the extraction phase after exposure of the extraction phase to the sample matrix for the sampling time, V_f is the volume of the fiber, K_{fs} is the fiber coating/sample distribution coefficient of the analyte, and q_0 is the amount of standard that is preloaded in the extraction phase. Standard-in-fiber calibration is suitable for both homogenous and heterogeneous samples and compensates well for effects of agitation, temperature, timing and biofouling; however, it needs a reproducible standard preloading procedure and requires external calibration at equilibrium to determine $K_{fs}V_f$.

1.3.3 Recent Development and Current Status of SPME in Bioanalysis

Two major recent fundamental advances in bioanalytical applications of SPME include the development of *in vivo* SPME, and the development of high throughput SPME using multi-well plate technology. *In vivo* SPME sampling relies on direct immersion of SPME coating into a living system in order to directly extract the analyte into the coating without having to remove a representative sample of biofluid or tissue from the living system. Therefore, this technique eliminates the need for blood withdrawal during pharmacokinetic studies and allows the study of biochemical processes directly *in vivo*. The first SPME *in vivo* study was reported by Heather et al. in 2003.⁷² A probe based on a polypyrrole extraction phase was developed and used for

extraction of benzodiazepines directly from a peripheral vein with subsequent LC-MSMS quantification. The limit of detection of the assay is 3~7 ng/mL for analysis of the benzodiazepines from whole blood and the method was used to monitor the PK profiles of diazepam and its metabolites in dogs. The results compared favorably with profiles determined from conventional methods. Since then, several SPME in vivo studies have been conducted and reported in different animal species including pigs, rats, and mice.⁷³⁻
⁷⁵ The biggest challenge for in vivo SPME is the limited commercial availability of biocompatible coatings that are suitable for a variety of biological applications. In addition, the lack of automation of the sample preparation processes makes in vivo SPME less attractive for high throughput drug discovery environment. In addition, a SPME method has to be developed beforehand for each compound before in vivo experimentation, which would further increase total analysis time.

Numerous applications of automated SPME-GC for the analysis of volatiles and semi-volatiles in clinical samples have been established over the years. The first automated in-tube SPME-LC method was developed by Eisert in 1997.⁷⁶ The extractions are achieved through several draw and eject cycles of the sample over the extraction phase, and subsequently the mobile phase is used to desorb and transfer the analytes to the analytical column for chromatographic separation and analysis. Despite the advantage of automation of in-tube SPME and the advantage that various GC capillary columns can be used as extraction phases, the approach does not permit parallel extraction processes and, therefore, has lower sample throughput because all samples and standards must be analyzed in sequence. In addition, in-tube SPME normally requires sample pre-treatment to filter samples containing particles or dilute biological samples to avoid capillary

column blocking. This will substantially increase the need in human participation in the whole sample preparation process. O'Reilly et al.⁷⁷ first proposed the approach of performing high throughput parallel SPME on a 96-well plate format. Since sample extraction and desorption are the most time consuming steps in SPME, a parallel multi-fiber approach is, obviously, the best way to use automated SPME to achieve high sample throughput. Based on this concept, the first commercially available automated SPME sample preparation system from Professional Analytical System (PAS) has been introduced using a 96-well plate format, an SPME multi-fiber device, two orbital shakers, and a three-arm robotic system. With the use of this system, high throughput analysis of benzodiazepines in human whole blood has been achieved. The method allowed the automated sample preparation of 96 samples in 100 minutes with a LLOQ of 4 ng/mL and accuracy from 87-113% with 800 μ L of blood sample.⁷⁸ Although the automation system significantly increased the extraction recovery by using SPME fibers in thin-film configuration, it required a large amount of the biological sample and extraction solvent in order to achieve satisfactory method precision and accuracy. The most dramatic advantages of SPME exist at the extremes of sample volumes as a solvent-less technique has been abandoned in the system.

SPME claims to have many distinct advantages over traditional sample preparation techniques such as PPT, LLE and SPE for bioanalytical applications. SPME is a simple, fast, solvent-less technique with less instrumentation and easy for automation; SPME provides very clean sample extracts as a small amount of sorbent is used and, thus, limits the possibility of co-eluting interferences; SPME has the ability to handle very complex, heterogeneous samples including whole blood without sample pre-treatment.

SPME is able to obtain free and total concentration information from a single biofluid sample by using appropriate calibration strategies. However, to date, the goal of using SPME technique as an alternative approach for quantitative determination of analytes especially in pharmaceutical bioanalysis has not been realized. Most of the applications are limited to research purposes. For example, compared with the rapid growth of SPE sorbents in the market, although many fiber coating techniques are now available in the literature⁷⁹ including sol-gel coating technology, electrochemical, and chemical procedures, and physical deposition of biocompatible materials, the progress of developing commercial available new SPME coatings is not substantial. In addition, although it is a very promising technique in the early drug discovery stage, in vivo SPME has not been widely accepted and the published results are generated mainly from one laboratory. Furthermore, few reported SPME assays have been fully validated according to FDA guidelines that could be used for routine drug analysis and researchers have seldom investigated matrix effects, which is essential for quantitative determination of drug analytes in various biological fluids. Based on the current practice of bioanalysis in drug discovery and development, it is necessary to explore and develop different automated SPME approaches that can be easily adapted to common commercially available liquid handling systems while maintaining the simplicity of the SPME technique.

1.3.4 SPME Modeling and Simulation

In SPME, equilibrium established must be among the concentrations of an analyte in the sample, in the headspace above the sample, and in the polymer coating on the fused silica fiber. Depending on the type of SPME coating, the extraction mechanism can be

distinguished as absorption for liquid coatings and adsorption for solid coatings. Independent from the nature of the coating, analyte molecules are initially attached to the surface of the coating. Whether they migrate to the bulk of the coating or remain at its surface depends on the magnitude of the distribution coefficient of the analyte in the coating. The amounts of the analytes extracted at equilibrium can be determined by laboratory experiments or by simplified mathematical models. However, SPME measurements are often performed under non-equilibrium conditions, in particular, for more hydrophobic chemicals for which equilibration times can be very long. It would be very useful to have a model that can be used to analyse measured concentrations in the fiber coating as a function of time. In addition, in a complex sample, the presence of another matrix or hydrophobic phase, such as protein or humic acids, may strongly influence the extraction efficiency and complicate the calibration procedure. An understanding of the possible mechanisms of binding matrix on the uptake of kinetics of analytes into the SPME fiber should be very valuable.

Several approaches have been proposed to mathematically model the kinetics of the absorption process to SPME liquid fibers such as PDMS. Vaes et al.⁸⁰ introduced "negligible depletion SPME" (nd-SPME) as a simple method to measure the free concentration of a compound in various matrixes. In nd-SPME, the concentration of analyte in the fiber coating (C_f) in time is related to the freely dissolved concentration in the aqueous phase ($C_{aq,free}$) through a first-order kinetic, one compartment model with absorption and desorption rate constants as parameters.

$$C_f = \frac{k_1}{k_2} C_{aq,free} (1 - e^{-k_2 t}) \quad \text{Equation 1.5}$$

This model is based on the assumptions that there must be equilibrium between the free and matrix bound fraction of the analyte, that the fiber should extract only a negligible amount of the free fraction, and that the matrix should not influence the uptake kinetics or adsorb to the fiber. The advantage of such an approach is its simplicity. The disadvantage is that the model is not explicitly based on processes like diffusion and partitioning of the analyte and on the experimental conditions like medium volume and fiber geometry. Therefore, the model hampers the development of a more fundamental understanding of the experimental data which can be used to optimize experimental conditions.

In 1997, Ai⁶⁸⁻⁶⁹ proposed a dynamic SPME model based on a diffusion-controlled mass-transfer process. In this approach, absorption into the fiber coating is a process governed by intra-fiber molecular diffusion and mass transfer between the bulk medium and the fiber by intra-layer molecular diffusion through a stagnant layer around the fiber with a finite thickness. The bulk medium is considered to be well stirred. The model can explain the influence of stirring or agitation on the uptake kinetics and it can also be applied to predict kinetics based on parameters such as the fiber-water partition coefficient, diffusion coefficient, and diffusion layer thickness. A layer thickness for this model can be estimated by assuming that the flow around the SPME fiber is steady and laminar. However, the layer thickness estimate requires additional parameters such as the speed of the fluid at the fiber surface, the fluid's kinematic velocity, and the diffusion coefficient of the analyte in the medium. The disadvantage of the model is that for other agitation conditions such as ultrasound agitation, or agitation by fiber itself, the accelerated flow regimes do not fulfill the requirements for estimating the stagnant layer thickness.

Instead of assuming that mass transfer from bulk medium to fiber is controlled by an explicitly modeled stagnant layer around fiber, others have introduced the concept that mass transfer is governed by the concentration difference between bulk medium and outer fiber surface.⁸¹⁻⁸² The model assumes that the diffusion of analyte in the fiber and in the boundary layer surrounding the coating is the rate limiting factor, and transport by diffusion is governed by Fick's law:

$$J(r,t) = -D \frac{\partial C(r,t)}{\partial r} \quad \text{Equation 1.6}$$

Here the diffusion mass flux J ($\text{mol dm}^{-2}\text{h}^{-1}$) at time t and at the radial coordinate r is proportional to the concentration gradient and D is the diffusion coefficient.

Furthermore, the model includes binding of the analyte to a matrix of bovine serum albumin (BSA). One limitation of using SPME to determine free concentration in biological matrixes is that binding matrixes should not interact with the SPME fiber. Binding matrixes may interact by adsorbing to the fiber surface, thus, possibly blocking the exchange of analyte across the fiber boundary. This may also lead to an overestimation of the concentration in the fiber coating as the matrix-bound analyte adsorbed to the fiber coating is measured along with the analyte in the fiber coating. Understanding the mechanism of the possible influence of a binding matrix on the uptake kinetics of compounds into the fiber is particularly important when considering SPME measurements performed under non-equilibrium conditions. Figure 1-4 shows a schematic depiction of the kinetic compartment considered in the model.

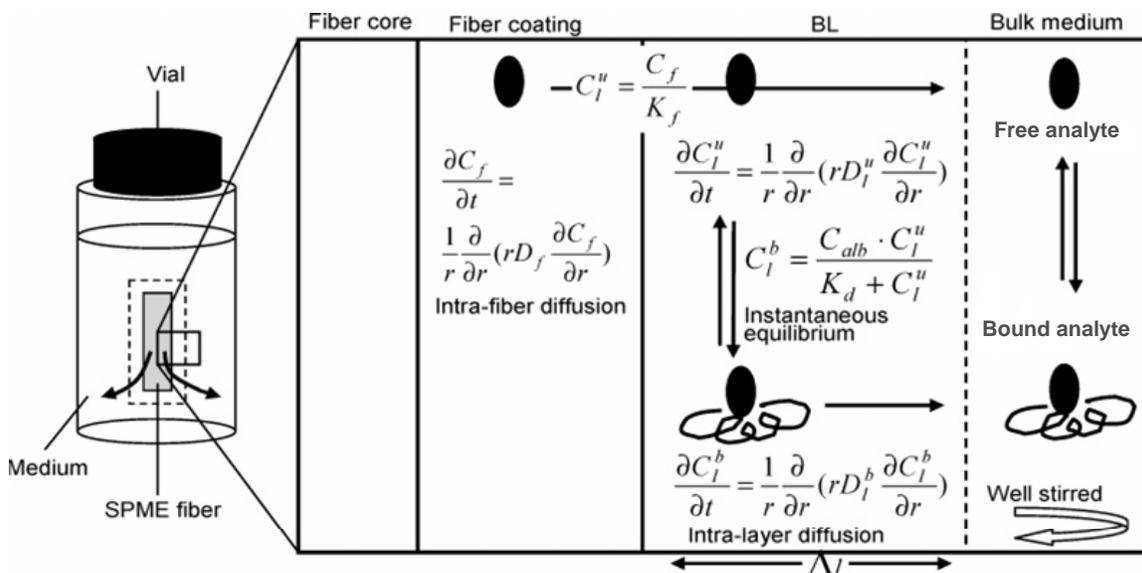


Figure 1-4 Simplified schematic depiction of the model compartments in radial coordinates (r).

It is assumed that the unbound and bound concentrations in the medium are in chemical equilibrium and that, in any instant at the fiber-medium interface, the concentration in fiber and the unbound concentration in medium are in equilibrium. In addition, continuity of fluxes over both the fiber coating and boundary layer interface and boundary layer and bulk medium interface is assumed. In this model, since the fiber coating thickness and the initial concentration of the analyte are known, the model requires the estimation of many parameters including diffusion coefficients, boundary layer thickness, fiber-medium partition coefficient, and association and dissociation constants. These parameters are obtained by fitting the model to the experimental data, however, the computation times or numerical stability can become prohibitive for some combinations of these parameter values.

An understanding of the fundamentals of thermodynamics and mass transfer of analytes in multiphase systems will provide insight and direction when developing SPME

methods and identifying parameters for rigorous control and optimization. For example, based on the dynamic model proposed by Ai,⁶⁸⁻⁶⁹ Chen et al.⁷⁰⁻⁷¹ demonstrated the isotropy of absorption and desorption in the SPME liquid coating fiber and proposed a new concept called "in-fiber standardization technique". Later this was termed the "kinetic calibration method". The method uses the desorption of the standards, which are preloaded in the extraction phase, to calibrate the extraction of the analytes. More recently, the one-calibrant kinetic calibration technique was developed to use the desorption of a single calibrant to calibrate all extracted analytes. The technique eliminates the requirement of several isotopic compounds, or high-concentration standards, and it simplifies the standard loading and quantitation procedures. Despite all the efforts and successfully applications of SPME modeling and simulation, there is still a need to develop simple and accurate models not only for liquid coating fibers, but for solid coating fibers as well. Increasing computation capabilities and advancements in the application of numerical techniques make it possible to include all transport steps in kinetic modeling and simulation. The effective use of the modeling approach will be helpful in SPME fiber selections and will minimize the number of experiments needed in SPME method development and, therefore, shorten the total cycle time.

1.4 Thesis Objectives

The overall objective of this thesis is to design a convenient format of SPME for high throughput bioanalysis in pharmaceutical industry and to validate the approach to the application.

Chapter 2 describes a proof-of-concept study of a multi-fiber SPME approach in 96-well format using commercially available Polydimethylsiloxane (PDMS) – divinylbenzene (DVB) fibers with a homemade module for simultaneous multi-fiber extraction and desorption. For the first time, a sensitive and selective HPLC-MSMS method has been developed and fully validated to determine a drug compound in clinical development with multi-fiber SPME in 96-well format. Proof-of-concept has been achieved based on the validation results and a head-to-head comparison with an LLE method for analyzing clinical samples.

Chapter 3 discusses the development of the in-tip SPME technique including preparation of fiber-packed and sorbent-packed in-tip SPME fibers. The in-tip SPME technique takes advantage of widely used commercially available automated liquid handling systems such as the Tomtec Quadra 96 workstation, and integrates the fiber SPME with the system in a very unique configuration. In-tip SPME is simple and easy to automate without introducing additional devices.

Chapters 4 and 5 report two applications of the automated in-tip SPME in bioanalysis. Chapter 4 is the quantitative LC-MS/MS determination of vitamin D₃ in human serum with derivatization. The in-tip SPME approach is compared with traditional LLE methods using either tubes or 96-well plate extraction. Chapter 5 reports the use of HILIC-MS/MS to determine three polar compounds, IMP, CIL and BLI, in biological fluids.

Chapter 6 reports a comprehensive evaluation of matrix effects using the automated in-tip SPME approach in bioanalysis.

Chapter 7 compares in-tip SPME with other automated SPME approaches and mainly focuses on the comparison between in-tip SPME and methods using the PAS SPME automation system. Strategies for in-tip SPME method development validation, potential applications, and future directions of in-tip SPME in bioanalysis are discussed.

Chapter 8 describes various model approaches to simulate the SPME extraction and desorption processes with both liquid and solid coating fibers. The simulation results are compared with experimental data and different applications of using SPME modeling and simulation are also discussed.

Finally, Chapter 9 summarizes the overall conclusion of the research work presented here and makes recommendations for future considerations.

Chapter 2

Proof of Concept Study: Comparison of SPME and LLE in 96-well Format for the Determination of a Drug Compound in Human Plasma by LC-MS/MS

2.1 Preamble and Introduction

2.1.1 Preamble

This chapter has been published as a paper: W. Xie, J. Pawliszyn, W.M. Mullett, B.K. Matuszewski, "Comparison of solid-phase microextraction and liquid-liquid extraction in 96-well format for the determination of a drug compound in human plasma by liquid chromatography with tandem mass spectrometric detection", *J. Pharm. Biomed. Anal.* **2007**, *45*, 599-608. The figures and tables are reprinted from this manuscript with the permission of Elsevier (Copyright Elsevier 2007).

The authors would like to point out that this research work was completed and submitted for publication to *Journal of Chromatography B* in 2004. Unfortunately, the Journal could not publish the paper unless the structure of the compound, which was in the clinical trials at the time, was released. The manuscript was finally published three years later in *Journal of Pharmaceutical and Biomedical Analysis* without the compound structure. In 2005, O'Reilly et al.* first proposed the approach of performing high throughput parallel SPME on a 96-well plate format in *Journal of Separation Science*.

* O'Reilly, J.; Wang, Q.; Setkova, L.; Hutchinson, J.P.; Chen, Y.; Lord, H.L.; Linton, C.N.; Pawliszyn, J. *J. Sep. Sci.* **2005**, *28*, 2010-2022.

2.1.2 Introduction

HPLC-MS/MS has gained widespread acceptance for the quantitative determination of drugs and metabolites in biological fluids because of the method's high selectivity and sensitivity compared to other techniques.⁸³⁻⁸⁷ In spite of the high selectivity and sensitivity achieved by HPLC-MS/MS, a rapid and accurate determination of trace amounts of drugs in very complex matrices such as plasma and urine is still quite challenging. This is largely due to the possibility of a severe matrix effect originating from co-eluting matrix components that may affect ionization of analytes of interest leading to ion suppression or enhancements.⁸⁸⁻⁹¹ In addition, the co-eluting metabolites of a drug being analyzed may give a MS/MS response in the channel used for drug quantification.¹⁰⁻¹² Therefore, isolation of analytes from biological matrices using an effective sample clean up technique is often critical to achieve assay selectivity.

LLE and SPE either on-line or off-line are two very commonly used approaches and are generally found to be sufficient to reduce or eliminate matrix effects and to provide reliable HPLC-MS/MS data.^{95,96} Although both LLE and SPE sometimes involve tedious and time-consuming extraction steps and often require evaporation and reconstitution steps prior to injection into the chromatographic system, up until now LLE and SPE are still the preferred methods for quantitative drug analysis in the pharmaceutical industry. With the development of new analytical instrument and techniques, high sensitivity of MS/MS detection may be achieved; therefore, in drug analysis the trend is to use a small volume of samples (usually less than 100 μ L) and simplify sample extraction procedures to improve overall method efficiency with low cost. Extracting very small volumes of samples using conventional methods, such as LLE

and SPE may be challenging. As an alternative method, SPME has shown great potential as a highly efficient sample preparation technique. Since its invention in 1990,⁶¹ SPME has been widely used in biomedical and pharmaceutical analysis, and several excellent reviews have been published on the topic.^{16,19,62,63,97} The great advantage of SPME over other extraction methods is that SPME is a solvent free extraction technique that combines sampling, extraction, concentration, and sample introduction in one step. The method could save sample preparation time and disposal cost, and can be used with a very small volume of samples. Despite the advantages of this technique, SPME has never been reported in the literature for routine drug quantitation from biological fluids in the pharmaceutical industry. Ulrich⁶² listed some principal disadvantages of SPME in biomedical analysis preventing its applications in drug analysis in biological fluids. In addition, limited selections of commercially available SPME fibers and difficulties with method automation for high-throughput sample analysis are also reasons why SPME is not widely accepted and used in clinical sample analysis.

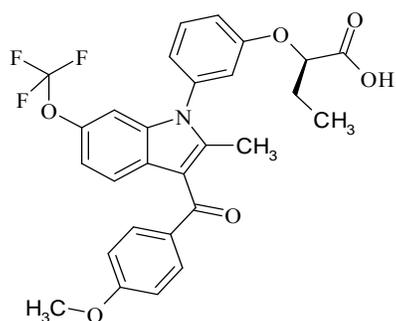
As part of a series of research studies conducted in our laboratory to explore the SPME technique in high-throughput drug analysis, we have developed and validated two extraction methods based on LLE and SPME to quantify a drug compound in human plasma from a clinical study. To increase sample throughput of SPME, the concept of 96-well format was introduced into sample preparation. In order to make a direct comparison between LLE and SPME, the same amount of plasma sample was processed over the same calibration curve range. The intraday precision and accuracy, the lower limit of quantitation, and the matrix effects of each method were evaluated, and results obtained from a healthy subject after single-dose and administration of 25 mg of drug using the

two different extraction methods were compared. To the best of our knowledge, this is the first example of implementation of SPME in 96-well format and validation of the SPME based method for quantitation of a drug in human plasma from a clinical study.

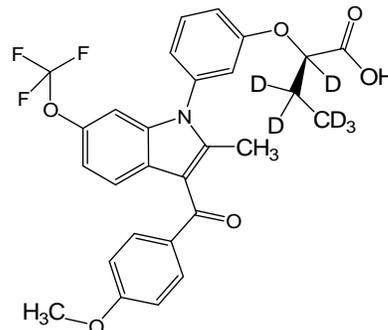
2.2 Experimental

2.2.1 Materials

A drug compound and its deuterated internal standard (d_6 -ISTD, Figure 2-1), were synthesized at Merck Research Laboratories (Rahway, NJ, USA). All solvents were HPLC or analytical grade and were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ammonium formate (HPLC grade), purchased from J.T. Baker (Phillipsburg, NJ, USA), formic acid (95%) and 85% o-phosphoric acid (Sigma, Milwaukee, WI, USA), were used as received. Deionized water was obtained by passing in-house water through a Millipore Milli-Q plus system (Bedford, MA, USA). Nitrogen (99.999%) was supplied by West Point Cryogenics (West Point, PA, USA). PDMS – DVB fibers (60 μ m) were purchased from Supelco (Bellefonte, PA USA). 96-Well collection plates (1.2 and 2.4 mL) and mats were purchased from Marsh Biomedical (Rochester, NY, USA). Different lots of drug free human plasma were obtained from Biological Specialties Corp. (Lansdale, PA, USA) and stored at -20 °C before use.



MK-0533



Internal Standard (d₆-ISTD)

Figure 2-1 Chemical structures of MK-0533 and its internal standard.

2.2.2 Instrumentation

A Perkin-Elmer (Norwalk, CT, USA) LC-200 micro pump and a Shimadzu SIL-HTC autosampler (Columbia, MD, USA) for 96-well plate were used in this work. The chromatographic separation of analytes was performed on a Restek BDS Hypersil C18 column (5 × 2.1 mm, 3 μm) with a 0.5 μm in-line filter. Mobile phase consisted of acetonitrile (ACN) : water (80:20, v/v) and was pumped at a flow rate of 0.2 mL/min. The total run time was 4 minutes. ACN : water (90:10, v/v) was used as a washing solvent for needle and flow path cleaning of the autosampler after each injection.

An Applied Biosystems-Sciex API 4000 triple quadrupole mass spectrometer (Foster City, CA, USA) equipped with a turbo ion spray (TIS) source operating in the negative ion ionization mode was used for all HPLC-MS/MS analysis. Multiple reaction-monitoring (MRM) mode was utilized for quantitation. In TIS experiments, the TIS probe temperature was maintained at 450 °C, and the nebulizing gas (air) pressure was set at 75

psi. The settings for the curtain gas, gas 1, and gas 2 were 10, 40, and 50 psi, respectively, and the ion spray voltage was -4200 V. Source and MS parameters were optimized by infusing a neat solution of drug compound prepared in ACN : water (50:50, v/v) at a flow rate of 20 $\mu\text{L}/\text{min}$ into a mobile phase pumped at 0.2 mL/min through the TIS interface. Multiple reaction monitoring of the precursor \rightarrow product ion pairs at m/z 526 \rightarrow 440 for drug compound and m/z 532 \rightarrow 440 for d_6 -ISTD was used for quantitation.

2.2.3 Preparation of Standard Solutions and QC Samples

A stock solution of drug compound (100 $\mu\text{g}/\text{mL}$) was prepared in ACN : water (50:50, v/v). This stock solution was further diluted with ACN : water (50:50, v/v) to give a series of working standards with concentrations of 5, 25, 50, 250, 500, 1,000, and 2,500 ng/mL. The d_6 -ISTD was also prepared as a stock solution (100 $\mu\text{g}/\text{mL}$) in ACN : water (50:50, v/v). A working standard solution of 1,000 ng/mL of d_6 -ISTD, prepared by diluting stock solution with ACN : water (50:50, v/v), was used for plasma samples analyses. All standard solutions were stored at 4°C. Plasma standards were prepared by adding 50 μL of each working standard to 250 μL of acidified human control plasma (15 μL of concentrated phosphoric acid per mL of plasma). The resulting plasma standard concentrations ranged from 1 to 500 ng/mL.

A stock solution for QC samples of drug compound was prepared separately by the same procedure using a separate weighing. QC samples were prepared by diluting the QC working solution with acidified human control plasma. QC samples at three concentrations (Low QC (2 ng/mL); Middle QC (100 ng/mL); High QC (400 ng/mL))

were used to evaluate assay precision and accuracy. All QC samples were divided into 1 mL aliquots in separate cryo tubes and stored at -20°C until analysis.

2.2.4 LLE Procedure

Standards, QC, and subject plasma samples were thawed at room temperature. 250 μL of subject plasma samples were added individually into a 2 mL deep 96-well plate with 50 μL of ACN : water (50:50, v/v) into blank and QC/subject plasma samples. Internal standard solution (50 μL) was added to each well of the plate, except to the well designated for the double blank plasma. The plate containing samples was placed onto a Tomtec Quadra 96 workstation (Hamden, CT, USA) for liquid transfer. After transferring 1.2 mL of hexane : isopropanol (80:20 v/v) extraction solution by Tomtec workstation, the plate was sealed with mat made of molded PTFE/silicone liner and was roto-mixed 20 minutes for LLE. The plate was then centrifuged for 15 minutes at 3,000 rpm and the top organic layer (100 μL) was aspirated and dispensed into a 1.2 mL 96-well collection plate by Tomtec workstation. The organic extract was evaporated to dryness under heated N_2 stream and reconstituted in 300 μL of ACN : 10 mM ammonium formate (80:20 v/v , adjusted pH = 3.3 using formic acid) solution and 2 μL were injected into the HPLC-MS/MS system.

2.2.5 SPME Procedure

Standards, QC, and subject plasma samples were thawed at room temperature. 250 μL of subject plasma samples were added individually into a 2 mL deep 96-well plate with 50 μL of ACN : water (50:50, v/v) into blank and QC/subject plasma samples.

Internal standard solution (50 μL) was added to each well of the plate, except the well designated for the double blank plasma. After transferring 500 μL of water to all wells on the plate using a Tomtec Quadra 96 workstation, the plate was sealed with mat made of molded PTFE/silicone line. A homemade plastic module (Figure 2-2) was used for SPME in 96-well format. The plastic module consists of three plates. The bottom plate was cut 1.3 cm deep at the bottom to fit the 2 mL deep 96-well plate and to seal the mat completely to avoid any leaking during rotation. In addition, 96 holes were drilled through the bottom plate with diameter slightly larger than that of SPME needle. The middle plate was used for two purposes. First, 96 wells were cut on the plate with holes drilled through to match the holes from the bottom plate, therefore, SPME needles could line up easily in each well but have to be manually penetrated through the mat. The height of the middle and bottom plates were measured accurately so that when fibers were manually pushed out of the needle, they would not reach the bottom of the 96-well plate (Figure 2-2). Secondly, after extraction, the fibers would be first withdrawn to the needles individually. Then the middle plate could be pulled out and all the SPME needles would come out of the mat altogether. The top plate was used as a cover so that the whole unit could be put on a Multi-tube vortexer for roto-mixing. During extraction procedure, eight PDMS/DVB needles were used at the same time, and the extraction time was optimized at 20 minutes. The same procedure was repeated except a new collection plate and a new mat were used with 1 mL of acetonitrile in each well for solvent desorption. After desorption for 10 minutes, the fibers were put into a standard desorption chamber individually for cleaning, and the same eight fibers were used for another eight samples extraction from the original 2 mL deep 96-well plate. Until desorption of all the samples

was done, the collection plate was evaporated to dryness under heated N₂ stream and reconstituted in 150 μL of the same solution as in LLE procedure, and 10 μL was injected into the HPLC-MS/MS system.

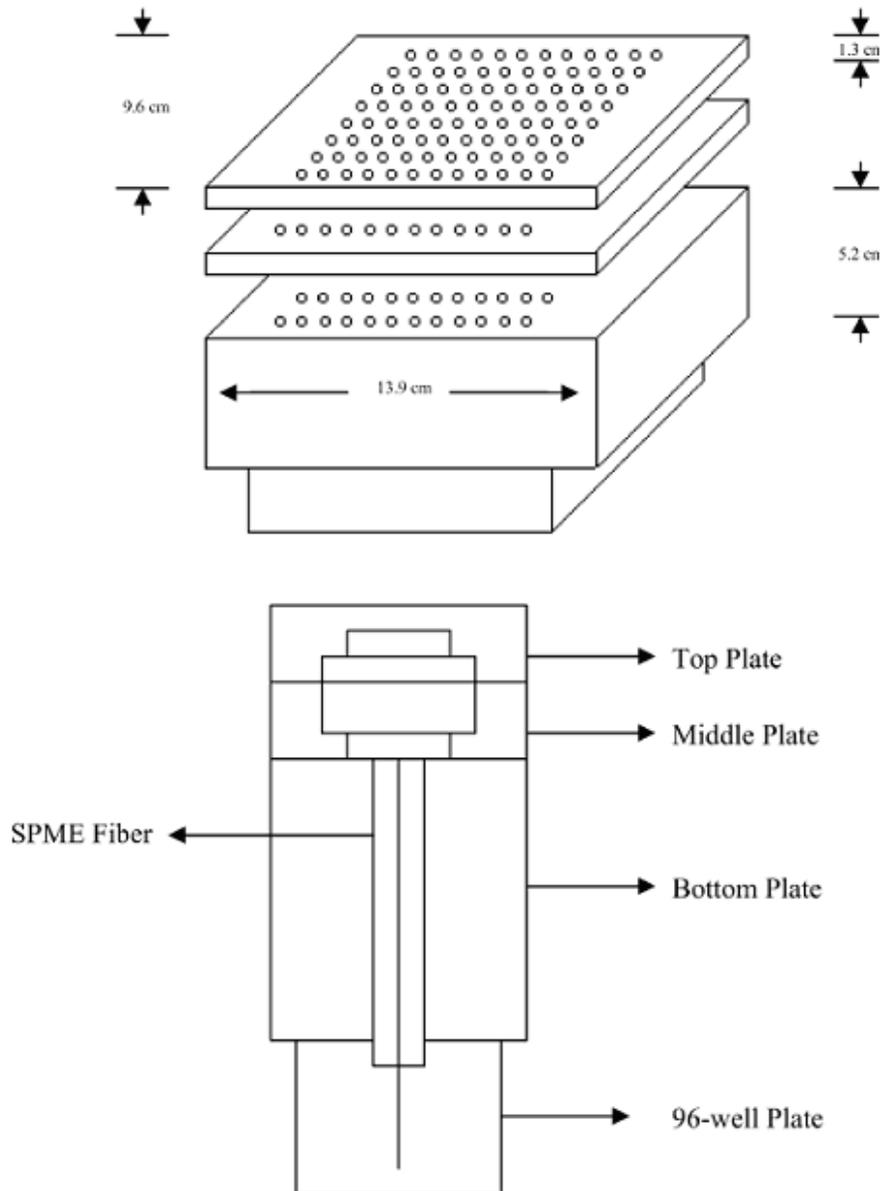


Figure 2-2 A home-made plastic module for SPME in 96-well format

2.2.6 Precision and Accuracy

The precision of the method was determined by the replicate analysis ($n = 5$) of drug compound in five different sources of human plasma at all concentrations utilized for the construction of calibration curves. The linearity of each calibration curve was confirmed by plotting the peak area ratio of the drug to internal standard *versus* drug concentration. The unknown sample concentrations were calculated from the equation $y = mx + b$, as determined by weighted ($1/x^2$) linear regression of the standard line. The accuracy of the method was determined as the percentage between the mean concentration observed and the nominal concentration. The precision of the method as measured by the CV was required to be <15% at the LLOQ and <10% at other concentrations used for constructing the standard curve.

2.2.7 Selectivity

The selectivity of the method was confirmed by processing control drug-free human plasma samples from six different sources to determine whether endogenous peaks were present at the MS/MS transitions used for monitoring the analyte and/or the internal standard. In addition, the “cross-talk” between MS channels used for monitoring the analyte and the internal standard was evaluated.

2.2.8 Recovery and Matrix Effects

Extraction recovery was evaluated for drug compound and its internal standard using standards spiked at three concentrations (5, 50, and 200 ng/mL) for drug compound, and for internal standard at a concentration of 200 ng/mL. Recovery was determined by

comparing the absolute peak areas of standards spiked into control human plasma and extracted to the control plasma extracted in the same manner and then spiked post-extraction with the analytes. Since a stable isotopically labeled compound was used as an internal standard, the potential “relative” matrix effects on ionization should not have any adverse effect on the quantitation of the drug compound in different plasma lots. The absence of the “relative” matrix effects was illustrated by the examination of the slopes of the calibration curves in five different lots of control plasma.

2.2.9 Stability

The stability of drug compound and its internal standard in the stock and working solutions was investigated. Storage stability of the drug compound in human plasma and the influence of freeze-thaw cycles were also examined by analyzing a set of QC samples at three concentrations. The calculated mean values should not deviate by greater than 15% of the nominal value.

2.3 Results and Discussion

2.3.1 Evaluation of the Stability of the Acyl-glucuronide of Drug Compound

Due to the presence of a carboxylic group moiety in the drug compound under study, the formation of the acyl-glucuronide metabolite of drug *in vivo* was likely and was confirmed after dosing animal species with the drug. This acyl-glucuronide could potentially hydrolyze to parent compound in sodium heparinized human plasma following sample collection. Therefore, it was necessary to evaluate the stability of this metabolite in human plasma during sample extraction and handling. Hydrolysis of the

glucuronide was found to be dependent on the temperature and pH of the sample. The addition of at least 10 μL of concentrated phosphoric acid per milliliter plasma was found to prevent the glucuronide hydrolysis. Up to 30 μL of concentrated phosphoric acid per mL of plasma could be added to heparinized human control plasma without denaturing plasma proteins. The acyl-glucuronide was found to be stable in acidified plasma stored at room temperature for at least 60 minutes. However, at room temperature, hydrolysis was observed in non-acidified heparinized human control plasma after 30 minutes and it became significant after 60 minutes. The stability of acyl-glucuronide was further assessed by spiking 200 ng of acyl-glucuronide standard (known to be contaminated with drug) in 1 mL of acidified human control plasma and analyzing the resulting drug concentrations following F-T cycles. The determined drug concentrations in these samples practically did not change following up to three freeze-thaw cycles.

2.3.2 Optimization of Chromatography and Extraction Conditions

Good peak shape and acceptable sensitivity were observed when initial attempts were made to detect drug compound by using turbo-ion spray interface in positive ion mode with conventionally buffered mobile such as ACN:10 mM ammonium formate (60:40 v/v , pH 3). However, poor reproducibility was obtained when five standard curve samples were extracted from acidified plasma and analyzed. It was interesting to find that utilization of a “buffer-free” mobile phase of ACN: water (80:20, v/v), in negative ionization mode using turbo-ion spray interface resulted in significant improvement in sensitivity and reproducibility, as long as the mobile phase used as the reconstitution solutions was adjusted to pH of about 3. After exploring many different kinds of reverse

phase columns, it was found that BDS Hypersil C18 column (5×2.1 mm, $3 \mu\text{m}$) produced the best results in terms of peak shape and retention of analytes. The acylglucuronide of drug compound eluted at the solvent front and was, thus, well separated from the analyte under the conditions utilized.

Different types and various compositions of organic solvents were tested to achieve better recovery of analyte from plasma during LLE. Due to the acidic nature of the compound, it was found that for most of solvents tested, better recoveries were obtained at a pH of 3. However, good reproducibility was also observed when the extraction solvent was composed of 80% hexanes and 20% IPA. The solubility of IPA in aqueous media plays a critical role during the extraction, but its volume should not exceed 20 percent. As both control and subject plasma samples were treated with concentrated phosphoric acid, no other buffers were necessary for pH adjustment before liquid-liquid extraction.

The biggest advantage of SPME is that it is a solvent-free extraction technique. The SPME fibers were directly immersed into the plasma for extraction and all commercial available fibers were tested under same conditions for extraction efficiency and sensitivity of detection. The extraction efficiency of SPME depends on the intermolecular interactions between the fiber coating and the analytes. It was found that PDMS-DVB gave the best results compared with other types of SPME fibers. For analytes extraction from biological fluids, SPME fibers with solid coatings generally produce better extraction efficiency compared with those with liquid coatings. This is due to the well-defined, dense crystalline structure of solid coating which significantly reduces the diffusion coefficients within the structure and extraction occurring through

adsorption on the surface of the fiber.⁹⁸⁻¹⁰¹ In order to make a direct comparison between SPME and LLE, attempts were focused on making SPME procedure as simple as possible. No salts were added to the samples and no pH adjustment of plasma samples were made, except the addition of 500 μL of water to each sample well in the 96-well plate to reduce viscosity of plasma sample caused by the presence of a concentrated acid and to make sure the fibers could be completely immersed in the sample solution during the plate rotation. Agitation was unnecessary for SPME extraction in this work as the whole plate was rotated consistently and the fiber position in the plate well was not critical, as long as the fiber was completely exposed from the protective needle. In order to achieve a 1 ng/mL of LLOQ, extraction time was optimized to 20 minutes and 10 minutes for desorption. Considering 96-well format was used for SPME sample preparation, the whole process was relatively fast and simple. Both methanol and acetonitrile were tested for solvent desorption and no difference between these two solvents was observed. Since acetonitrile was used for sample preparation and in the mobile phase; this solvent was also selected as the desorption solvent. Issues with sample carry-over were observed in this experiment. It was found that after a single solvent desorption, there were still more than 10% of the analytes remaining in the SPME coating, and further washing was necessary to reduce carry-over to an acceptable level before the same fiber could be used for subsequent sample extraction. A standard desorption chamber from Supelco was used for fiber cleaning. Mobile phase was flushed through the fiber until no carry-over was observed. It took about 4 to 8 minutes to eliminate carry-over depending on analyte concentration. Further experiments are needed to reduce the time and to increase the efficiency of the desorption procedure. In the work present in this

paper, eight commercially available PDMS-DVB fibers were used at the same time for 96-well format extraction. The excellent precision and accuracy of the method indicated that the concept of 96-well format could be successfully applied to SPME using large number of fibers.

2.3.3 Method Validation

The two methods based on LLE and SPME were validated in human plasma over the concentration range of 1 to 500 ng/mL of drug. Assessment of the intraday variability of each method was conducted in five different lots of acidified human control plasma spiked with drug compound. The resulting method precision and accuracy data are presented in Tables 1 and 2. For LLE, the intra-day precision of the method was 0.8% at LLOQ, and was equal to or lower than 3.3% at all other concentrations used for the construction of the calibration curve. Method accuracy was found to be within $\pm 2\%$ of the nominal concentration for all the standards evaluated. For SPME, the intra-day precision was 6.9% at LLOQ, and was equal to or lower than 5.7% at all other concentrations. Method accuracy was found to be within $\pm 5\%$ of nominal concentrations. The correlation coefficient for the mean standard curves constructed from five different lots of acidified human plasma for LLE and SPME was 0.9997 and 0.9986, respectively.

Table 2-1

Intraday precision and accuracy data for the determination of drug compound in five different lots of acidified human control plasma using LLE

Nominal conc. (ng/mL)	Mean calculated conc. (ng/mL) ^a	Precision C.V.% ^b	Accuracy (%) ^c
1	0.995	0.8	99.5
5	5.05	2.4	101.0
10	10.16	1.1	101.6
50	50.90	0.7	101.8
100	100.18	1.6	100.2
200	196.00	3.3	98.0
500	489.80	1.0	98.0

^a Mean concentrations calculated from the weighted linear least-squares regression curve constructed using all five replicate values at each concentration.

^b Expressed as coefficient of variation (C.V.%) of peak area ratios.

^c Expressed as [(mean calculated concentration)/(nominal concentration)] × 100%.

Table 2-2

Intraday precision and accuracy data for the determination of drug compound in five different lots of acidified human control plasma using SPME

Nominal conc. (ng/mL)	Mean calculated conc. (ng/mL) ^a	Precision C.V.% ^b	Accuracy (%) ^c
1	1.004	6.9	100.4
5	4.82	2.7	96.4
10	10.16	1.2	101.6
50	51.84	0.5	103.7
100	101.60	1.1	101.6
200	202.00	1.0	101.0
500	476.00	5.7	95.2

^a Mean concentrations calculated from the weighted linear least-squares regression curve constructed using all five replicate values at each concentration.

^b Expressed as coefficient of variation (C.V.%) of peak area ratios.

^c Expressed as [(mean calculated concentration)/(nominal concentration)] × 100%.

2.3.4 Selectivity

Assessment of the selectivity of a method is critical and needs to be confirmed in the presence of *in-vivo* metabolites of an analyte. Metabolites that are chromatographically not separated from the analyte of interest may be converted to a parent drug during sample preparation and/or undergo partial fragmentation in the ion source at elevated temperatures giving the same molecular ion as for the parent drug. The major metabolites of drug compound were evaluated for the “cross-talk” in channels used for monitoring both drug and the internal standard. No interference or “cross-talk” from these metabolites was observed. In addition, the “cross-talk” between channels used for monitoring both drug and the internal standard was evaluated by the analysis of standard samples containing individual compounds separately at the concentrations of 500 and 200 ng/mL for drug and internal standard, respectively, and monitoring the response in other MS/MS channel used for quantification. No response was observed in the channel of the other analytes at their retention times. Figure 2-3 shows the representative extraction ion chromatograms obtained from human control plasma blank, human control plasma spiked with 200 ng/mL of internal standard, human control plasma spiked with 1 ng/mL of drug and 200 ng/mL of internal standard, respectively, and 500 ng/mL of drug only.

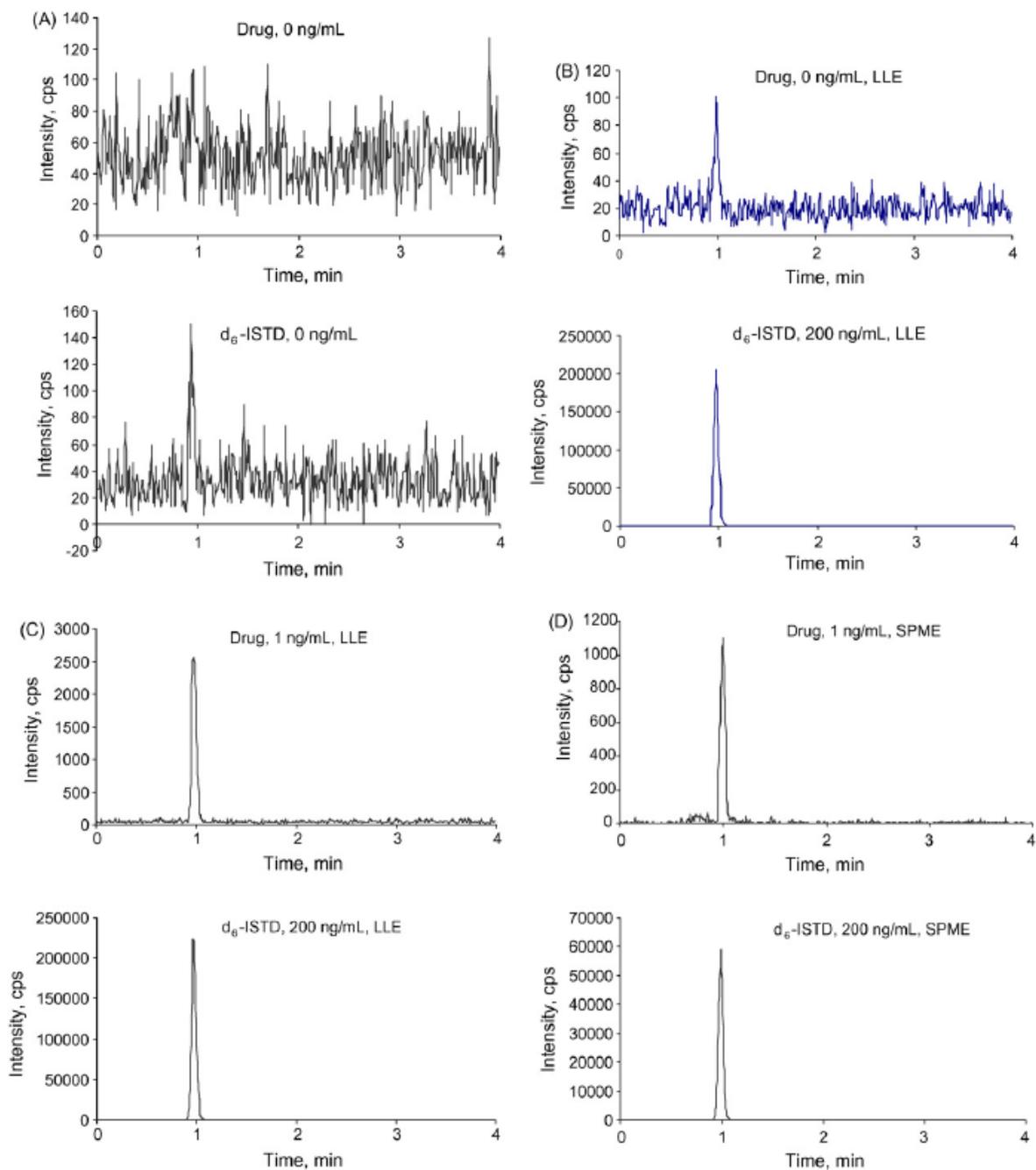


Figure 2-3 Representative extracted ion chromatograms of (A) double blank; (B) single blank, spiked with 200 ng/mL of d_6 -ISTD; (C) lower limit of quantification (LLOQ), 1 ng/mL of drug with 200 ng/mL of d_6 -ISTD using LLE; (D) LLOQ, 1 ng/mL of drug with 200 ng/mL of d_6 -ISTD using SPME; (E) 500 ng/mL of drug only using LLE

2.3.5 Recovery and Assessment of the Matrix Effects

In the 96-well LLE procedure, extraction recovery and the effect of the plasma matrix on ionization was evaluated for drug compound using standards spiked at concentrations of 5, 50, and 200 ng/mL and for d₆-ISTD spiked at a concentration of 200 ng/mL. Recoveries were determined by comparing the peak areas of standards spiked into five different lots of acidified human control plasma and extracted as per LLE procedure to acidified human control plasma extracted in the same manner and then spiked post-extraction with a known amount of the drug. “Absolute” matrix enhancement/suppression of ionization was evaluated by comparing the peak area of acidified human control plasma samples extracted and then spiked with a known amount of each analyte, to neat standards injected directly in the same reconstitution solvent. Results are shown in Table 2-3. Based on the intraday precision and accuracy results (Table 2-1) and the slope data (Table 2-4) that were obtained using five different lots of human control plasma, the use of a stable isotope labeled analogue as the internal standard largely compensated for any variation in matrix effects and/or recovery between the different lots of human control plasma. Therefore, “relative” matrix effects⁹³ on ionization from five different lots of human control plasma were negligible. A general practice in determining the recovery in SPME is to compare the peak areas obtained from the extracted, spiked plasma samples with those obtained by direct injection of standard solutions. Due to the relatively small surface area of the stationary phase of the SPME fiber and the use of different extraction mechanisms compared with LLE, the recoveries observed in SPME are generally one order of magnitude lower than those obtained by LLE, which was also found to be the case in this work. However, special attention was given to the evaluation

of the “relative” matrix effects. It was expected that variation in the “absolute” matrix effects in SPME would be larger than that in LLE, as SPME fibers were directly immersed in plasma samples. Based on the intraday precision and accuracy results (Table 2-2) and the slope data (Table 2-4) that were obtained using five different lots of human control plasma, it was found that the “relative” matrix effects were not observed, and the utilization of a stable isotope labeled analog as the internal standard played a critical role in compensating for any variation in “absolute” matrix effects and/or recovery between different lots of human control plasma. In cases when an analog rather than a stable isotope labeled internal standard are utilized, the careful assessment of the “relative” matrix effects is necessary when LLE, SPME, or other sample preparation procedures are utilized.

Table 2-3

Extraction recovery and assessment of matrix effects for drug compound and ISTD in acidified human control plasma using LLE

Standard conc. (ng/mL)	Drug		ISTD	
	Extraction recovery (%) ^a	Matrix effect (%) ^b	Extraction recovery (%) ^a	Matrix effect (%) ^b
5	92.8	125.1		
50	80.1	112.2		
200	78.9	125.0		
200			83.5	118.5

^a Extraction recovery was calculated by dividing the mean peak areas of analyte ($n = 5$) and ISTD ($n = 15$) spiked into plasma before the extraction by the respective mean peak areas of analyte ($n = 5$) and ISTD ($n = 15$) spiked after the extraction.

^b Matrix effect was calculated by dividing the mean peak areas of analyte ($n = 5$) and ISTD ($n = 15$) spiked after extraction by the respective mean peak areas of the analyte ($n = 5$) and ISTD ($n = 15$) standards in the mobile phase injected directly.

Table 2-4

Human control plasma lot number	Slope	
	LLE	SPME
1	0.01060	0.00922
2	0.01060	0.00950
3	0.01070	0.00955
4	0.01070	0.00942
5	0.01070	0.00944
Mean	0.01066	0.00943
Standard Dev.	0.00005	0.00013
Precision ^a (%)	0.5	1.4

^a Coefficient of variation, $n = 5$.

2.3.6 Analyte Stability

The stability of drug stock solution was evaluated by comparing freshly prepared standards solutions from a new standard weighing to similarly prepared solutions stored for 60 days at 4 °C. The peak areas of the new standard solutions were found to be within 98-102% of the 60 days old standard solution peak areas, confirming the stability of drug in stock solutions for 60 days. QC samples ($n = 5$ at each concentration) were subjected to three freeze-thaw cycles consisting of a thaw to reach room temperature and then refreezing at -20 °C. These samples, together with a set ($n = 5$ at each concentration) of human QC samples that were not subjected to additional freeze-thaw cycles, were then defrosted and analyzed. In all cases, the results for the samples that were subjected to additional freeze-thaw cycles were within $\pm 8\%$ of the nominal value. The results are shown in Table 2-5.

Table 2-5

Freeze–thaw (F/T) stability of drug compound in acidified human control plasma

Nominal conc. (ng/mL)	Mean ^a determined conc. (ng/mL) after 1 F/T cycles using LLE	Accuracy (%) ^c	Mean ^a determined conc. (ng/mL) after 3 F/T cycles using LLE	Accuracy (%) ^c	Mean ^a determined conc. (ng/mL) after 1 F/T cycles using SPME	Accuracy (%) ^c
2	1.904 (1.6) ^b	95.2	2.158 (4.4)	107.9	1.888 (2.7)	94.4
100	97.9 (1.5)	97.9	96.5 (4.0)	96.5	102.4 (0.9)	102.4
400	378.6 (0.9)	94.7	384.4 (5.4)	96.1	394.8 (1.5)	98.7

^a $n = 5$.^b Numbers in parentheses are coefficients of variation (%C.V.).^c Expressed as [(mean determined concentration)/(nominal concentration)] \times 100.**Table 2-6**

Initial intraday and interday analysis of plasma quality control (QC) samples from clinical studies using LLE

	Low QC (ng/mL)	Middle QC (ng/mL)	High QC (ng/mL)
Nominal concentration	2.0	100	400
Initial mean ($n = 5$)	1.904	97.9	378.6
Accuracy ^a (%)	95.2	97.9	94.7
C. V. ^b (%)	1.6	1.5	0.9
Daily runs			
Mean ($n = 84$)	1.992	98.0	391.4
Accuracy (%)	99.6	98.0	97.9
C.V. (%)	4.3	4.4	4.4

Initial date of preparation QC samples.

^a Expressed as [(mean calculated concentration)/(nominal concentration)] \times 100%.^b Coefficient of variation.

2.3.7 Clinical Sample Analysis

The method using LLE approach has been implemented in a clinical study, and thus far, more than two thousands plasma samples have been analyzed. Inter-day precision and accuracy of the method for the clinical samples analysis were determined by analyzing QC samples at low, medium, and high concentrations. Table 2-6 demonstrated the means, precision, and accuracy for QC samples prepared before the analysis of the study samples and for QC samples analyzed in replicate with the daily runs of the clinical samples. The precision for daily runs (n=84) was less than 4.5% with accuracy ranging from 97.9-99.6%.

In order to compare the clinical data obtained using LLE vs SPME technique, samples from one post-dose subject from the clinical study were reanalyzed using both approaches. Concentration-time profiles of drug in plasma of the same healthy subject after single-dose administration of 25 mg of drug obtained using LLE and SPME methods are presented in Figure 2-4. The two data sets obtained using two widely different extraction methods are in excellent agreement, clearly demonstrating that SPME could be used in the case under study as an alternative approach for multi-sample analysis in pharmacokinetic studies.

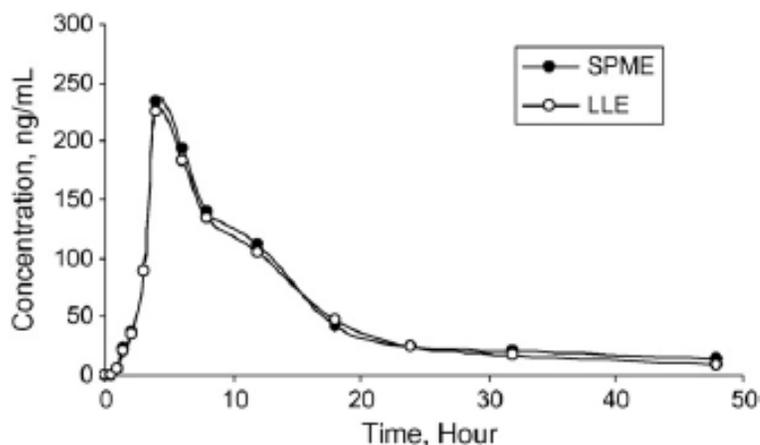


Figure 2-4 Concentration-time profile of drug compound in plasma of a healthy subject after single-dose administration of 25 mg of drug using LLE and SPME techniques.

2.3.8 Comparison between LLE and SPME

The same LLOQ of 1 ng/mL when 0.25 mL of human plasma was processed was achieved using both LLE and SPME methods. Both procedures were validated in the same concentration range of 1 - 500 ng/mL. The linearity of the calibration curves, the intra-day precision, and accuracy were all satisfactory in both methods. Recoveries of analytes using LLE were at least 10 times higher than those obtained by SPME and overall sensitivity of detection was much better from LLE than that from SPME. In the reported study, the overall sample preparation time for LLE was less than half of that required for SPME with the same number of clinical samples. However, the advantages of using SPME were also very evident. First, the procedure is simple and organic solvent consumption is far less than that of LLE. Secondly, evaporation and reconstitution steps required in LLE prior to injection to the chromatographic system could be avoided in SPME, which may be particularly desirable for the quantification of labile analytes that are stable in biological fluids, but may decompose during the evaporation process. In

addition, there is a great potential that the sample preparation time could be significantly reduced if the SPME process is automated. In our case, if more than eight fibers were used for extraction at a time, the total sample preparation time of SPME would be comparable or shorter than that of LLE. On the other hand, the disadvantages of SPME cannot be overlooked. There are only a few commercially available SPME fibers. In comparison with LLE, SPME is a relatively non-selective extraction method, and great effort is needed to increase extraction recovery and efficiency. In addition, extra clean-up procedures are necessary for repeat analyses using the same fiber. Quantitation is more prone to errors due to changes of the matrix in SPME than in other conventional extraction methods, and matrix effects should be thoroughly investigated during method validation.

2.4 Conclusions

For the first time, highly selective and sensitive HPLC-MS/MS methods with LLE and SPME approaches in 96-well format were developed and validated for the determination of a drug compound in human plasma. Both methods achieved a LLOQ of 1 ng/mL using 0.25 mL of plasma sample. The applicability of the method using liquid-liquid extraction was demonstrated by analysis of a drug compound in more than two thousands human plasma samples from a clinical study. The potential for implementation of SPME approach in multi-sample drug analysis was also successfully demonstrated, and the results obtained from the analysis of a drug in plasma samples from a healthy subject after single-dose and administration of 25 mg of drug using the LLE and SPME methods were practically the same.

Chapter 3

Development of High Throughput In-tip SPME Fibers

3.1 Preamble and Introduction

3.1.1 Preamble

Experimental results described in this chapter about fiber-packed in-tip SPME has been published as a paper: W. Xie, W.M. Mullett, C.M. Miller-Stein, J. Pawliszyn, "Automation of in-tip solid-phase microextraction in 96-well format for the determination of a model drug compound in human plasma by liquid chromatography with tandem mass spectrometric detection", *J. Chromatogr. B* **2009**, 877, 415-420. The figures and tables are reprinted from this manuscript with the permission of Elsevier (Copyright Elsevier 2009)

Experimental results described in this chapter about sorbent-packed in-tip SPME will be published in *Bioanalysis* in the September Special Issue on Biological Sample Preparation as an invited paper.

3.1.2 Introduction

Since its introduction in 1990, SPME has been widely used in many areas of analytical chemistry, such as food, environmental, and biological analysis as a solvent-free extraction technique which combines sampling, sample clean-up, and pre-concentration into a single step. The most significant advantages of SPME include the potential for fast methods, easy handling, automation, minimal requirements for necessary equipment, and low solvent usage. However, its disadvantages can limit the

viability of the method as much as its advantages promote it. In 2000, Ulrich gave a critical review⁶² of SPME in biomedical analysis which summarized and concluded that SPME was not a universal sample preparation method: as a non-exhaustive extraction technique, in complex matrices, the extraction time was relatively long and recoveries were considerably lower than those reported from other extraction methods, such as LLE and SPE. Therefore, it is difficult to analyze target analyte at very low concentrations due to interferences from endogenous substances in biological fluids. Analyte carry-over is also possible in SPME methods due to the repeated use of a single fiber and the requirements for longer desorption time decreases the advantages of the SPME methods. Besides these principle disadvantages, the limited range of commercially available SPME stationary phases and lack of high throughput capability are also major factors that prevents its application in routine use as an alternative approach for quantitative determination of analytes, especially in pharmaceutical bioanalysis.

To solve these problems and overcome the challenges of SPME technique, research has been focused on the areas of new SPME coatings and devices that offer higher extraction efficiency and selectivity, automation for high throughput analysis, and new SPME method optimization and calibration approaches. Although the development of new coatings is one of the most active areas of SPME studies, the progress of developing commercially available new SPME coatings is not substantial compared with the rapid development of SPE sorbents in the market. Up until now, commercially available fibers include PDMS, poly(acrylate) (PA), carboxen/poly(dimethylsioxane) (CAR/PDMS), carbowax/templated resin (CW/TPR), and PDMS/DVB, all of which only roughly cover the scale of polarity. The existing fibers can have issues with instability

and swelling in organic solvent, lack of lot-to-lot reproducibility, breaking of the fibers, stripping of the coatings, and a relatively high expense. Several SPME coating procedures have been explored in the past, including sol-gel technology, in-tube extraction, electrochemical procedures, and physical deposition, and the most recent developments in SPME coatings have been summarized in some reviews.^{79,98,102} Many in-house made SPME coatings have been discovered and tested in various applications, such as a new type of molecularly imprinted polymers (MIPs), fibers that were directly synthesized using silica capillaries as molds with silica being etched away after polymerization and tested for the SPME of triazines from environmental and food samples.¹⁰³ Other examples include a novel Pt fiber coated with single-walled carbon nanotubes prepared by electrophoretic deposition and applied to the determination of phenols in aqueous samples¹⁰⁴ and a new line of biocompatible coatings immobilized on the metal fiber core and consisting of a mixture of proprietary biocompatible binder and various types of coated silica particles with an assessment in vitro.¹⁰⁵ An interesting trend of developing new SPME coatings is to use methacrylate-based monoliths, which are widely used as efficient stationary phases for all types of chromatography separations since their introduction in the early 1990s. The polymer monoliths are normally prepared through bulk free-radical copolymerization of a monovinyl monomer with a cross-link monomer in the presence of porogenic solvent and an initiator by thermal or irradiation. The pore properties and the surface area of the polymer monoliths could be freely controlled by the type and composition of the porogenic solvent and by the percentage of cross-linkers. The main advantages of polymer monoliths are the simplicity of preparation with a wide range of chemistries for reactions and enhanced mass transfer. In

addition, entrapping chromatographic beads and sorbents for preparing fritless columns using methacrylate-based monoliths is one of the favorable features of the technique. The initial applications of polymer monoliths are mainly focused on protein separation and dedicated to use in conventional SPE; ^{106,107} more recent polymer monoliths are used as sorbent for in-tube SPME for drug analysis.¹⁰⁸ The successful applications of these in-tube SPME methods using polymer monoliths demonstrated the feasibility of this new coating approach, however, due to the limitations of high throughput in in-tube SPME, as each sample is processed serially, it is generally agreed that parallel SPME fibers using a multi-well plate approach would be the best option for high throughput bioanalytical applications of SPME.^{109,110}

To accomplish SPME automation and to maintain the simplicity of the technique, which is the biggest advantage compared with other conventional extraction methods, we have developed a new approach of using pipette tip-based SPME in a 96-well plate format. The automation of in-tip SPME technique can be easily achieved by commercially available system using 96-well extraction plates and a robot without the need to for introducing additional devices. In this study, we aim to introduce a simple and high throughput in-tip SPME fiber procedure based on polymer monoliths using photopolymerization. Different from other reported SPE based microtips,¹¹¹ the polymer monoliths in-tip SPME fibers are designed for non-exhaustive extraction and could be used as disposable tips which will completely eliminate carry-over effect encountered often in repeated use SPME fibers. The open configuration for extraction also better facilitates viscous samples as commonly encountered with bioanalysis. Besides sorbent-

packed in-tip SPME, an alternative technique using SPME fiber instead of polymer materials has also been developed.

To the best of our knowledge, this is the first study to use an automation device to prepare 96 fibers at one time. A polymerization mixture consisting of ethylene glycol dimethacrylate (EDMA), dimethoxy- α -phenylacetophenone (DMPA), and 1-decanol was used to prepare the sorbent-packed in-tip SPME fibers. The optimization procedures that affect polymer morphology, such as compositions of the cross-linkers and porogens, polymerization time and fiber thickness, and the extraction efficiency by immobilized extraction sorbent such as Oasis HLB particles were investigated. Also, the reproducibility of automated in-tip SPME fiber preparation, as well as sample processing parameters, such as sample extraction and desorption times and volumes, aspiration, and dispense speed were evaluated. Finally, the performance of the sorbent-packed and fiber-packed in-tip SPME was assessed with model drug compounds and compared with those from conventional sample preparation methods such as SPE and LLE.

3.2 Experimental

3.2.1 Materials

EDMA, DMPA, and 1-decanol were purchased from Sigma-Aldrich (Milwaukee, WI, USA), and were used as received. MK-0533 and MK-0974 and their deuterated internal standards (Figure 3-1, structures of MK-0533 and its internal standard are shown in Chapter 2) were synthesized at Merck Research Laboratories (Rahway, NJ, USA). Oasis HLB with particle size 60 μm and Oasis HLB (5 mg) $\mu\text{Elution}$ SPE plates were obtained from Waters (Milford, MA, USA). All solvents were HPLC or analytical grade

and were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ammonium formate (HPLC grade), purchased from J.T. Baker (Phillipsburg, NJ, USA), formic acid (95%) and 85% o-phosphoric acid (Sigma, Milwaukee, WI, USA), were used as received. Deionized water was obtained by passing in-house water through a Millipore Milli-Q plus system (Bedford, MA, USA). Nitrogen (99.999%) was supplied by West Point Cryogenics (West Point, PA, USA). Blank GC capillary was obtained from Restek (Bellefonte, PA, USA). PDMS – DVB fibers (60 μm) were purchased from Supelco (Bellefonte, PA USA). 96-Well collection plates (1.2 and 2.4 mL) and mats were purchased from Marsh Biomedical (Rochester, NY, USA). Different lots of drug free human plasma were obtained from Biological Specialties Corp. (Lansdale, PA, USA) and stored at $-70\text{ }^{\circ}\text{C}$ before use.

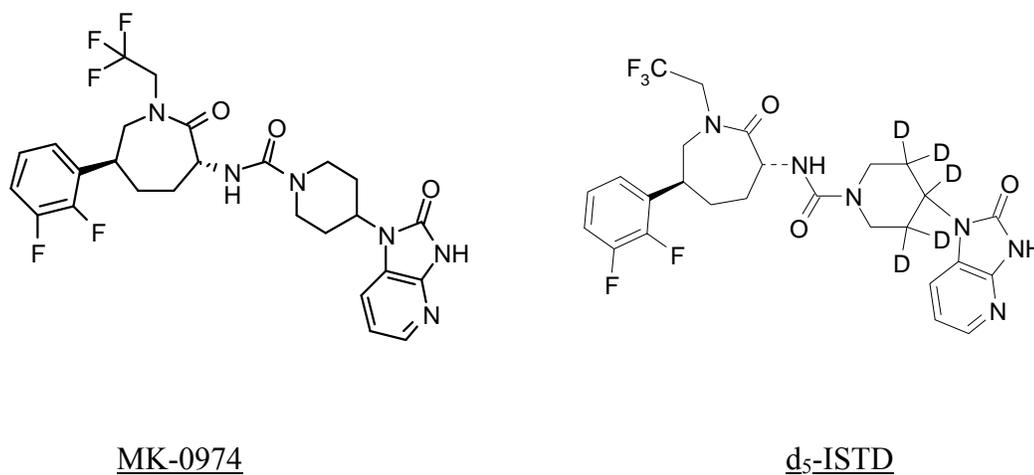


Figure 3-1 Chemical structures of MK-0974 and its internal standard.

3.2.2 Instrumentation

A Tomtec Quadra 96 workstation (Hamden, CT, USA) was used in high throughput polymer monoliths in-tip SPME fibers preparation and the automated sample extraction and desorption process. Photo-polymerization was achieved using UVP Compact UV lamps purchased from Fisher Scientific. A LEO 1530 field emission scanning electron microscopy (SEM) (Carl Zeiss NTS GmbH, Oberkochen Germany) was used to acquire SEM images of the coating using an acceleration voltage of 15 kV. A Perkin-Elmer (Norwalk, CT, USA) LC-200 micro pump and a CTC PAL Leap autosampler (Carrboro, NC, USA) for 96-well plates was used in this work. The chromatographic separation of analytes was performed on a Thermo-Hypersil Keystone (Bellefonte, PA, USA) FluoPhase RP column (150 × 2.1 mm, 5 μm) and a Restek BDS Hypersil C18 column (50 × 2.1 mm, 3 μm), respectively. Mobile phase consisted of (A) ACN: water (60:40, v/v) with a flow rate of 0.2 mL/min in a total run time of 4.5-min was used for MK-0974; and (B) ACN: water (80:20, v/v), flow rate 0.2 mL/min, total run time 3-min for MK-0533. 50% ACN with 0.1% formic acid and a "cocktail" of ACN/IPA/acetone (50:40:10, v/v/v) were used as washing solvents for the autosampler. An Applied Biosystems-Sciex API 3000 triple quadrupole mass spectrometer (Foster City, CA, USA) equipped with a TIS source operating in the positive ion ionization mode was used for all HPLC-MS/MS analysis. MRM mode was utilized for quantitation. The transitions monitored were m/z 567 → m/z 219 and m/z 528 → m/z 135 for MK-0974 and MK-0533, respectively; m/z 572 → m/z 224 and m/z 534 → m/z 135 for the their internal standards, respectively. In TIS experiments, the turbo ion spray probe temperature was maintained at 450 °C and the ion spray voltage was at 5000 V. Source

and MS parameters were optimized by infusing a neat solution of drug compound prepared in ACN:water (50:50, v/v) at a flow rate of 20 $\mu\text{L}/\text{min}$ into a mobile phase pumped at 0.2 mL/min through the turbo ion spray interface.

3.2.3 Preparation of Sorbent-packed In-tip SPME Fibers

Polymer monolith in-tip SPME fibers were prepared in two steps. First, in-tip SPME fiber modules were prepared for photopolymerization (Figure 3-2A). Polyethylene (PE) frits (25 μM , 6.3 mm in diameter) purchased from Innovative Microplate (Chicopee MA, USA), and non sterilized polypropylene pipette tips purchased from Tomtec Inc were used to prepare the modules. A piece of GC capillary tubing (0.01"-0.03" outer diameter) was cut into pieces (about 6.5 cm) and punctured into the middle of the PE frit to ensure a secure fit of the tubing. The PE frit with the capillary tubing was carefully inserted into the pipette tip so that the PE frit sat tightly at the top of the pipette tip with the capillary exposed by 2-3 mm outside the tip. The distance from the PE frit to the top of the pipette tip was about 11 mm to avoid extrusion from tip loading. Up to 96 tips were prepared in the same way and were loaded on a Tomtec Quadra 96 Workstation. The whole process took about 60 minutes and the PE frits with capillaries were reusable for future in-tip fibers preparation.

Secondly, the 96 tips were filled with a polymerization mixture for photopolymerization. A mixture of cross-linker (EDMA, 0.8 g), initiator (DMPA, 0.008 g), and porogen (1-decanol, 1.2 g) was prepared in a 4 mL glass vial, vortex-mixed thoroughly, and ultrasonicated for 10 s. About 100 μL of the mixture was transferred to each well in a 96-well plate with 600 μL 12 \times 8 removable tube strips from ArcticWhite

LLC (Bethlehem, PA, USA). For Oasis HLB in-tip fibers, the polymerization mixture was mixed thoroughly with Oasis HLB particles in a ratio of 1 mg of particle / 4 μ L of mixture. The plate was placed at one position on the deck of the Tomtec Workstation and UV light lamps were placed at another position for photopolymerization. The Tomtec Workstation was programmed as such so that the tips with capillaries exposed were lowered to the bottom of the wells, but ensured no contact. 10 μ L of polymerization mixture solution was aspirated simultaneously from the 96-well plate to 96 pipette tips and then moved to the UV lamps position, the 96 tips were photo-illuminated at 365-nm for 10 minutes. After photopolymerization, the 96 tips were un-loaded from the workstation, the capillary tubing was manually removed from the bottom of each tip, and all the tips were immersed into a reservoir with methanol overnight and dried under vacuum. The whole photopolymerization process was performed at room temperature. The freshly prepared tips were ready for SPME experiments (Figure 3-2B).

3.2.4 Preparation of Fiber-packed In-tip SPME Fibers

Polyethylene (PE) frits (25 μ M, 6.3 mm in diameter) purchased from Innovative Microplate (Chicopee MA, USA), and non-sterilized polypropylene pipette tips purchased from Tomtec Inc (Hamden, CT, USA) were used to prepare the in-tip SPME fibers. A hole was drilled in the middle of the PE frit with a needle of the same diameter as the protective needle of a SPME fiber; ensuring a secure fit of the fiber by the PE frit. The SPME fiber was carefully exposed just outside the protective needle and cut precisely to remove the sealing septum and the hub from the SPME fiber. The distance

from the end of the fiber to the bottom of the pipette tip should be 11 mm, as well as the distance from the PE frit to the top of the pipette tip.

3.2.5 SPME Automation

In-tip SPME extraction and desorption process for all experiments was fully automated using a Tomtec Quadra 96 workstation (Figure 3-2C). In summary, 96-well sample extraction and desorption plates, as well as tip washing plate, waste plate, and reservoirs containing desorption solvent were placed on the deck of the Tomtec Quadra 96 workstation. The Tomtec Quadra 96 workstation was programmed as such so that the whole process ran in a sequence of tip loading, extraction, washing, and desorption. The sample extraction and desorption process was accomplished through repeated aspirating and dispensing of sample solution and desorption solvent, respectively (Figure 3-2D). After sample extraction and desorption, the plate was either directly injected to the HPLC-MS/MS system or evaporated and reconstituted if enhanced sensitivity was required.

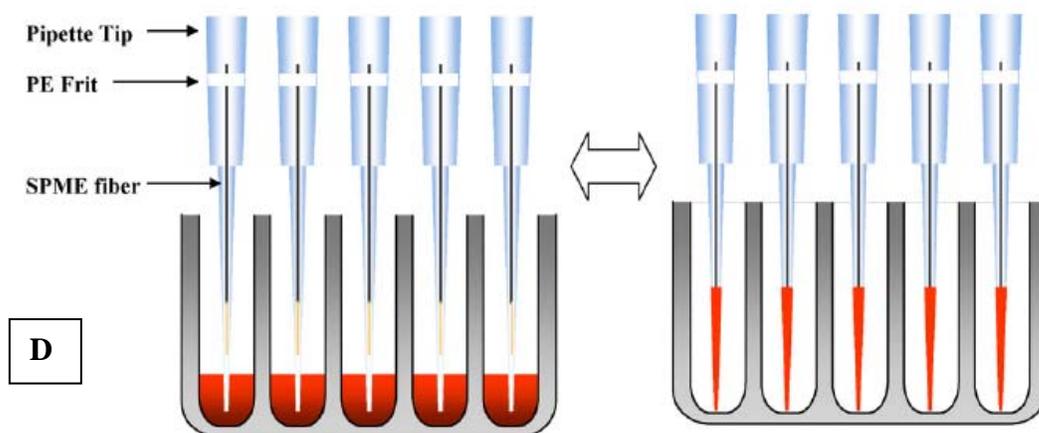
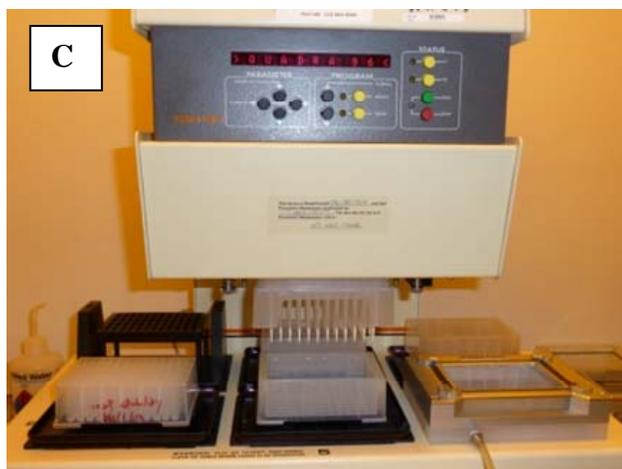
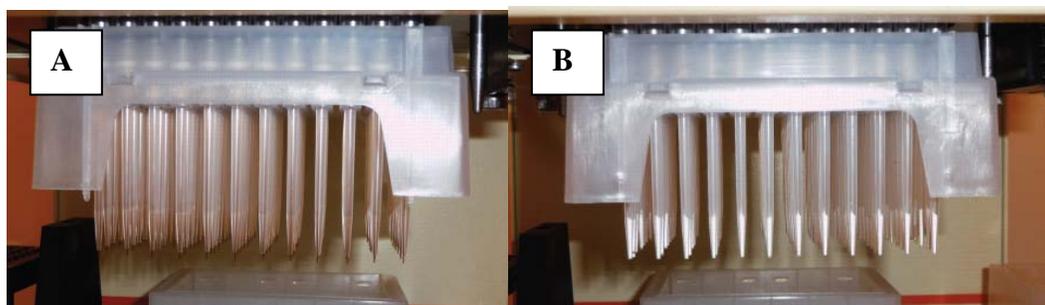


Figure 3-2 (A) In-tip SPME fiber module of 96 tips with capillary tubing inside each pipette tip; (B) In-tip SPME fibers coated with Oasis HLB poly(EDMA) monoliths in 96 tips; (C) Tomtec Workstation; and (D) Demo of in-tip SPME sample extraction and desorption.

3.2.6 Standards and Sample Preparation

Stock standard solutions for MK-0974 and d₅-ISTD were prepared at concentrations of 200 μM. A series of MK-0974 working standard solutions at concentrations of 5, 10, 20, 200, 800, 2000, 4000, and 5000 nM were diluted from the stock standard. A working d₅-ISTD standard solution was prepared similarly by diluting the 200 μM stock solution to 800 nM. A solvent of ACN/water (50:50, v/v) was used for standard preparation and dilution. The standards were prepared and used at room temperature and were stored at 4°C. A stock solution for QC samples of MK-0974 was prepared separately by the same procedure using a separate weighing. QC samples were prepared by diluting the QC working solution with human control plasma to obtain the corresponding QC samples containing 15, 400, and 4000 nM of MK-0974. The QC samples were divided into 500 μL aliquots in 12×75mm conical polypropylene tubes with caps (Sarstedt, NC, USA) and stored at -70 °C; clinical samples were stored under identical conditions.

For MK-0974 in-tip SPME preparation, 200 μL of 0.1 M acetic acid was added to 50 μL of control plasma, QC, and clinical samples in a 2-mL 96-well plate. 50 μL of working standard was added to control plasma and 50 μL of 50% ACN/water to the single blank, QC, and plasma samples, or 100 μL to the double blank to balance the total volume with 50 μL of internal standard working solution in each well, except double blank. The plate was sealed with mat and vortex-mixed 10 minutes before in-tip SPME processing on Tomtec Quadra 96 Workstation. The steps in the sample extraction procedure are: Precondition Oasis HLB polymer monolith in-tip SPME fibers with 2×50 μL of ACN followed by 2×50 μL of water; aspirate and dispense 100 μL of plasma

sample 20 times for extraction; wash in-tip SPME fibers with 2×50 μL water followed by aspirate and dispense 10 times in a 1.2 mL 96-well collection plate with 100 μL of ACN for desorption; Dry the collection plate completely under nitrogen at 20 to 40°C and reconstituted the residue into 120 μL of 50% ACN and injected 15 μL to LC-MS/MS system.

Standards and sample preparation for MK-0533 as well as in-tip SPME procedures have been described previously in Chapter 2.

3.3 Results and Discussion

3.3.1 Preparation of Polymer Monolith In-tip SPME Fibers

The focus of this work was to develop a simple, flexible, low-cost, and reproducible procedure for the preparation of in-tip SPME fibers with polymer monoliths using photo-polymerization technique. A model system based on published research work¹¹²⁻¹¹⁴ has been selected and modified with EDMA as a cross-linker, DMPA as an initiator, and 1-decanol as a porogenic solvent. The optimization procedures that affect polymer morphology, such as compositions of the cross-linkers and the types of porogens, as well as polymerization time were evaluated. Factors that could impact extraction efficiency such as fiber thickness and coating volumes, as well as chromatographic beads or sorbents immobilized by the polymer monoliths were also investigated.

Unlike polymer monoliths in chromatographic applications where the monoliths must have low flow resistance, engineering pressure drop is not a major concern in developing polymer monolith in-tip SPME fibers when capillary tubing is applied to form a flow channel through the monolithic materials. This unique way of preparing in-tip

SPME fibers distinguishes themselves from other tip-based micro extraction devices in such that extraction is non-exhaustive and the mechanism of extraction is based on the adsorption of analyte to the surface of the polymer monoliths. An extraction phase with large surface area to volume ratio will greatly enhance the sensitivity of SPME method and thus becomes one of the main objectives in in-tip SPME fibers preparation. Based on many previous studies on polymer monoliths, it has been found that the character of the porogenic solvent, the concentration of cross-linkers, and the ratio between total monomer to total porogen have a direct impact on the porous properties of the polymer monoliths, and the percentage of initiator should not exceed 1 wt% of the total polymerization mixture.¹¹⁵

Porogens play a critical role in obtaining the desirable porous structure without changing the rigidity of the polymer monoliths. The mechanism of pore formation using porogens is based on the solubility of the porogenic solvents to the final polymer. During the polymerization process, the polymer chains are formed initially in a homogenous solution and precipitated immediately when they become insoluble in the reaction medium depending on the types of porogenic solvents used. If the porogenic solvent is a thermodynamically "good" solvent for the final polymer, then the phase separation occurs later and the pores will be smaller.¹¹⁶⁻¹¹⁸ A variety of solvents including methanol, propanol-2, 1-decanol, ethyl ether, and THF were evaluated to prepare in-tip polymer monoliths with same amount of cross-linker and 1wt% initiator. For EDMA, all solvents yielded a white solid porous monolith except that THF formed a transparent solid, which indicated the formation of an extremely small pore structure and, as a result, the capillary tubing was difficult to withdraw from the pipette tips without breaking the final polymer.

The percentage of EDMA was adjusted from 30% to 70% in order to evaluate its impact to porous properties and chemical composition of the monoliths. Because of the early formation of highly cross-linked microglobules, an increase in percentage of cross-linker could result in decrease in average pore size,¹¹⁶ Therefore, it was expected that the extraction efficiency would be increased due to increase of the surface area. As shown in Figure 3-3, the analyte relative abundance increased slightly when the percentage of EDMA increased from 30 to 60 %, and the relative abundance increased dramatically when the EDMA reached 70%. However, the biggest challenge at such a high concentration of EDMA was to pull out the capillary tubing from the pipette tips. This problem was encountered as before when THF was used as the porogenic solvent. The final compositions of the polymer mixture (EDMA : 1-decanol, 40:60 w/w) were selected based on the formation of rigid monoliths with large surface areas and, in the meantime, the capillary tubing could be easily withdrawn from the pipette tips without breaking the final monolithic materials. SEM images in Figures 3-4A, 3-4B and 3-4C illustrate the impact of the composition of cross-linker to the pore size of the polymer monoliths.

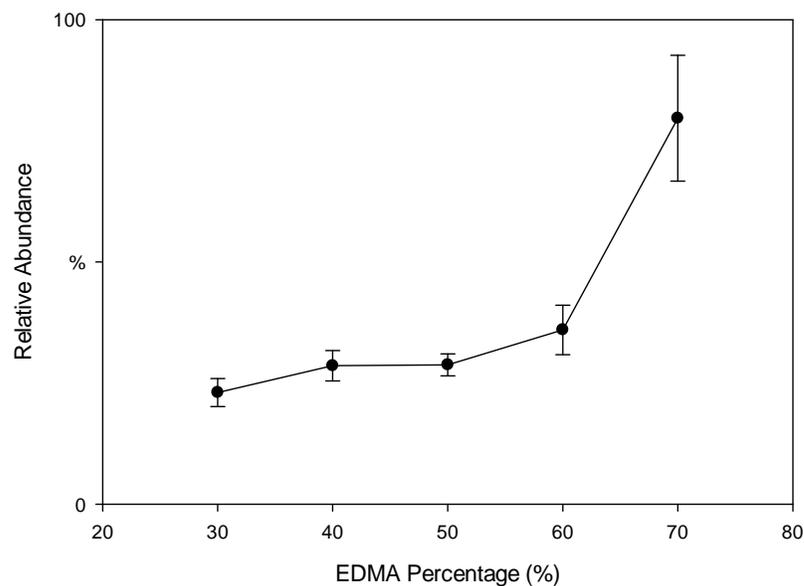


Figure 3-3 Dependence of cross-linker (EDMA) percentage on extraction efficiency using MK-0974 as a model compound at a concentration of 2000 nM spiked in human control plasma. SPME procedure is described in the experimental section. Error bars represent %RSD of five extractions using five different fibers.

Both thermal and UV-initiated polymerization can be used for polymer monoliths. Typically, thermally-initiated polymerization uses AIBN as initiator and the polymerization takes a longer time, about 24 h.¹¹⁹ In contrast, the UV-initiated polymerization process is usually performed at room temperature and can be accomplished within a few minutes.¹²⁰ As a commonly used initiator, DMPA was selected in the current study. It was found that the polymerization conversion was achieved within 5 minutes by observation of the polymerization mixture in pipette tips changing from clear solution to dense liquid and, finally, to white solid; complete conversion was achieved in 10 minutes. Photo-polymerization time from 10 to 30 minutes was tested (Figure 3-5A) and resulted in comparable analyte recoveries. This

indicated that longer photo-polymerization time did not impact the porous properties of the monoliths and the polymerization conversion was complete. Thus, 10 minutes was applied throughout all future experiments. The thickness of polymer monoliths could be adjusted by using capillary tubing with different diameters. Three different sizes of capillary tubing with diameters of 0.01", 0.02", and 0.03" were tested during polymer monoliths preparation. As shown in Figure 3-5B, there was an apparent linear relationship between the observed analyte recovery and the size of the capillary tubing, with about 20% on average. However, because of the limited dimensions of the pipette tips, it was not feasible to use capillary tubing with a diameter larger than 0.03". The increased extraction recovery was mainly due to the larger surface area obtained from using the larger diameter capillary tubing when same amount of polymerization mixture was aspirated to the pipette tips, although the in-tip SPME fiber thickness was larger with 0.01" than with 0.03" capillary tubing. The capillary tubing with diameter of 0.02" was selected for in-tip SPME fibers preparation because the inter-fiber reproducibility was much better than that using 0.03" and it was much easier to handle when the capillary tubing was withdrawn from the pipette tips. The loading volume of the polymerization mixture was also evaluated and the extraction recovery increased substantially when the volume of the polymerization mixture aspirated to the pipette tips increased from 5 μ L to 15 μ L (Figure 3-5C), which provided an alternative option to increase assay sensitivity, if needed. One of the favorable features of polymer monoliths is that they could be used as a medium to entrap chromatographic beads or sorbents in the photo-polymerization process for analyte separation and extraction. Oasis HLB sorbent with a particle size of 60 μ m was thoroughly blended with EDMA/DMPA/1-decanol mixture and the extraction

recovery was evaluated at different ratios between the volume of polymerization mixture and the amount of Oasis HLB particles. Figure 3-5D demonstrates that the extraction recovery almost doubled when Oasis HLB particles were immobilized within the polymer monoliths in a ratio of 1 mg of particle / 4 μ L of mixture. Oasis HLB in-tip SPME fibers were used in MK-0974 assay validation and sample analysis in order to make a direct comparison between in-tip SPME and SPE approaches as the clinical assay was developed using Oasis HLB (5 mg) μ Elution SPE plates.¹²¹

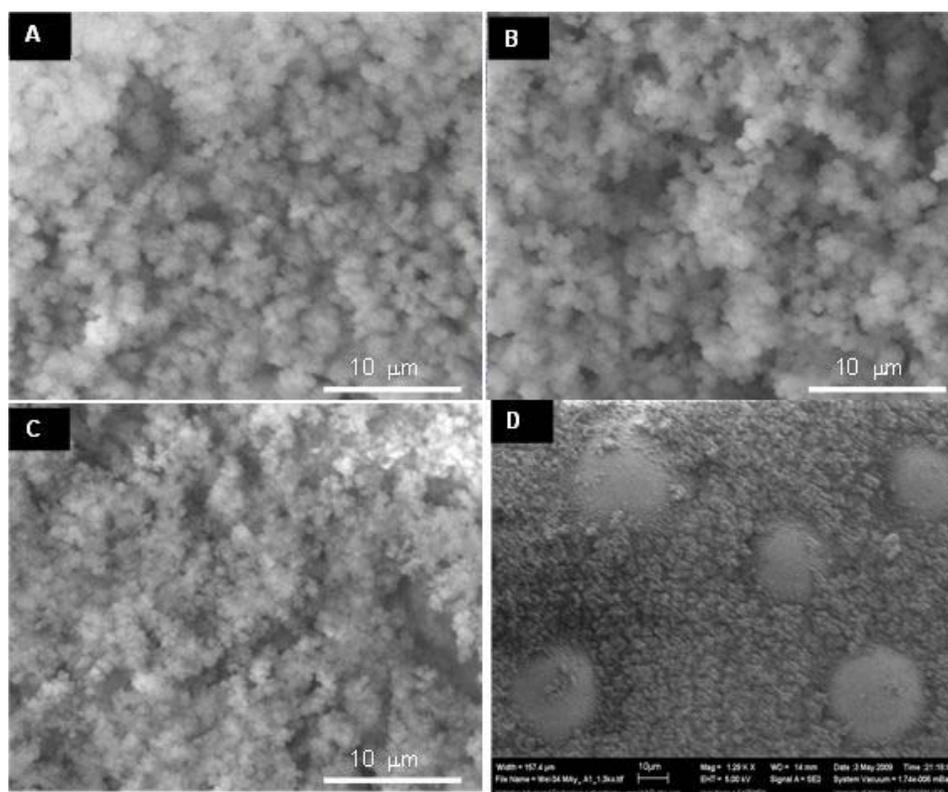


Figure 3-4 SEM images of in-tip SPME fiber coated with poly(EDMA) monoliths with surface morphology using magnification = 4000 \times in (A) EDMA (40%); (B) EDMA (60%); (C) EDMA (70%) and (D) EDMA (40%) with distribution of Oasis HLB particles.

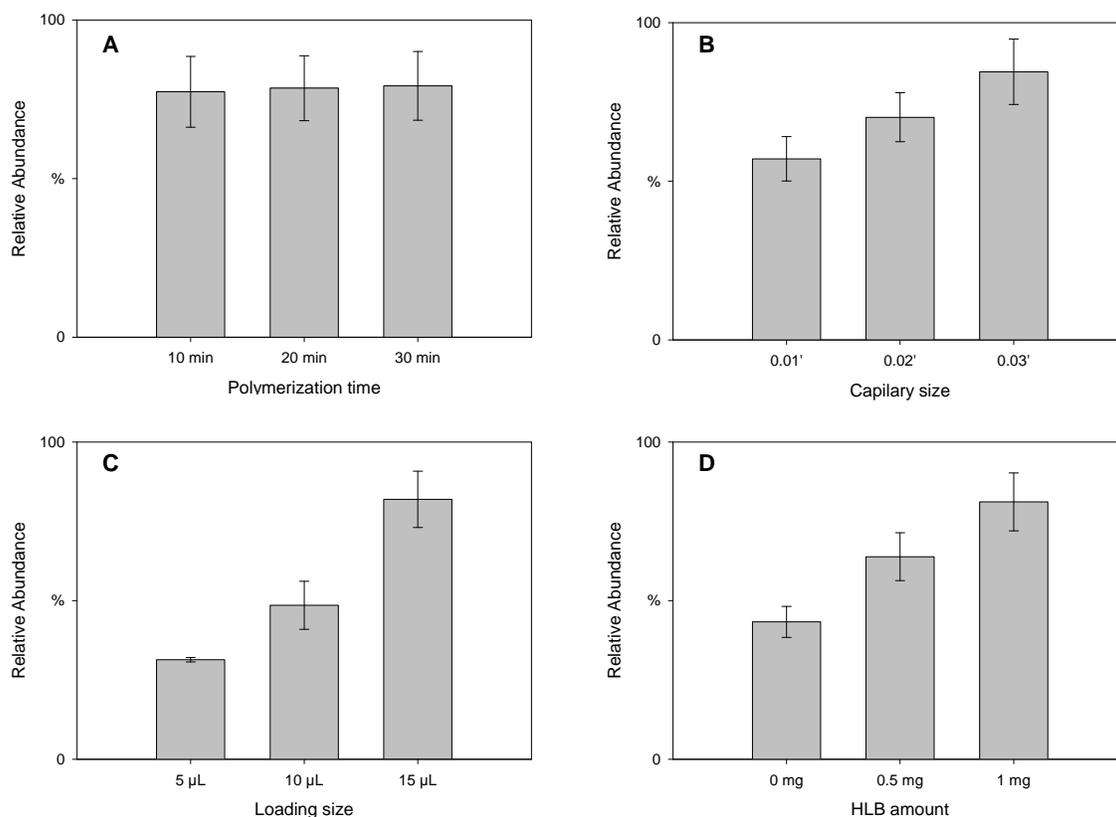


Figure 3-5 Evaluation of polymerization time (A): 10, 20, and 30 minutes; capillary size (B): 0.01', 0.02', and 0.03'; polymer mixture loading size (C): 5, 10, and 15 μL ; and Oasis HLB amount (D): 0, 0.5, and 1 mg on extraction efficiency for MK-0974 at 2000 nM spiked in human control plasma. SPME procedure is described in the experimental section. Error bars represent %RSD of five extractions using five different fibers.

During polymer monolith in-tip SPME fibers preparation, it was important that the 96 pipette tips with polymerization mixture aspirated to the sharp end of the tips and received uniform illumination from the UV lamps; therefore, the lamps were placed right below the sharp ends of the pipette tips at a distance about 5 cm without any angle. It was very difficult to line up the capillary tubing right in the middle of each tip; this is illustrated in Figure 3-6 where capillary tubing was withdrawn from the pipette tips after photo-polymerization. It is seen very clearly that the positions of the flow channels created from the capillary tubing were random. However, the relative standard deviations

(R.S.D.) from each measurement using the five different in-tip SPME fibers shown in Figures 3-3 and 3-5 ranged from 8 to 16%, which demonstrated that the position factor might contribute to the variations of the measurements, but did not affect the overall performance of the fibers. This was further verified from the inter-fiber reproducibility experiments. The flexibility of the capillary tubing positions in pipette tips made the whole preparation process quick and easy. Compared with the preparation procedure for polymer monolith in-tip SPME fibers using homogeneous polymerization mixture of EDMA/DMPA/1-decanol, it was a big challenge to prepare fibers with a slurry mixture such as Oasis HLB particles blended with EDMA/DMPA/1-decanol. The sample plate containing the slurry polymerization mixture needs to be vortex-mixed thoroughly before aspirating to the pipette tips and this mixture transfer step must be performed very quickly so that the Oasis HLB particles are distributed homogeneously across the monolithic bed (Figure 3-4D).

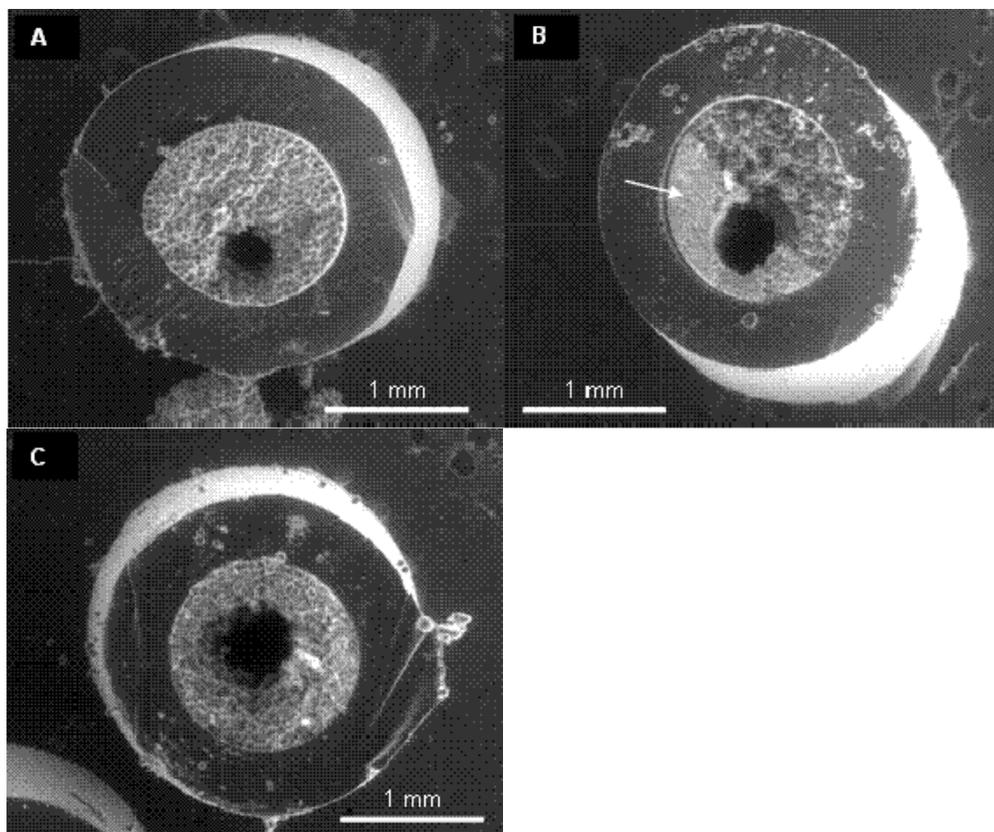


Figure 3-6 SEM images of cross-sections of three different in-tip SPME fibers coated with poly(EDMA) monoliths using magnification 40 ×.

3.3.2 Performance of Polymer Monolith In-tip SPME Fibers

The evaluation of the performance of the polymer monolith in-tip SPME fibers mainly focused on fiber to fiber reproducibility, extraction efficiency, and matrix effects, fiber carry-over, and pre-treatment.

Considering the small volume of the extraction phase in SPME, obtaining high fiber to fiber reproducibility is challenging for in-house tailor-made and commercial devices. It would be ideal to ensure excellent fiber reproducibility as many factors can contribute to the extraction variability using SPME. Fiber to fiber reproducibility becomes less of an issue during quantitative SPME method development when an

internal standard compensates for differences between individual fibers. 96 polymer monolith in-tip SPME fibers were simultaneously evaluated by performing an extraction of the test compound (MK-0974) at 2000 nM in human plasma with 5 aspiration/dispense cycles. The absolute analyte response and the accuracy of each measurement are shown in Figure 3-7. The relative standard deviation (R.S.D.) of the absolute peak areas from 96 fibers was 15.4%, but the accuracy results were excellent with a R.S.D. of 5.6% when isotopic labeled internal standard was applied. The utilization of automation to prepare polymer monolith in-tip SPME fibers greatly improved the inter-fiber reproducibility as well as sample throughput since the Tomtec Workstation could aspirate equal amounts of polymerization mixture to the 96 pipette tips at the same time with good precision. According to previous studies,^{112, 122} similar tip-based micro-extraction devices were made based on capillary force and, therefore, it was difficult to control the total volume of polymerization mixture in each individual pipette tip. The major contributing factor to the variability of the in-tip SPME fibers was the thickness differences of the monolith coatings due to the variable positions of the capillary tubing in the pipette tips, although a number of other factors could have contributed to this variability as well.

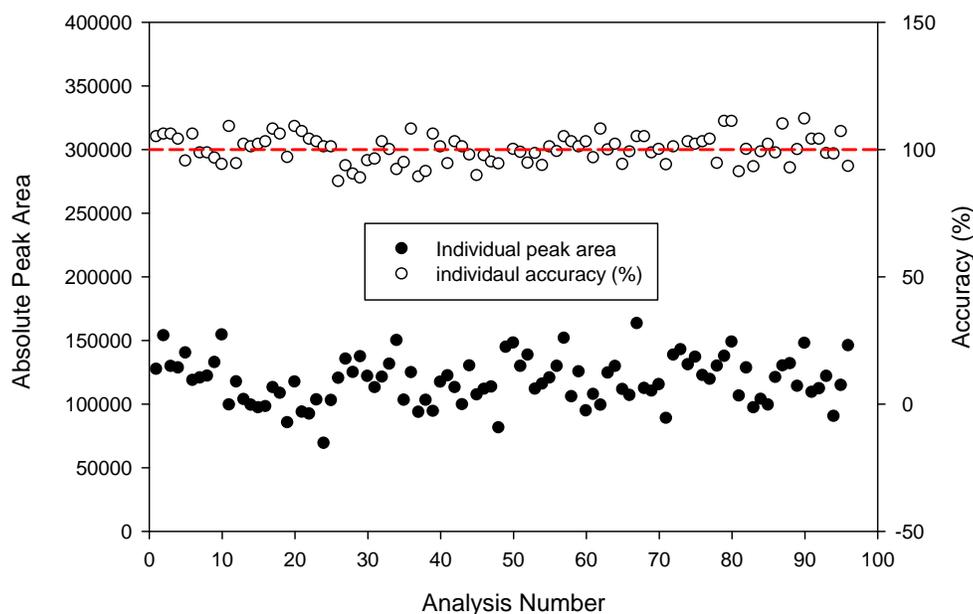


Figure 3-7 Distributions of absolute peak area and accuracy of MK-0974 extracted from 96 Oasis HLB polymer monolith in-tip SPME fibers at concentration of 2000 nM spiked in human control plasma.

One of the disadvantages of the SPME technique is the relatively low extraction efficiency compared with other types of extraction methods such as LLE and SPE. In most cases, the absolute recovery is normally less than 1% and this could be problematic in SPME assay development if sensitivity is an issue. According to SPME fundamental principles, the amount of analyte extracted by SPME is proportional to the volume of the extraction phase, and sensitivity of the SPME methods could be improved by an increase in the volume of the extraction phase. In this study, the absolute extraction recovery for MK-0974 was about 20% determined at three different concentrations. Although it was impossible to make direct comparisons of fiber coatings between polymer monolith in-tip SPME fibers and other traditional fibers with coating dimensions of 1 cm coating length

and 10-100 μm coating thickness, it was believed that the total surface area of polymer monoliths was much larger than those of traditional fibers due to the inner surface area of the flow channel as well as outer surface area of the polymer monoliths because the polymer materials were physically attached to the surface of the pipette tips allowing sample solutions to flow through both sides during aspiration/dispense procedure. In addition, the homogeneous distribution of Oasis HLB particles in polymer monolithic materials enhanced the weak intermolecular interactions and hydrophobic interactions between analytes and extraction sorbent, which improved extraction efficiency of in-tip SPME fibers over other traditional SPME fibers.

Matrix effects must be considered during any SPME quantitative bioanalytical assays development and validation especially when the fibers are directly immersed into biological matrix for sample extraction. The matrix effects, which are defined as interference from matrix components that are unrelated to the analyte, could cause significant errors in precision and accuracy of the SPME-LC-MS/MS assays. Because of the equilibrium extraction characteristics of the much smaller volume of sorbent, SPME is often considered as a sample preparation technique which provides sample clean-up as effective or better than SPE with no or minimal matrix effects because the absolute amounts of analytes of interests as well as potential interferences extracted by SPME are much smaller. However, this conception was not supported in the evaluation of matrix effects using different SPME coatings including Oasis HLB polymer monolith, PDMS-DVB, C18, and C30 phase coated SPME fibers in our other study [unpublished data]. It was found that SPME was less effective at removing phospholipids, a major cause of matrix effects, than LLE and SPE regardless of the types of coatings when SPME was

performed at equilibrium in biological fluids. The explanation to this phenomenon was that, unlike LLE and SPE methods, SPME combines sampling, sample clean-up, and pre-concentration into a single step, such that SPME fibers are not "washed" to remove the loosely bound species such as phospholipids co-extracted in the sample preparation process. In MK-0974 in-tip SPME method validation, matrix enhancement/suppression of ionization was evaluated by comparing the absolute peak areas (n=5 at each concentration) of post-spiked plasma samples to that of neat standard at the same concentration, and the absolute matrix effects⁹⁰ were about 89%. In contrast, in a different method using Oasis HLB (5 mg) μ Elution SPE plate, no ion suppression/enhancement was observed with absolute matrix effect values close to 100%.

Compared with other extraction methods, SPME is relatively simple and can be done at a low cost mainly because SPME fibers could be used many times. However, the advantage has, also, its limitations as carry-over effects occur very easily in SPME methods because of the repeated use of one single fiber. Additional efforts are often necessary to handle this problem, which will reduce the speed of the whole sample preparation process. A detailed investigation of carry-over effects was performed to evaluate if the fibers could be used repeatedly during method development. It should be pointed out that carry-over is also compound dependent. In the current study when MK-0974 was used as the model compound, it was found that carry-over effects could be controlled within 2% by using a high percentage of organic solvent such as ACN (>90%) in the desorption solvent (1 mL) and increasing desorption time (>40 cycles of aspiration/dispense); fibers could be used repeatedly at least five times without any loss in fiber performance. To eliminate carry-over effects in high throughput quantitative

biological analysis using SPME, fibers should be used only once. Fiber pre-conditioning is a necessary step in most SPME assays in order to ensure the best performance of the fibers, in general, this essential step will add up approximately 30 minutes to sample extraction and may not be acceptable for routine sample analysis. For polymer monolith in-tip SPME fibers, pre-conditioning could be achieved in less than one minute by 2 aspiration/dispense cycles with 50 μ L of 50% ACN or methanol.

3.3.3 In-tip SPME Procedures

Analyte extraction using automated in-tip SPME was accomplished by repeatedly aspirating and dispensing sample solution through pipette tips utilizing an automation system such as Tomtec Workstation, and equilibrium could be established between the SPME phase and the analyte extracted after a certain number of aspirate/dispense cycles. During in-tip SPME procedures, several important factors need to be considered including extraction and desorption time, sample and desorption volume, extraction speed, as well as tip positions, which could all impact the outcome of the automation process.

It is desirable to perform extraction at equilibrium in SPME to reach maximum sensitivity. The types of fibers and the thickness of the coatings, the properties of the analytes, and the agitation speed could all contribute to the time to reach equilibrium and, in many cases, this process could be extremely long. In this study, it was found that the extraction equilibrium was reached after nearly 60-min (720 cycles) for MK-0533 in plasma samples, while for MK-0974, although equilibrium was achieved under the tested conditions, the extraction recovery was increased from 7% to 38% when aspiration/dispense cycles changed from 5 to 320 cycles. Results from this and recent

studies in our laboratory both demonstrate that automated SPME could be used without impacting precision with pre-equilibrium extraction time as long as sufficient time was allowed to establish uniform agitation.⁷⁸ Unlike exhaustive sample preparation methods, the amount of analyte extracted at equilibrium using SPME is dependent on sample volume, and an increase in sample volume will lead to a non-linear increase in the amount extracted. However, this trend was not obvious in the extraction of MK-0974 using polymer monolith in-tip SPME when sample volume was tested at 25, 50, and 100 μL , respectively. The relative recovery of the analyte was approximately constant and independent of the sample volume; this was properly due to the short pre-equilibrium sampling times. The utilization of different volumes of desorption solvent did not make any differences to the release of the analyte from the polymer monolith sorbent and, therefore, it was better to use as little organic solvent as possible. Because it was difficult to handle very small volumes using instruments such as the Tomtec workstation, it was recommended to use a volume above 50 μL during aspirate and dispense cycles for in-tip SPME approach.

Besides the aspiration/dispense volume and cycle, the aspiration and dispense speed is another parameter that can be evaluated and optimized with the Tomtec Quadra 96 workstation. Three aspiration and dispense speeds are available, from low (speed 1), to medium (speed 2), to high (speed 3). Three different PDMS-DVB fibers were used at three different well locations for each speed while other parameters were constant such as aspiration volume and cycles. In this experiment, aspiration and dispense speed was maintained equally and no combinations were tested, such as aspiration at speed 1 and dispense at speed 2, etc. It was found that there were no major differences between the

different speeds, with % C.V. from 10.4 to 13.1% (Figure 3-8). Since high speed is relatively faster than medium and low speed in terms of shortened extraction and desorption time, high speed is selected for future experiments. In addition, higher flow velocities generated during rapid draw/eject cycles facilitates more rapid diffusion of the analytes into the fiber coating; thereby reducing equilibration time.

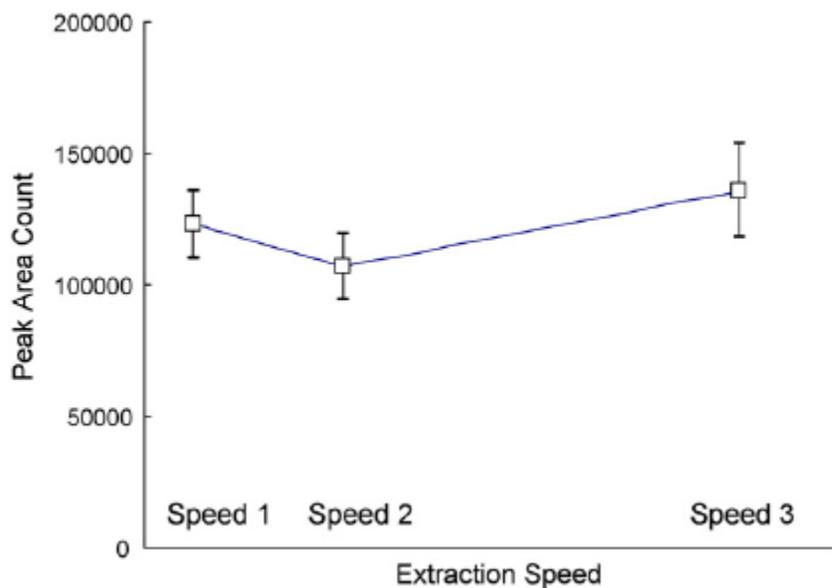


Figure 3-8 In-tip SPME extraction speed profile. Conditions: in-tip SPME was performed in accordance to the method outlined in the experimental section. Three in-tip SPME fibers were extracted simultaneously at three different wells with aspiration/dispense volume at 100 μ L. Desorption conditions for all time points were the same.

The tip position of in-tip SPME fibers within wells of a 96-well plate was not as critical as that reported in the literature when different extraction phase geometries of SPME were applied in parallel fiber automation.^{65,78} This was very obvious as in either the thin film or blade approach, the SPME fibers had to be immersed completely into the sample solution to make sure uniform contact between the coating surface and sample

solution while the sample plate was agitated at a moderate speed, normally 850 rpm to avoid a splashed solution. In in-tip SPME, extraction was accomplished by repeated aspirating and dispensing of sample solution through pipette tips, and the tip position was very flexible as long as sample solution could be completely aspirated and dispensed. The relative standard deviation in analyte extraction and desorption was largely based on the performance of the automation system. However, because of the viscosity of blood and plasma sample, in addition to diluting the biological sample with water, it is suggested that the tips be lowered to the bottom of the well during aspiration and raised to the top of the well after aspiration so that the sample solution could be completely dispensed into the well plate before the next cycle. It might be helpful to aspirate an air gap of 50 μ L before and after sample solutions, to expel all sample residues from the pipette tips.

3.3.4 Applications of Drug Analysis

A sensitive and selective SPME-LC-MS/MS method using Oasis HLB polymer monolith in-tip SPME fibers was developed and validated to determine MK-0974 in human plasma over the concentration range of 5-5000 nM. Assessment of the intraday variability of the method was conducted in five different lots of human control plasma spiked with MK-0974. The resulting method precision and accuracy data are presented in [Table 3-1](#). The intraday precisions (%C.V.) was 13.0% at LLOQ, and was equal to or lower than 7.2% at all other concentrations used for the construction of the calibration curve. Method accuracy was found to be within $\pm 4.3\%$ of the nominal concentration for all the standards evaluated. The correlation coefficient for the mean standard curves constructed from five different lots of human plasma was 0.9996. The selectivity of the

assay was assessed in six different lots of human control plasma and no interfering peak was observed in the retention time window of MK-0974 and the internal standard at the conditions specified in the experimental section. In addition, no “cross-talk” was observed between MS channels used for monitoring MK-0974 and the internal standard. Extraction recovery and matrix effects were evaluated at nominal concentrations of 5, 200, and 5000 nM for MK-0974 and working concentration of 800 nM for internal standard and the results are listed in Table 3-2. Recovery of the extraction was determined by comparing the absolute peak areas (n= 5 at each concentration) of pre-spiked analyte to that of post-spiked analyte, and the mean recoveries were 20.2% and 17.5% for MK-0974 and internal standard, respectively. Although slight ion-suppression was observed in "absolute" matrix effects determination, the precision of standard line slopes in five different lots of human plasma expressed as coefficient of variation C.V.% did not exceed 3%, which indicated that the in-tip SPME method could be considered practically free from the "relative" matrix effect liability.¹²³ The stability of MK-0974 and its internal standard was also investigated in human plasma and the influence of freeze-thaw cycles were examined by processing a set of QC samples at concentrations of 15, 400, and 4000 nM. The results are shown in Table 3-3. In all cases, the results for the samples that were subjected to additional freeze-thaw cycles were within $\pm 4\%$ of the nominal value.

Table 3-1 Intraday Precision and Accuracy Data for the Determination of MK-0974 in Five Different Lots of Human Control Plasma Using In-tip SPME

Nominal Conc. (nM)	Mean ^a Calculated Conc.(nM)	Precision ^b C.V.%	Accuracy ^c (%)
5	4.99	13.0	99.8
10	9.82	5.3	98.2
20	20.86	7.2	104.3
200	202.72	4.0	101.4
800	790.32	5.6	98.8
2000	1950.14	3.0	97.5
4000	3975.98	3.7	99.4
5000	5030.71	3.4	100.6

- ^a Mean concentrations calculated from the weighted linear least-squares regression curve constructed using all five replicate values at each concentration.
- ^b Expressed as coefficient of variation (C.V.%) of peak height ratios.
- ^c Expressed as [(mean calculated concentration)/(nominal concentration)] × 100%.

Table 3-2 Extraction Recovery and Assessment of the “Absolute” Matrix Effects During the Determination of MK-0974 and Internal Standard in Human Control Plasma Using In-tip SPME^a

MK-0974 Concentration (nM)	% Extraction Recovery ^b	% Matrix Effects ^c
5	19.2 (12.0)	94.8 (5.9)
200	19.7 (10.7)	84.9 (2.1)
5000	21.6 (5.5)	88.0 (7.4)
Mean	20.2 (6.3)	89.2 (5.7)
d ₅ -ISTD Concentration (800 nM)	17.5 (16.0)	92.4 (9.7)

- ^a Determined in 5 different lots of control human serum
- ^b Extraction recovery was calculated by dividing the mean peak area of analyte spiked before extraction by the respective mean peak area of analyte spiked after extraction and multiplying by 100.
- ^c Matrix effect was calculated by dividing the mean peak area of an analyte spiked after extraction by the mean peak area of the neat analyte standard and multiplying by 100.

Table 3-3 Freeze-Thaw (F/T) Stability of MK-0974 in Human Control Plasma

Nominal Conc. (nM)	Mean Determined Conc. (nM) After 1 F/T (n=5)	Accuracy ^b (%)	Mean Determined Conc. (nM) After 3 F/T (n=5)	Accuracy ^b (%)
15	14.4 (3.7)	96.2	15.6 (4.7)	103.7
400	398.8 (2.8)	99.7	410.3 (3.1)	102.6
4000	3736.6 (1.6)	93.4	3848.3 (4.7)	96.2

^a Numbers in parentheses are coefficients of variation (%CV).

^b Expressed as [(mean determined concentration)/(nominal concentration)] × 100%.

Since an assay validation has been performed in Chapter 2 for MK-0533 using PDMS-DVB SPME fibers, assessment of the intraday variability of the automated in-tip SPME approach was only conducted in three different lots of acidified human control plasma over the concentration range of 5 to 2000 ng/mL of MK-0533. The resulting method precision and accuracy data is presented in Table 3-4. The intra-day precision was 13.7% at LLOQ, and was equal to or lower than 9.1% at all other concentrations used for the construction of the calibration curve. Method accuracy was found to be within ±8% of the nominal concentration for all the standards evaluated. The correlation coefficient for the mean standard curves constructed from three different lots of acidified human plasma was 0.9957.

Table 3-4 Intraday Precision and Accuracy Data for the Determination of MK-0533 in Three Different Lots of Acidified Human Control Plasma Using In-tip SPME.

Nominal Conc. (ng/mL)	Mean ^a Calculated Conc.(ng/mL)	Precision ^b C.V.%	Accuracy ^c (%)
5	4.73	13.7	94.5
10	10.72	9.1	107.2
50	52.78	1.0	105.6
100	97.13	5.1	97.1
200	211.75	4.2	105.9
500	510.50	3.5	102.2
1000	948.25	2.4	94.8
2000	1825.00	2.0	91.3

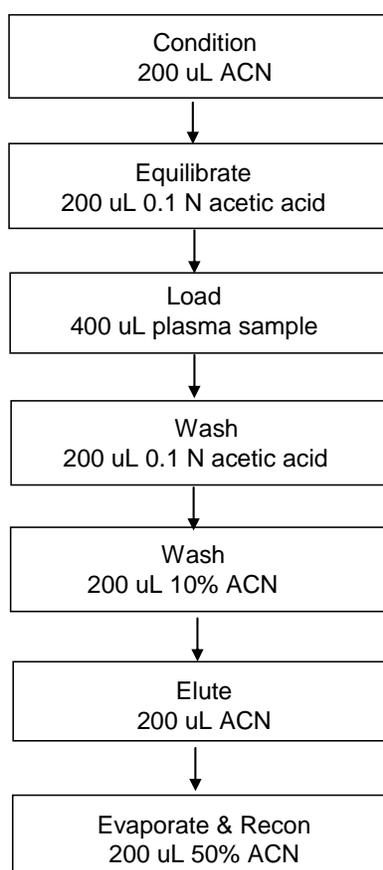
^a Mean concentrations calculated from the weighted linear least-squares regression curve constructed using all three triplicate values at each concentration.

^b Expressed as coefficient of variation (C.V.%) of peak area ratios.

^c Expressed as [(mean calculated concentration)/(nominal concentration)] ×100%.

A LC-MS/MS assay using Oasis HLB (5 mg) μ Elution plates was originally developed and validated for the determination of MK-0974 in human plasma. A detailed comparison of sample preparation procedure between SPE and in-tip SPME methods was illustrated in Figure 3-9. In-tip SPME clearly demonstrated its advantages of simple, fast and utilization of less organic solvent, let alone the extreme low cost compared with μ Elution SPE plates. In addition, in-tip SPME approach using Oasis HLB polymer monolith achieved similar intraday precision and accuracy as that from SPE method, and this was shown in Figure 3-10 where the relative deviations from nominal concentrations obtained from the five-curve validation of two different methods were overlapped across the concentration range.

SPE Assay (Oasis HLB μ Plate)



In-tip SPME Assay

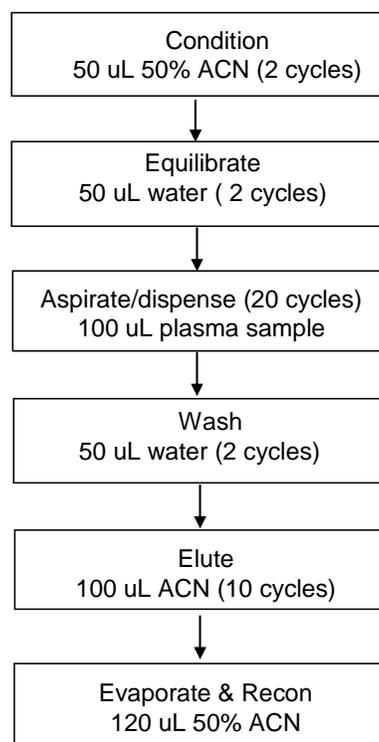


Figure 3-9 Comparison of SPE assay using Oasis HLB μ Plate and in-tip SPME assay using Oasis HLB polymer monolith

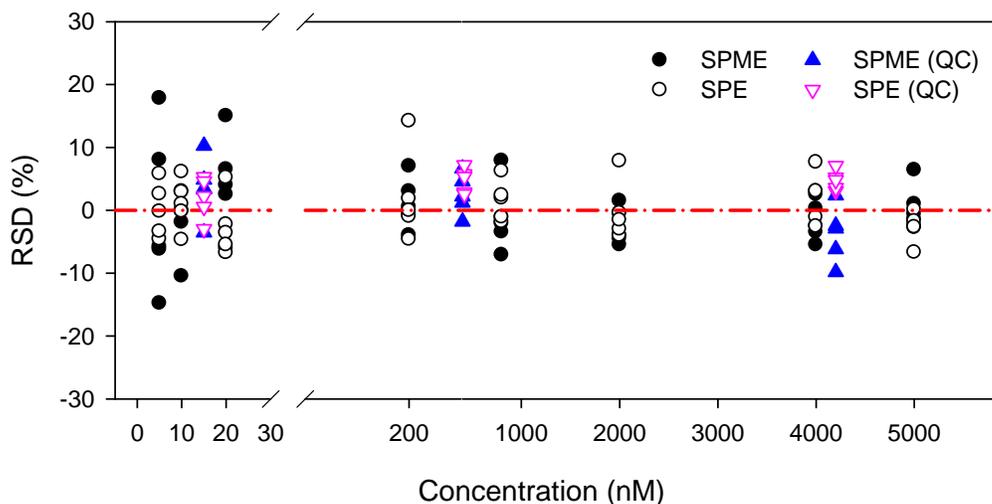


Figure 3-10 Comparison of in-tip SPME and SPE methods with relative deviations from nominal concentrations in five curve validations and QC samples using different lots of plasma.

The SPE method has been implemented in sample analysis up to phase three clinical studies with several thousands of samples already analyzed. In order to compare the clinical data obtained using SPE versus in-tip SPME technique; clinical samples from several post-dose subjects were pooled and re-analyzed using both approaches. Individual and mean concentration-time profiles from three different subjects after oral administration of 400 mg of MK-0974 obtained from two methods are presented in Figure 3-11, and individual and mean pharmacokinetic parameters such as $AUC_{0-\infty}$, AUC_{0-last} , C_{max} , T_{max} and apparent half-life ($t_{1/2}$) are reported in Table 3-5. The two data sets obtained from two different sample preparation methods are in good agreement, clearly demonstrating that in-SPME could be used as an alternative approach for multiple sample analysis in pharmacokinetic studies.

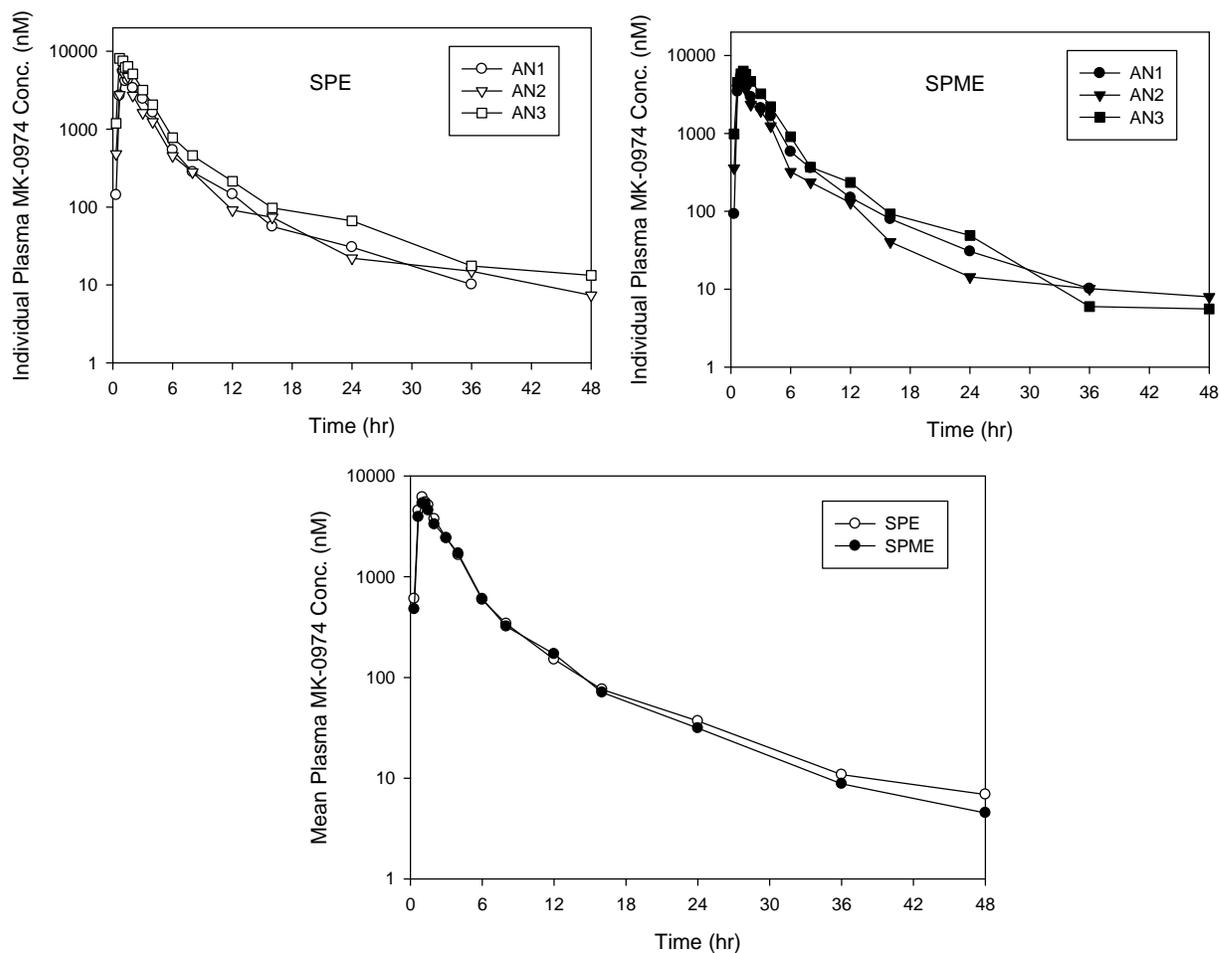


Figure 3-11 Individual and mean plasma concentration-time profiles of MK-0974 following oral administration of 400 mg MK-0974 in healthy subjects obtained from SPE and in-tip SPME, respectively.

3.4 Conclusions

It is important and challenging to develop a simple, low-cost, and reproducible procedure to prepare SPME coatings that could be used for high throughput drug analysis. The overall data from this study demonstrated that using in-tip SPME coated with polymer monoliths through photo-polymerization was a feasible approach with variation of the absolute peak areas from 96 fibers at 15.4%, and accuracy of 5.6% when

isotopic labeled internal standard was applied for the extraction of a drug compound from human plasma. Many factors, such as the compositions of the cross-linkers and porogens, polymerization time, and fiber thickness could impact the performance of the polymer monolith in-tip SPME fibers. The advantages of polymer monoliths fibers are: (i) simple and easy to fabricate at a low-cost, (ii) enhanced extraction recovery and (iii) no carry-over effects if used as disposable fibers. In addition, the SPME method selectivity could be greatly improved with a wide range of chemistries for reactions and enhanced mass transfer in polymer monoliths preparation.

Table 3-5 Individual and Mean MK-0974 Plasma Pharmacokinetic Parameters Following Administration of Single Oral Doses of 400 mg MK-0974 in Pooled Healthy Subjects Using SPE and SPME Approaches

AN	AUC _{0-∞} (nM•hr)		AUC _{0-last} (nM•hr)		C _{max} (nM)		T _{max} (hr)		Apparent t _{1/2} (hr) [§]		Ratio AUC _{0-∞}	Ratio AUC _{0-last}	Ratio C _{max}
	SPE	SPME	SPE	SPME	SPE	SPME	SP E	SPM E	SPE	SPME	SPE/SPM E	SPE/SPM E	SPE/SPME
1	15581	15899	15483	15807	5560	5410	1.0	1.0	6.7	6.3	0.98	0.98	1.03
2	14201	12981	14089	12809	6105	4745	1.3	1.0	10.5	15.0	1.09	1.10	1.29
3	24317	21018	24121	20960	8107	6310	0.7	1.3	10.2	7.2	1.16	1.15	1.28
AM	18033	16633	17898	16525	6591	5488	1.0	1.1	8.8	8.2	1.08	1.08	1.20
SD	5486	4068	5434	4123	1341	786	0.3	0.2	2.6	3.1	0.09	0.09	0.15

AM: Arithmetic Mean; SD: Standard Deviation; [§] Harmonic Mean and Pseudo-Standard Deviation for Apparent t_{1/2}

Chapter 4

Applications of In-tip SPME (Part I): Quantitative LC-MS/MS Determination of Vitamin D₃ in Human Serum with Derivatization: A Comparison of In-tube LLE, 96-well Plate LLE and In-tip SPME

4.1 Preamble and Introduction

4.1.1 Preamble

This chapter has been accepted for publication in *Journal of Chromatography B* (2011) in a Special Issue on Analytical Derivatizations.

4.1.2 Introduction

It is well known that vitamin D plays a critical role in the control of calcium and phosphate metabolism in the human body by increasing calcium absorption in the intestines, mobilizing calcium from bone, and decreasing its renal excretion. In humans, there are two sources of vitamin D: dietary ergocalciferol (vitamin D₂), derived from ergosterol in plants, and cholecalciferol (vitamin D₃, **I**) generated in the skin from 7-dehydrocholesterol by the action of ultraviolet irradiation. Vitamin D₃ has no biological activity, but is converted to the biologically active metabolite, 25-hydroxyvitamin D₃ (calcifediol) by oxidation in the liver, and this metabolite then is further converted to a series of other metabolites of varying activity in the kidney, the most of which is 1 α ,25-dihydroxyvitamin D₃ (calcitriol). The biosynthesis of vitamin D is illustrated in Figure 4-1.

Determinations of vitamin D₃ and its metabolites at relatively high concentrations (≥ 2 ng/mL) have been reported in the literature.¹²⁴⁻¹²⁹ These methods were mostly based on HPLC with ultraviolet (UV) detection, a highly complex sample preparation procedure, and were all potentially non-selective in the presence of metabolites and related compounds present in serum samples. For example, Holick et al.^{130,131} used a LLE followed by SPE and HPLC with UV detection to quantitate vitamin D₃ in human serum following oral doses of 12.5 mg or greater. The precision of this method was 19% at the claimed LLOQ of 2 ng/mL, with poor and variable recovery (50-70%).

The development and validation of reliable quantification methods for vitamin D₃ in clinical samples at low sub-nanogram concentrations (0.1 – 1 ng/mL) using a small volume of serum is difficult and has not been described in the literature. Concentrations of vitamin D₃ after microgram (μg) doses to human subjects are very low (≤ 0.5 – 5 ng/mL), and the determination of **I** in the presence of a number of metabolites circulating in human biofluids exhibiting similar chemical and HPLC behavior as the parent compound may be highly non-selective. In addition, both **I** and metabolites are potentially unstable in the presence of UV light and elevated temperatures.¹³²

Similarly to the determination of the majority of drugs, metabolites, and variety of other analytes in complex matrices, the HPLC with MS/MS detection has proven to be a powerful tool for the analysis of vitamin D related compounds in biological samples. Atmospheric pressure ionization methods, such as electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) were used for structural identification by tandem mass spectrometry of vitamin D metabolites in biological fluids at relatively low concentrations and facilitated their quantification.¹³³⁻¹³⁶ However, because vitamin

D₃ and its metabolites have no easily ionizable polar functional groups in their molecules, the ionization efficiency of these compounds using either ESI or APCI is very limited, and the required sensitivity for trace determination of these compounds in biological fluids is difficult to achieve.

In recent years, a variety of analogs and metabolites of **I** have been derivatized with dienophiles to form a Diels-Alder cycloaddition products.^{137,138} Higashi et al.,¹³⁹⁻¹⁴¹ for example, described the determination of vitamin D₃ metabolites in plasma based on chemical derivatization. When reacted with 4-substituted 1,2,4-triazoline-3,5-dione (TAD) type compounds (dienophiles, Cookson reagents), the *s-cis*-diene moiety of the hydroxyvitamin D₃ analogs rapidly and quantitatively reacts with the derivatizing reagent to form Diels-Alder cyclo-addition products. The sensitivity of detection of metabolites of **I** increased dramatically after the introduction of the high proton-affinity atoms from these dienophilic reagents. For example, the derivatives of 25-(OH)D₃ with 4-(4-nitrophenyl)-1,2,4-triazoline-3,5-dione (NP-TAD) provided 30-fold higher sensitivity of detection compared to an underivatized compound in the negative ionization mode using APCI. Although many applications have focused on identification and determination of vitamin D₃ metabolites, quantitative and selective analytical methods for the determination of vitamin D₃ by HPLC-MS/MS after derivatization with dienophiles directly in human plasma or serum at sub-nanogram/mL concentrations were not developed and described in the literature. The development of new, more sensitive, selective, reliable, and high throughput methods for the quantitative determination of vitamin D₃ was required to meet the high demand for pharmacokinetic (PK) data in support of clinical studies at very low (μg) oral doses of vitamin D₃. For this purpose, in

our studies, the reaction between **I** and a dienophile, 4-phenyl-1,2,4-triazoline-3,5-dione (P-TAD), was utilized. This reaction was never used for the development of quantitative methods for **I** in plasma or serum.

In order to map out the concentration-time profile following a single low oral dose of 70 µg of vitamin D₃ to human subjects, a method (method A) based on chemical derivatization of **I** with P-TAD after conventional tube LLE, and HPLC with MS/MS detection has been initially developed and validated in our laboratory. This method required processing 1 mL of serum and achieved the LLOQ of 0.5 ng/mL. However, the method had a relatively low sample throughput due to the labor intensive and time-consuming sample preparation steps required. Later, a higher throughput, highly sensitive and selective HPLC-MS/MS method B was also developed. In method B, both the LLE and derivatization of vitamin D₃ and its stable isotope-labeled internal standard (D₆-**I**, IS, **II**) were both performed in the 96-well plate format. This method required only 0.4 mL of serum for processing and achieved the same LLOQ (0.5 ng/mL) as in method A. As part of a series of studies conducted in our laboratory to explore the SPME technique in high throughput drug analysis, we developed a new approach of using in-tip SPME¹⁹ in 96-well format for determination of various drugs in biofluids. Compared with traditional extraction methods such as LLE and SPE, SPME has the advantage because of its simplicity and solvent-less characteristic. In order to further demonstrate the feasibility of using the in-tip SPME approach in high throughput clinical sample analysis and to explore the potential advantages and limitations of SPME technique in routine bioanalysis, we developed another method for the quantification of vitamin D₃ in human serum using in-tip SPME (method C) followed by chemical derivatization of **I** and the IS

(II) with P-TAD requiring only 0.1 mL of human serum, but with a higher LLOQ of 5 ng/mL. In all three methods, the derivatives formed (III and IV, respectively) were determined using HPLC-MS/MS in the positive ionization mode. Head-to-head comparisons were made among three different methods including precision and accuracy, sample throughput, recovery, and matrix effects, and the advantages and limitations of each method were discussed.

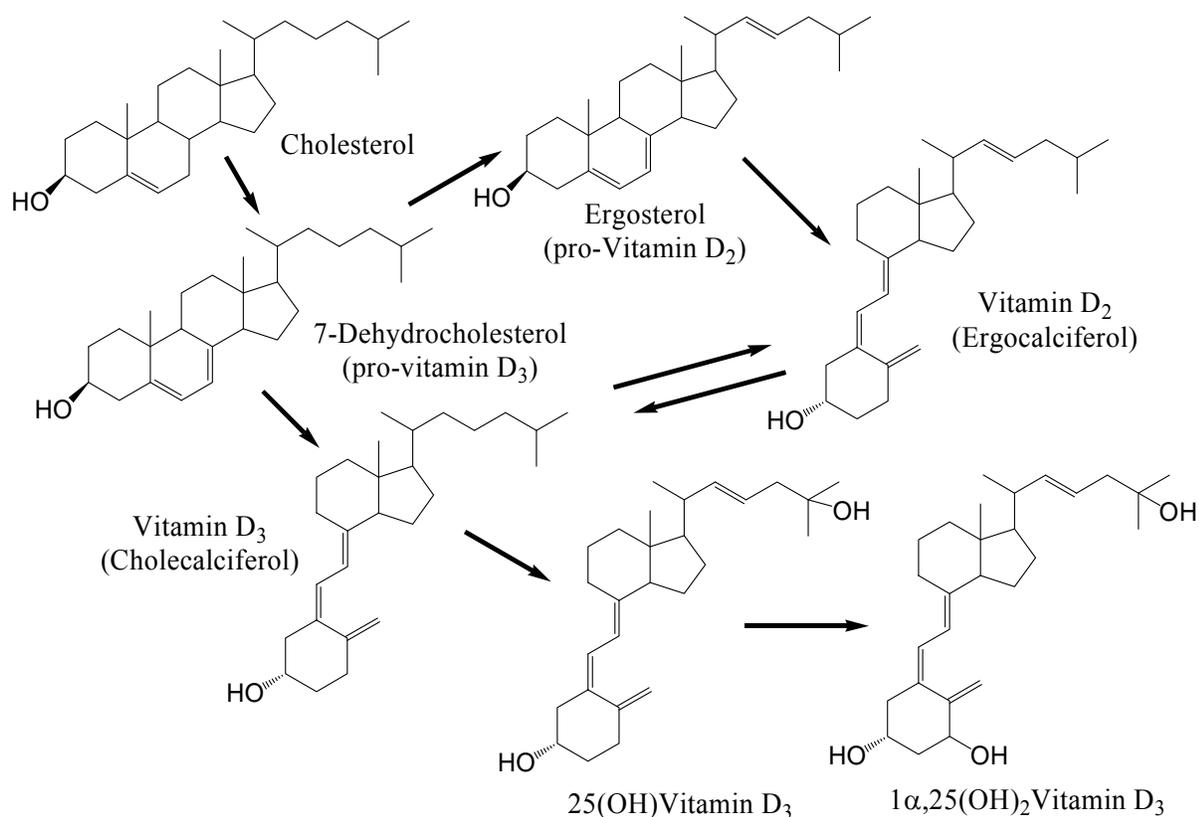


Figure 4-1 Biosynthesis of vitamin D.

4.2 Experimental

4.2.1 Materials

Vitamin D₃ (**I**) was purchased from Sigma Chemical Company (St. Louis, MO, USA), and its deuterated internal standard (D₆-**I**, **II**) was synthesized at Merck Research Laboratories (Rahway, NJ, USA). A reference compound **V** and its deuterated internal standard (D₈-**V**) were also synthesized at Merck Research Laboratories. All solvents were HPLC or analytical grade and were purchased from Fisher Scientific (Fair Lawn, NJ, USA). The derivatizing reagent (4-phenyl-1,2,4-triazoline-3,5-dione; P-TAD), EDMA and DMPA were purchased from Aldrich Chemical Company (Milwaukee, WI, USA). Ammonium acetate (HPLC grade) purchased from J.T. Baker (Phillipsburg, NJ, USA), formic acid (95%) and 1-decanol obtained from Sigma (Milwaukee, WI, USA) was used as received. Oasis HLB with particle size 60 μm was obtained from Waters (Milford, MA, USA). Deionized water was obtained by passing in-house water through a Millipore Milli-Q plus system (Bedford, MA, USA). Nitrogen (99.999%) was supplied by West Point Cryogenics (West Point, PA, USA). 96-Well collection plates (1.2 and 2.4 mL) and mats were purchased from Marsh Biomedical (Rochester, NY, USA). Different lots of human control serum were obtained from Biological Specialties Corp. (Lansdale, PA, USA) and stored at -20 °C before use.

4.2.2 Instrumentation

A Perkin-Elmer (Norwalk, CT, USA) binary 250 pump and a Varian (Palo Alto, CA, USA) ProStar 96-well plate autosampler were used in this work. The chromatographic separation of analytes was performed on a Phenomenex Synergi Polar-

RP column (50 × 2 mm, 4 μm) with a 0.5 μm in-line filter. Mobile phase consisted of methanol (MeOH):10 mM ammonium acetate (88:12, v/v) and was pumped at a flow rate of 0.3 mL/min. The total run time was 5 minutes. Two mL of MeOH was used as a washing solvent for needle and flow path cleaning of the autosampler after each injection.

An Applied Biosystems-Sciex API 3000 triple quadrupole mass spectrometer (Foster City, CA, USA) equipped with a heated nebulizer (HN) source operating in the positive ionization mode was used for all HPLC-MS/MS analysis. MRM was utilized for quantification. In HN experiments, the heated nebulizer probe temperature was maintained at 400 °C, and the nebulizing gas (air) pressure was set at 80 psi. The settings for the curtain and collision gases were 8 and 4 psi on the API 3000 mass spectrometer, respectively.

4.2.3 Preparation of Standard Solutions and QC Samples

A stock solution of **I** (100 μg/mL) was prepared in methanol. This stock solution was further diluted with methanol to give a series of working standards with concentrations of 0.005, 0.01, 0.025, 0.05, 0.1, 0.15, and 0.25 μg/mL. The internal standard (**II**) was also prepared as a stock solution (100 μg/mL) in methanol. A working standard solution of 0.1 μg/mL of **II**, prepared by diluting stock solution with methanol, was used for serum samples analyses. All standard solutions were stored at 4°C.

A stock solution for QC samples of **I** was prepared separately by the same procedure using a separate weighing. QC samples were prepared by diluting the QC working solution with human control serum. QC samples at three concentrations (Low QC, Middle QC, and High QC) were used to evaluate assay precision and accuracy. All

QC samples were divided into 1.25 mL aliquots in separate cryo tubes and stored at -20°C until analysis.

A stock solution of reference compound **V** ($10\ \mu\text{g/mL}$) was prepared in 50% ACN in water (v/v). Working standards were obtained by further dilution of the stock solution of **V** with 20% ACN in water (v/v). The internal standard (**D₈-V**) was also prepared as a stock solution ($10\ \mu\text{g/mL}$) in 50% ACN in water (v/v). A working standard solution of $100\ \text{ng/mL}$ of **D₈-V**, prepared by diluting stock solution with 50% ACN in water (v/v), was used for all measurements. All standard solutions were stored at 4°C . The purpose of preparing standard solution **V** and **D₈-V** was to determine σ_C and α in an empirical model to evaluate the effect of an ion-current ratio on measurement precision.

Because of light sensitivity, all standard preparation and sample extractions were performed under yellow light.

4.2.4 Sample Processing

4.2.4.1 In-tube LLE (Method A)

Standards, QC, and subject serum samples were thawed at room temperature. One mL serum samples were basified with pH 9.8 (1 mL) carbonate buffer, then extracted twice with 7 mL MTBE using 15 mL conical disposable glass tubes. The tubes were placed in a dry ice/acetone bath until the aqueous layer froze. The organic extracts were manually transferred to a clean 15 mL centrifuge tube and were evaporated to dryness under heated N_2 stream. The dried residue was reacted with 0.2 mL of derivatizing agent, P-TAD ($0.25\ \text{mg/mL}$ in ACN) for 30 minutes. Upon completion of the reaction, the excess of P-TAD was reacted with 0.5 mL of methanol. The mixture was dried under

heated N₂ stream and reconstituted in 150 µL of mobile phase. Fifteen µL of this solution was injected into the HPLC-MS/MS system. Serum standards were prepared the same way as serum samples by adding 100 µL of each working standard and 100 µL internal standard to 1 mL of human control serum. The resulting serum standard concentrations ranged from 0.5 to 25 ng/mL.

4.2.4.2 96-well Plate LLE (Method B)

Standards and QC serum samples were thawed at room temperature. A 400 µL aliquots of subject or control serum samples were added individually into a 2 mL deep 96-well plate followed by 40 µL of 50 % of methanol in water (v/v). 40 µL of the internal standard solution was added to each well, except to the well designated for the double blank serum. The plate containing samples was placed onto a Tomtec Quadra 96 workstation (Hamden, CT, USA) for liquid transfer. After transferring 50 µL of 0.2 M sodium carbonate buffer solution (pH = 11) and 1.28 mL of MTBE by Tomtec workstation, the plate was sealed with mat made of molded PTFE/silicone liner and was roto-mixed for LLE. The plate was then centrifuged and the top organic layer was aspirated and dispensed by Tomtec workstation into a 1.2 mL 96-well collection plate. The serum samples were extracted again with 1.28 mL of MTBE. The organic extract was evaporated to dryness under heated N₂ stream. The dried residue was reacted with 0.2 mL of derivatizing agent, P-TAD (0.125 mg/mL in ACN) for 30 minutes. Upon completion of the reaction, the excess of P-TAD was reacted with 0.2 mL of methanol. The mixture was dried under heated N₂ stream and reconstituted in 150 µL of mobile phase. Fifteen 15 µL of this solution was injected into the HPLC-MS/MS system.

4.2.4.3 In-tip SPME (Method C)

Preparation of In-tip SPME Fibers

A detailed preparation procedure has been described previously.¹⁴²

SPME Conditions

Standard and QC serum samples were thawed at room temperature. 100 μL of subject serum samples were added individually into a 2.4 mL deep 96-well plate spiked with 100 μL of methanol:water (50:50, v/v). Standard curve samples were prepared by spiking 100 μL of appropriate standard into 100 μL of human control serum. Internal standard solution (100 μL) was added to each well of the plate. After adding 50 μL of 0.2 M sodium carbonate buffer solution (pH = 11) to all wells on the plate, the plate was sealed with mat made of molded PTFE/silicone line and vortex-mixed thoroughly on a VWR multi-tube vortexer for 2 minutes. In-tip SPME extraction and desorption process was fully automated on a Tomtec Quadra 96 workstation (Hamden, CT, USA). Briefly, in-tip SPME fibers in 96-well format were loaded at position 1 (tip plate) on the deck of the Tomtec Quadra 96 workstation. Washing solvent, water, and 5% methanol (5% methanol : 95% water, v:v) reservoirs were placed at position 2 and 3, and eluting solvent methanol was at position 4, respectively. An empty 1.2 mL deep 96-well plate was placed at position 5 for desorption. The mat was carefully removed from the 2.4mL sample plate and the plate was placed at position 6 for extraction. The Tomtec Quadra 96 workstation was programmed as follows: after tips were picked up and washed subsequently with 50 μL of methanol and 50 μL of water, 300 μL of the solution containing serum was repeatedly aspirated and dispensed for 40 minutes from extraction plate at position 6.

When extraction was completed, in-tip SPME fibers were washed once with 50 μL of water and 10% methanol, respectively, then 50 μL (twice) of methanol was aspirated from methanol reservoir with 50 μL air gap and dispensed into the empty 1.2 mL desorption plate. The 1.2 mL deep 96-well plate was evaporated to dryness under heated N_2 stream and the dried residue was reacted with 0.2 mL of derivatizing agent, P-TAD (0.125 mg/mL in ACN) for 30 minutes. Upon completion of the reaction, the excess of P-TAD was reacted with 0.2 mL of methanol. The mixture was dried under heated N_2 stream, reconstituted in 150 μL of mobile phase and 15 μL was injected into the HPLC-MS/MS system.

4.2.5 Validation Procedures

4.2.5.1 Precision and Accuracy

The precision of the method was determined by the replicate analysis ($n = 5$) of I in five different sources of human serum at all concentrations utilized for the construction of calibration curves. The linearity of each calibration curve was confirmed by plotting the peak height ratio of the derivatized drug (III) to the derivatized internal standard (IV) vs. drug concentration. Calibration curve calculations were made by subtracting the mean peak height ratio of derivatized drug (III)/derivatized IS (IV) of triplicate analysis of the control blank serum samples used for the construction of standard lines from the respective peak height ratios of each standard concentration. The unknown sample concentrations were calculated from the equation $y = mx + b$, as determined by weighted ($1/x$) linear regression of the standard line. The accuracy of the method was determined as the percentage between the mean concentration observed and the nominal

concentration. The intra-day accuracy was required to be within $\pm 15\%$ at all concentrations. The precision, expressed by the coefficient of variation (%C.V.), was required to be $<15\%$ at the LLOQ and $<10\%$ at all other concentrations.

4.2.5.2 Selectivity

The selectivity of the method was confirmed by processing control drug-free human serum samples from six different sources to determine whether endogenous peaks interfered at the mass transitions chosen for the derivatized analyte and/or the internal standard. In addition, the “cross-talk” between MS channels used for monitoring the analyte and the internal standard was evaluated.

4.2.5.3 Recovery and Matrix Effects

Extraction recovery for **I** and **II** was evaluated using standards spiked at three concentrations of analytes. Recovery was determined by comparing the absolute peak heights of the standards in control human serum extracted and derivatized according to the described sample processing procedures to control serum extracted in the same manner and then spiked post extraction with the analytes and derivatized. Matrix enhancement/suppression of the ionization or "absolute" matrix effect was evaluated by comparing the absolute peak heights of the standards in post spiked extraction samples to neat derivatized standards injected directly in the same reconstitution solvent. Since a stable isotope labeled internal standard was used, a potential “relative” matrix effect on ionization should not have any adverse effect on the quantitation of **I** in different serum lots. This was evaluated and confirmed by the determination of slopes of the calibration curves in five different lots of control serum as suggested in the literature.¹²³

4.3 Results and Discussion

4.3.1 Derivatization of Vitamin D₃ and Optimization of MS/MS Conditions

Vitamin D₃ (**I**) that is formed from cholesterol by opening the B-ring of the secosteroid, is generated in the skin from 7-dehydrocholesterol under UV radiation (Figure 4-1). Therefore, it is necessary to evaluate the potential interference from 7-dehydrocholesterol in the determination of **I** in human serum. Initially, an attempt was made to develop a method based on the MS/MS detection of underivatized **I** using both TISP and HN interfaces. As illustrated in Figure 4-2A, an intense MS/MS response for a neat standard of **I** was observed when precursor and product ion pair at m/z 385 \rightarrow 367 was monitored. However, this response was significantly higher ($\sim 5x$) when the same amount of **I** was extracted from the control serum. Two additional peaks, which were separated from **I**, were also detected in the same MS/MS channel as for **I** (Figure 4-2B). To separate the interference peak which caused the enhancement of **I** as well as other endogenous serum impurities, further experiments were conducted using more selective chromatographic conditions. Under these HPLC conditions, **I** was separated from a large interference peak (Figure 4-3) and other interferences detected at the same MS/MS channel as **I**. However, due to a long (10.5 min.) retention time of **I** (k' =25) a significant peak broadening was observed decreasing the sensitivity of detection. The interference peak was further identified as 7-dehydrocholesterol, which had the same molecular weight as vitamin D₃ and fragmented to the same product ion (m/z 367).

The MS/MS detection of underivatized **I** was also attempted using APPI interface. Similarly as in the case of TISP and HN interfaces, a strong MS/MS response for neat standard (Figure 4-4A) of **I** was observed. However, when serum extracts containing **I**

were analyzed under the same conditions, a large number of interfering peaks were observed at the same MS/MS channel as used for monitoring **I**. The chromatograms of serum extract presented in Figures 4-4B and 4-4C illustrate how non-selective MS/MS detection can be. In spite of the utilization of the MS/MS detection, a large number of interfering compounds possessing the same MS/MS ions as the compound of interest are present. The need for an efficient chromatographic separation in such cases cannot be overemphasized. In order to improve the sensitivity and selectivity of detection and potentially produce a precursor → product ion pair that was distinguishable from 7-dehydrocholesterol, it was decided to evaluate derivatization of **I**.

As reported in the literature, because a cisoid diene system is a feature common to most vitamin D related compounds; all these compounds may react with dienophilic reagents such as triazolinediones to form Diels-Alder cycloaddition products at room temperature. These derivatized products will greatly enhance the sensitivity of their MS/MS detection. At the same time, these derivatives may also distinguish themselves from other interferences due to a significant increase in the molecular mass and their detection in the higher mass range together with a different fragmentation in the ion source. A variety of analogs and metabolites of vitamin D compounds have been derivatized with different triazolinediones,^{143, 144} including P-TAD, and these derivatization reactions were used predominantly for the qualitative assessment and structural confirmation of the presence of metabolites and/or analogs. An analog of **I** was quantified in plasma using an electron- capture derivatization reagent PFB-TADO, a pentafluorobenzyl (PFB) analog of P-TAD, and the potential for derivatization of **I** with this reagent was discussed.¹⁴⁴ However, the reaction with PFB-TAD and/or with P-TAD

was never used for the development of a quantitative method for **I** in plasma or serum samples.

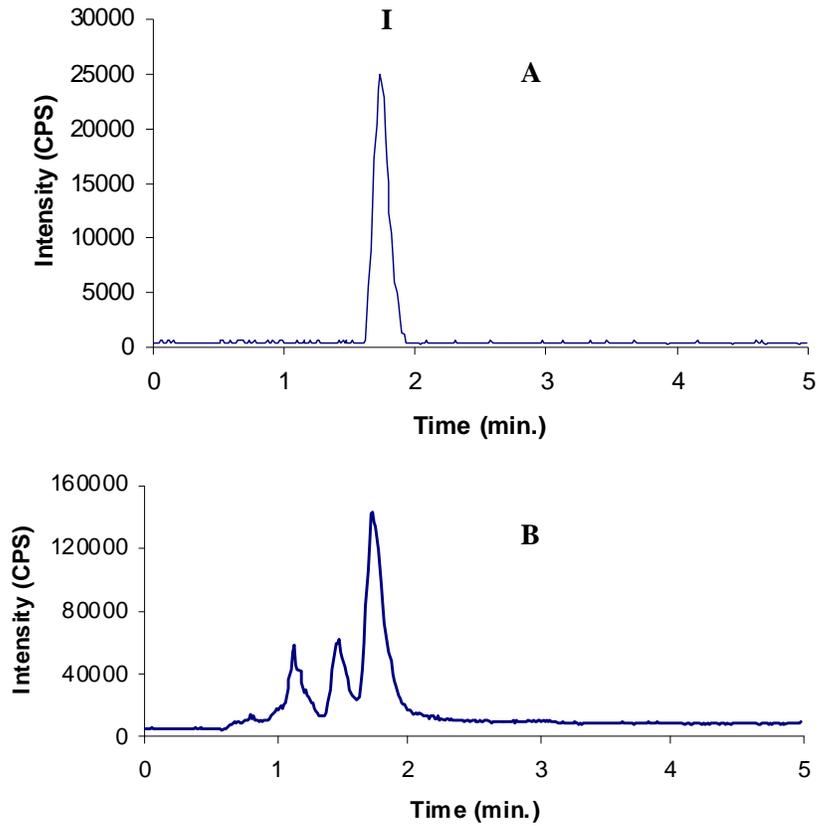


Figure 4-2 Representative chromatograms of 20 ng/mL neat standard of **I** (A) and a human control serum extract (B) monitored at m/z 385 \rightarrow 367, using MeOH:water containing 10 mM ammonium acetate (92:8, v/v) as a mobile phase

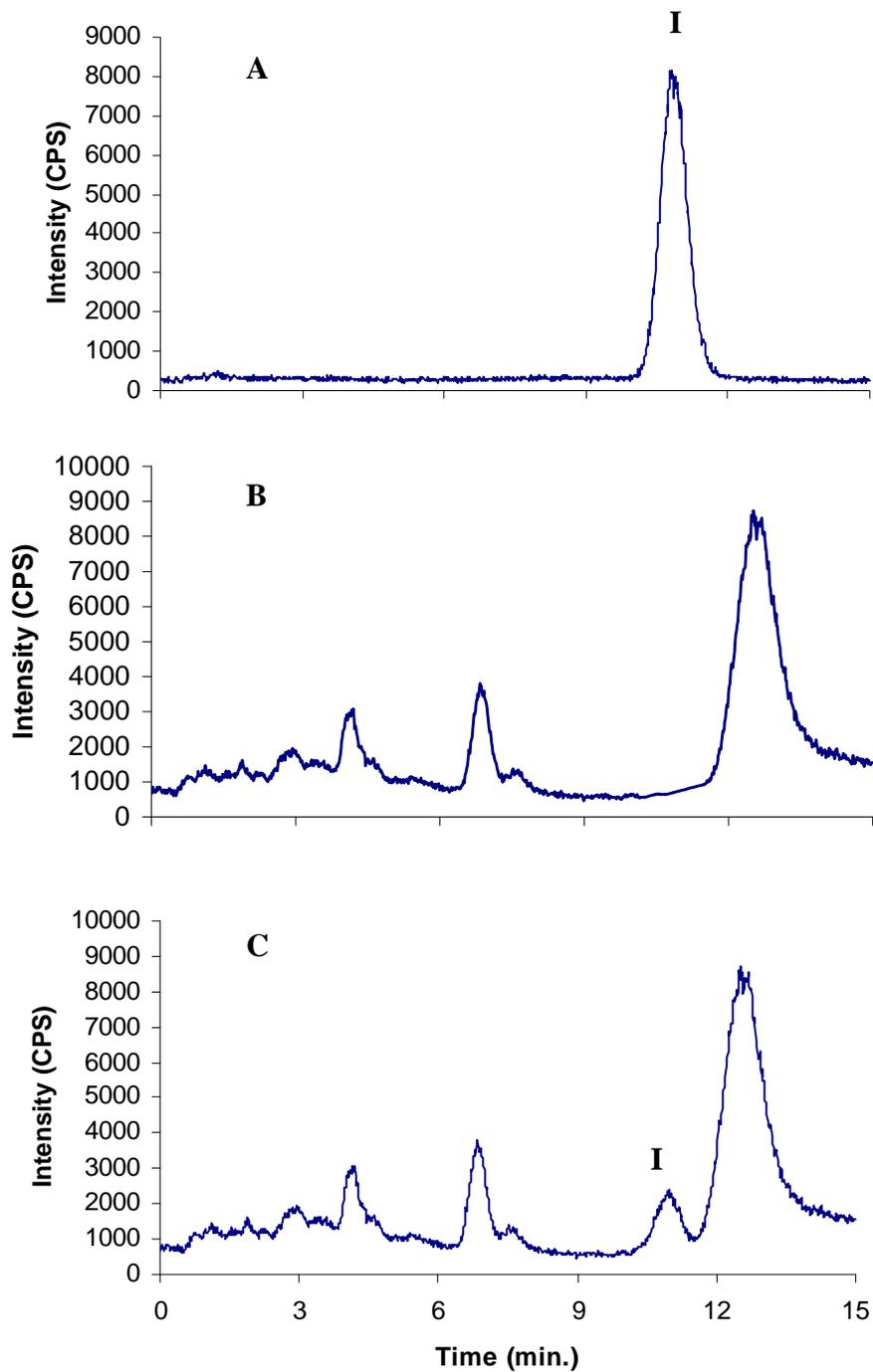


Figure 4-3 Representative chromatograms of 40 ng/mL neat standard of **I** (A), a human control serum extract (B), and human control serum spiked with 20 ng/mL of **I** (C) using methanol:water containing 10 mM ammonium acetate (60:40, v/v) as a mobile phase (C)

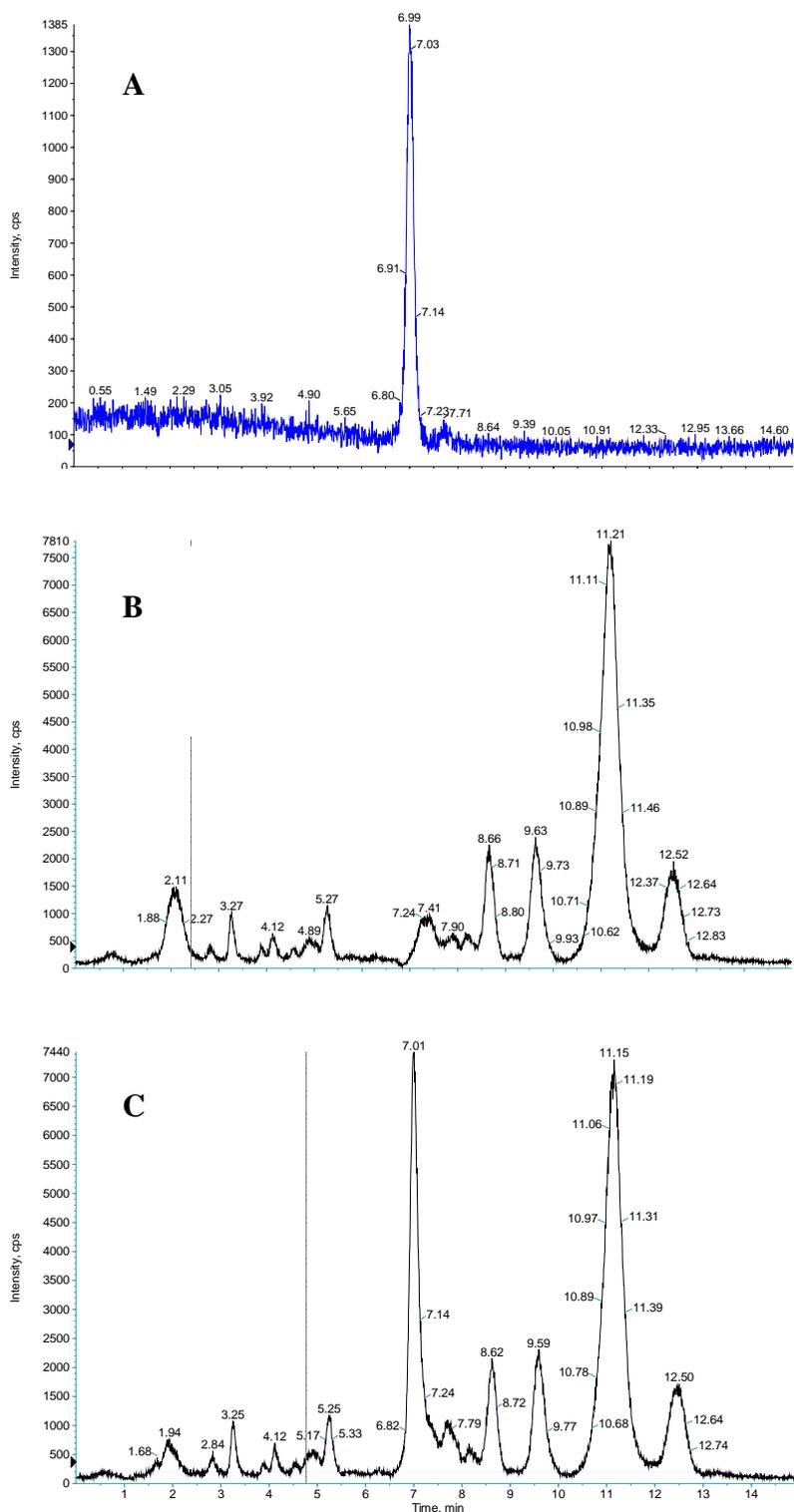


Figure 4-4 Representative chromatograms of 0.5 ng/mL neat standard of **I** (A), a human control serum extract (B), and human control serum spiked with 10 ng/mL of **I** (C) using the APPI interface.

In MS/MS infusion experiments, MeOH-free methylene chloride was chosen as the reaction solvent, as P-TAD is very sensitive to air and moisture. One $\mu\text{g/mL}$ of the P-TAD-vitamin D₃ derivative was prepared as follows: a P-TAD solution (0.29 M) in methylene chloride was reacted at room temperature with 0.1 M of **I** also in methylene chloride at 1:1 molar ratio. The reaction was stopped by adding water and the post-reaction mixture was extracted with methylene chloride. The organic extract was evaporated to dryness and the residue was reconstituted in methanol. **I**, **II**, and the biological precursor 7-dehydrocholesterol were successfully derivatized (Figure 4-5). The derivatized **I** was detected in the positive ionization mode using a heated nebulizer probe and a major product ion at m/z 298 was observed. The same experiment was performed using 7-dehydrocholesterol, however, a fragment ion at m/z 298 was not observed. Therefore, MRM transition at m/z 560 \rightarrow 298 was selected for selective quantification of **I** (Figure 4-6). The different fragmentation pattern between the derivatized **I** (**III**) and 7-dehydrocholesterol demonstrated that the derivatization enhanced not only sensitivity but also selectivity of the determination of **I**. Precursor – product ion at m/z 566 \rightarrow 298 was chosen for the derivatized internal standard (**IV**). Optimization of source and MS parameters were obtained by infusing neat solutions of the synthesized derivatives at a flow rate of 20 $\mu\text{L/min}$ into a mobile phase pumped at 0.5 mL/min using the heated nebulizer interface at 400°C.

It is important to note that during derivatization of the human serum extracts, an excess concentration of the P-TAD reagent is provided since many endogenous components may be co-extracted and may consume the P-TAD reagent affecting the

derivatization yield of an analyte of interest and the sensitivity and reliability of the method. Therefore, the amount of derivatizing reagents used needs to be optimized.

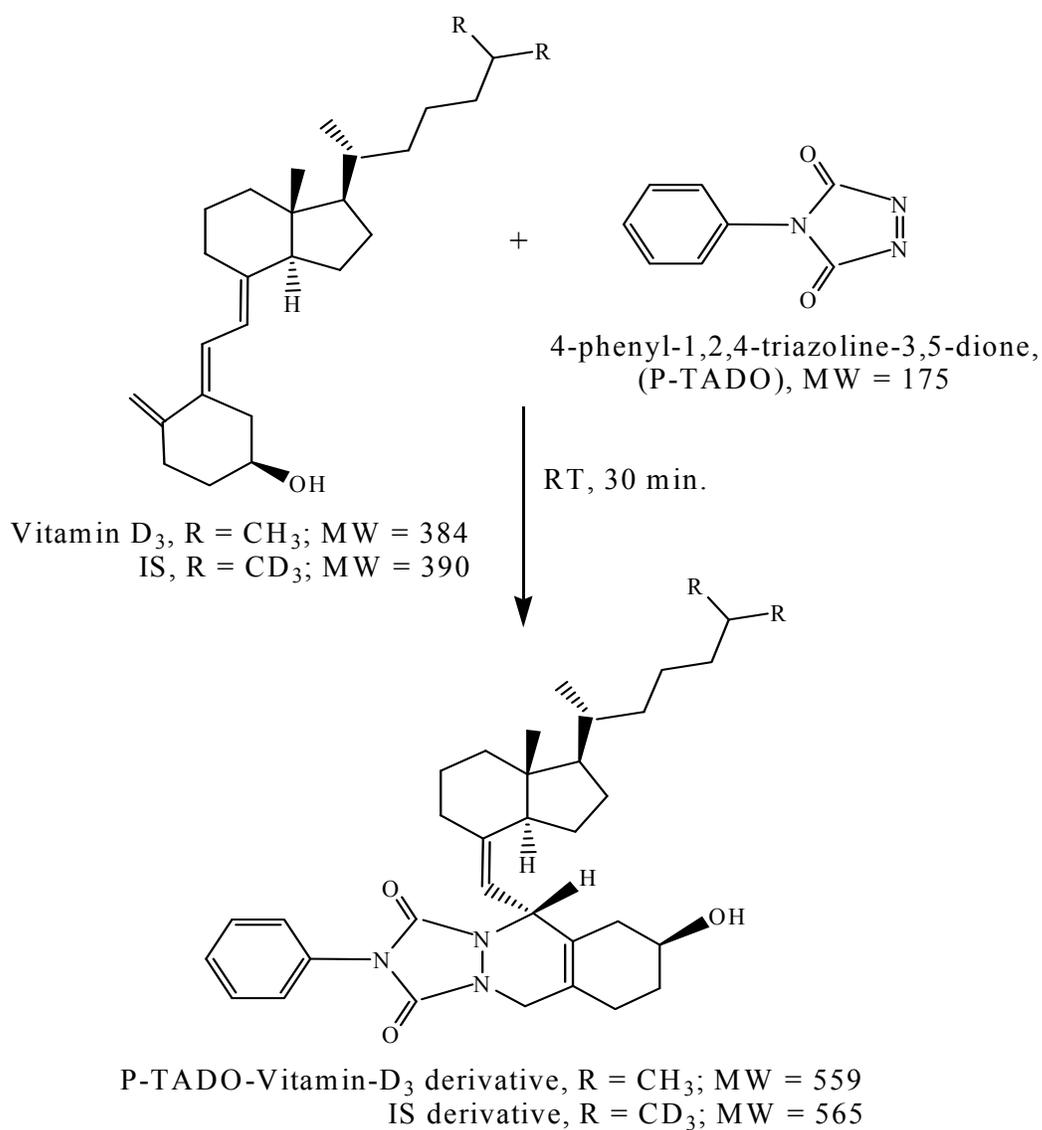


Figure 4-5 Derivatization of vitamin-D₃ (**I**) and D₆-I (**II**) with P-TAD and the formation of P-TAD-vitamin-D₃ (**III**) and P-TAD-D₆-vitamin D₃ (**IV**)

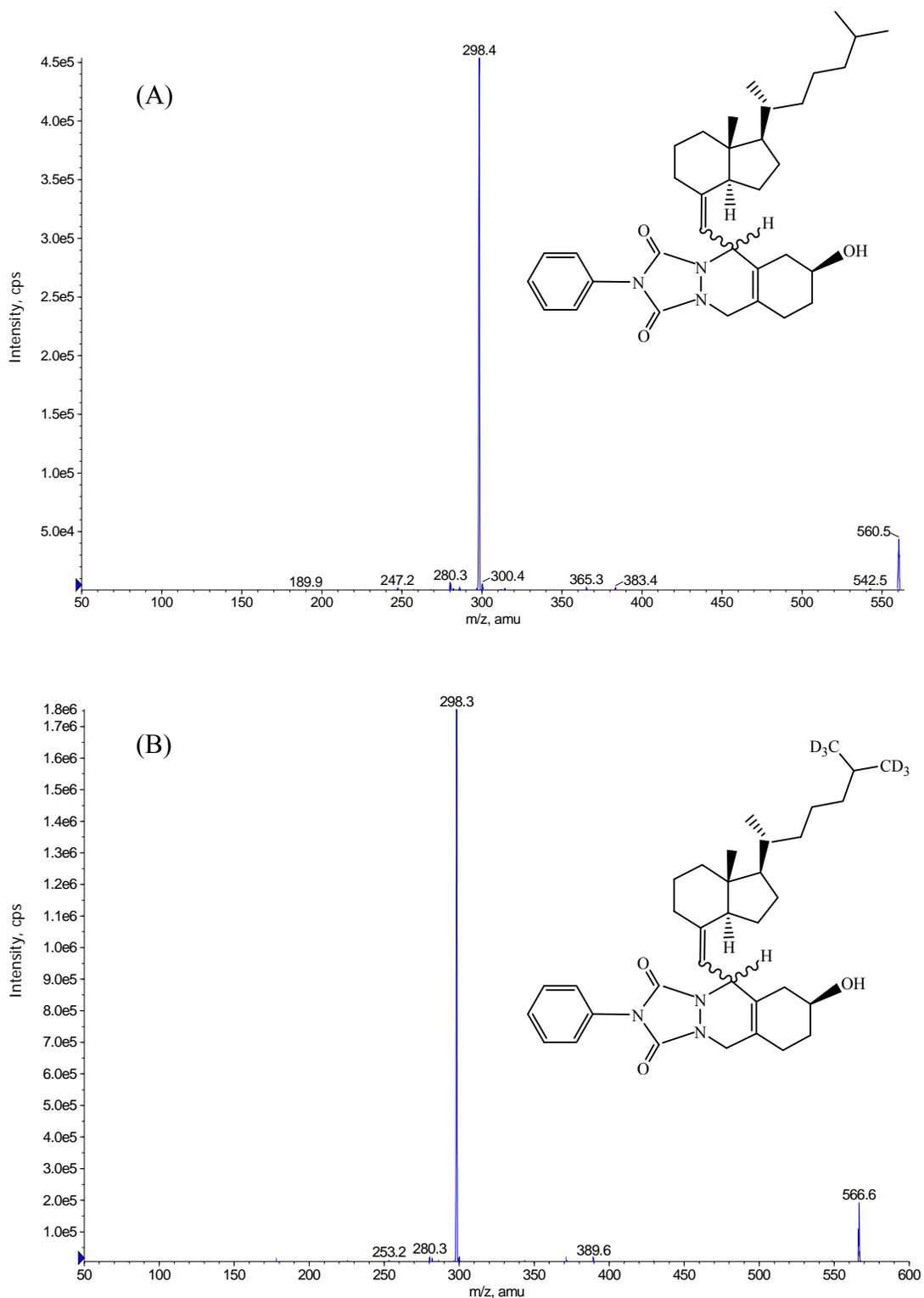


Figure 4-6 The product ion mass spectra of the protonated molecules of P-TAD-vitamin-D₃ (**III**, chromatogram A) and P-TAD-D₆-vitamin D₃ (**IV**, chromatogram B) derivatives (M+H)⁺, m/z = 560 and 566, respectively, under the optimized MS/MS conditions used in the assay.

4.3.2 Automated In-tip SPME Method Evaluation and Optimization

As an innovative technique that allows the integration of sampling and extraction in a single step, SPME has the advantages of (1) less solvent used, (2) easy handling, (3) little equipment needed, and (4) faster speed of analysis. However, the technique has not yet been accepted as an alternative approach for quantitative determination of analytes in pharmaceutical bioanalysis for many reasons. First of all, because of the fiber and in-tube configurations, SPME suffers from low-throughput and lack of automation which limits its application in analysis of a large number of similar samples. Secondly, as pointed out by Ulrich,⁶² there are some major disadvantages of SPME that include longer desorption time, carry over effects due to repeated use of the same fiber, and the method being prone to errors due to considerably lower recovery in comparison with more classical extraction methods. These issues have not been completely addressed and resolved since SPME invention.

In the current study, a simple, flexible, low-cost, and reproducible procedure for the preparation of in-tip SPME fibers with polymer monoliths using photopolymerization technique was developed. The porous polymers have many advantages including the simplicity of their fabrication and the wide range of chemistries for reactions. More importantly, the relative large surface areas of polymer monolith in-tip SPME fibers dramatically increase the absolute extraction recoveries (up to 30%) while in general, the recoveries reported for SPME are considerably lower (<1%). In addition, in-tip SPME is very easily coupled with commercially available liquid handling systems for automated sample preparation without introducing any additional equipment while maintaining its simplicity.

Factors that were involved in fiber fabrication included concentration of the initiator, total monomer to total porogen ratio, porogen type, and optimized photopolymerization time. The optimized reagent composition of 40 wt% of EGDMA and 60 wt% of 1-decanol with 1 wt% of DPA was obtained for preparing monolith in terms of rigidity and homogeneity. The scanning electron microscopy (SEM) micrographs of the monolithic structure indicated that the UV irradiation time of 10 minutes was sufficient of initiating monolithic polymerization of the solution in the tips. For the SPME automation to be successful in parallel extraction format, good reproducibility of the amount of analyte extracted by different fibers is necessary. In this study, 96 in-tip SPME fibers were fabricated at the same time by photopolymerization using a Tomtec Quadra 96 workstation. Fiber-to-fiber reproducibility was found to be within %C.V. of 15.4% using absolute peak area counts of a standard analyte. However, the precision increased significantly to 5.6% C.V. with the use of a stable isotope labeled internal standard that was extracted simultaneously with the analyte. This indicated that the inter fiber reproducibility is not critical in quantitative analysis in terms of accuracy and precision as long as an internal standard is able to compensate for the inter fiber variability in extraction capacity.

During in-tip SPME method development, parameters such fiber coating, sample volume, extraction conditions (pH or ionic strength), extraction and desorption time, desorption solvents, and calibration methods were thoroughly investigated. For in-tip SPME, samples were aspirated/dispensed across the solid phase media and the extraction equilibrium was accomplished through multiple aspirate/dispense cycles. Unlike conventional SPME methods, where agitation is a big issue, especially in automated

SPME-LC, the uniformity of agitation could be easily achieved for in-tip SPME using an automated liquid handling system such as the Tomtec Workstation. Extraction conditions were based on those from LLE and serum sample volume was chosen at 100 μ L. In order to achieve the maximum recovery in a reasonable period of time, 480 cycles was chosen which required about 40 minutes. Because of the small monolithic bed volume of the in-tip SPME fiber, extracted analytes could be released efficiently from the extraction sorbent with minimal elution volume. An elution scheme of 2 \times 50 μ L of solvent was sufficient for the quantitative release of analytes. Carryover effect was eliminated as polymer monolith in-tip SPME fibers were used as disposable.

4.3.3 Methods Validation

Method A and B were validated in human serum in the concentration range of 0.5 to 25 ng/mL of **I**, whereas the in-tip SPME method was validated in the concentration range of 5 to 250 ng/mL. The assessment of the intraday variability of each method was conducted in five different lots of human control serum spiked with **I**. The resulting method precision and accuracy data is presented in Table 4-1. For the in-tube LLE method A, the intra-day precisions (%C.V.) was 2.8% at LLOQ, and was equal to or lower than 6.0% at all other concentrations used for the construction of the calibration curve. Method A accuracy was found to be within \pm 2% of the nominal concentration for all the standards evaluated. In 96-well plate LLE method B, the intra-day precisions (%C.V.) was 5.9% at LLOQ, and was equal to or lower than 5.2% at all other concentrations used for the construction of the calibration curve. Method accuracy was found to be within \pm 3% of the nominal concentration for all standards evaluated. In in-

tip SPME method C, the intra-day precisions (%C.V.) was 10.9% at LLOQ, and was equal to or lower than 8.7% at all other concentrations used for the construction of the calibration curve. Method accuracy was found to be within $\pm 8\%$ of the nominal concentration at all standards concentrations evaluated. The correlation coefficient for the mean standard curves constructed from five different lots of human serum was 0.9997, 0.9996, and 0.9980 for methods A, B, and C, respectively.

Table 4-1 Intraday Precision and Accuracy Data for the Determination of Vitamin D₃ in Five Different Lots of Human Control Serum Using In-tube LLE (Method A), 96-well Plate LLE (Method B), and In-tip SPME (Method C), respectively

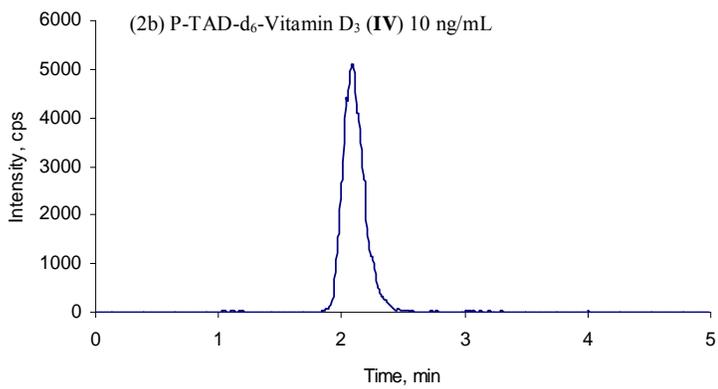
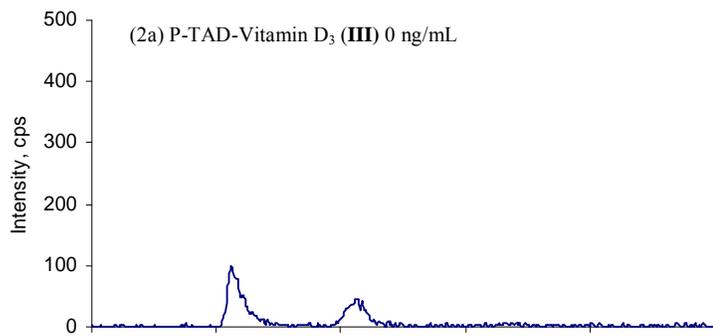
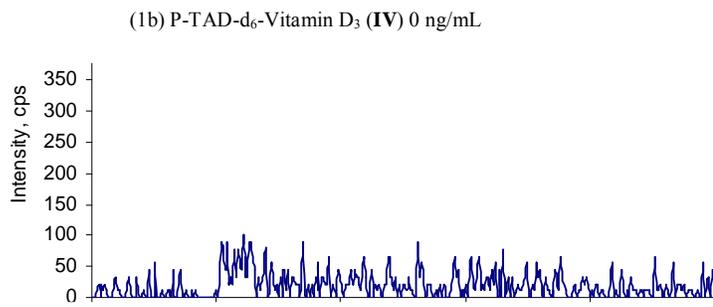
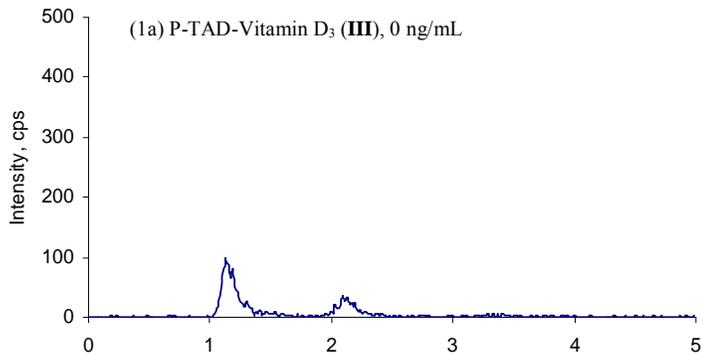
Nominal Conc. (ng/mL)	Accuracy (%) ^a [%C.V.] (n= 5)	Accuracy (%) ^a [%C.V.] (n= 5)	Nominal Conc. (ng/mL)	Accuracy (%) ^a [%C.V.] (n= 5)
	Method A	Method B		Method C
0.5	100.0 [2.8]	102.0 [5.9]	5	103.6 [10.9]
1	100.0 [2.4]	97.3 [4.2]	10	99.2 [8.7]
2.5	98.8 [6.0]	100.6 [5.2]	25	100.4 [5.6]
5	101.6 [3.5]	99.8 [1.6]	50	92.8 [2.8]
10	99.1 [0.9]	101.2 [2.3]	100	101.3 [2.2]
15	99.7 [3.0]	98.9 [2.2]	150	104.8 [3.2]
25	100.3 [2.0]	100.2 [3.1]	250	98.0 [6.4]

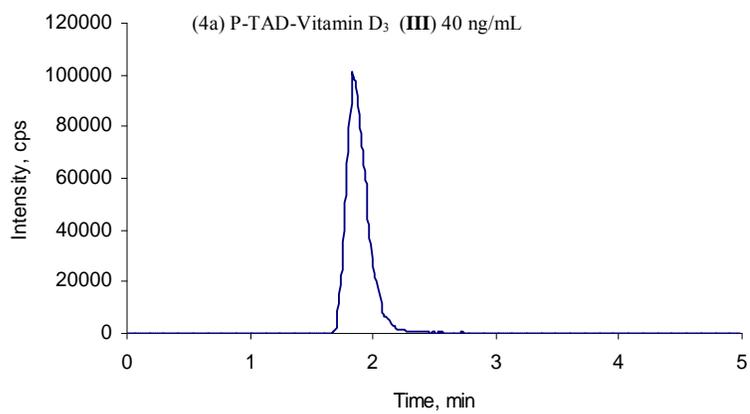
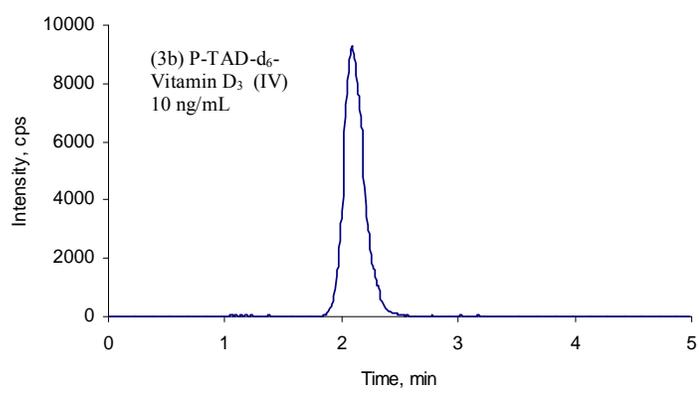
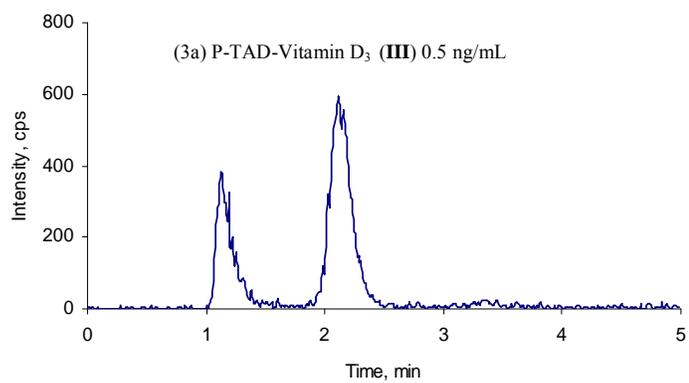
^a Expressed as [(mean calculated concentration)/(nominal concentration)] $\times 100\%$

4.3.4 Selectivity

Assessment of the selectivity of the method is critical and needs to be confirmed in the presence of *in-vivo* metabolites of the analyte. Some metabolites may be converted to parent drug during sample preparation and/or undergo partial fragmentation in the ion

source at high temperatures giving the same molecular ion as for the parent drug. The major metabolites 25-hydroxyvitamin D₃ and 1, 25-dihydroxyvitamin D₃ of **I** were evaluated for the “cross-talk” in channels used for monitoring both **I** and the internal standard. No interference or “cross-talk” from these metabolites was observed. In addition, the “cross-talk” between channels used for monitoring both **I** and the internal standard was evaluated by the analysis of standard samples containing individual compounds separately at the concentrations of 25 and 10 ng/mL for **I** and internal standard, respectively, and monitoring the response in other MS/MS channel used for quantification. No response was observed in the channel of the other analytes at their retention times. Also, no interference or “cross-talk” was observed from these compounds in the channels used for monitoring derivatized **I** and the internal standard (**III** and **IV**, respectively). Figure 7 shows the representative extracted ion chromatograms obtained from human control serum blank (Figure 4-7(1a) and 4-7(1b)), human control serum spiked with 10 ng/mL of **II** (Figure 4-7(2a) and 4-7(2b)), human control serum spiked with 0.5 ng/mL of **I** and 10ng/mL of **II** (Figure 4-7(3a) and 4-7(3b)), and human control serum spiked only with 40 ng/mL of **I** (Figure 4-7(4a) and 4-7(4b)). No “cross-talk” between channels used for the determination of **I** and **II** was observed. The small peak (<10% of the LLOQ) in the channel used for monitoring derivative **III** in blank serum sample was due to the endogenous **I** present in the serum sample. In clinical samples, when necessary and if present in pre-dose human serum in any quantifiable concentrations, this endogenous peak was subtracted from the total concentration of **I** determined in that sample.





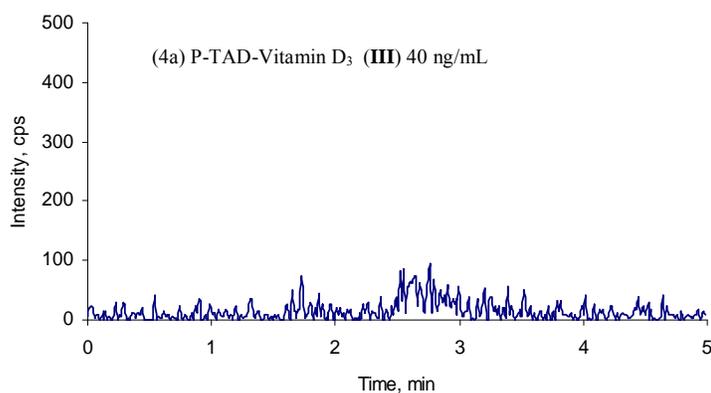


Figure 4-7 Representative chromatograms of human control serum obtained by multiple reaction monitoring at m/z 560 \rightarrow 298 for derivatized vitamin D₃ and m/z 566 \rightarrow 298 for derivatized D₆-vitamin D₃ using 96-well plate LLE

4.3.5 Recovery and Assessment of the Matrix Effects

Extraction recovery and the effect of the serum matrix on ionization and derivatization efficiency was evaluated for **I** and the internal standard using standards spiked at concentrations of 0.5, 10, and 25 ng/mL for **I**, and at 10 ng/mL for **II** in both methods A and B. In method C, this evaluation was performed at concentrations of 5, 100, and 250 ng/mL for **I**, and 100 ng/mL for **II**. Recoveries were determined by comparing the peak height of standards spiked into three different lots of human control serum and extracted as per sample preparation to human control serum extracted in the same manner, then spiked post-extraction with a known amount of the drug. The mean recoveries of **I** and **II** were 82% and 84%, respectively, in method A; 69% and 63%, respectively, in method B, and 29% and 24%, respectively, in method C (Table 4-2). Recovery in all methods was consistent over the entire range of the standard curve indicating that

extraction of the analytes in each method was independent on the concentration. In order to assess the "absolute" matrix effect on the ionization and derivatization process, the absolute peak heights of control human serum samples extracted and then spiked with a known amount of analytes and derivatized were compared to neat standards derivatized and injected directly in the same solvent. Results are shown in Table 4-2. Considering the experimental uncertainties, the "absolute" matrix effects in methods A and C were much less significant than those observed in method B with ion enhancement about 140%. However, the use of stable isotope labeled **I** as the internal standard fully compensated for any variation in matrix effect and/or recovery between different lots of human control serum. Therefore, an "absolute" matrix effect on ionization or any differences in derivatization efficiency of **I** and internal standard would not have any adverse effect on the precision and accuracy of any of the methods presented. The absence of a "relative" matrix effect^{89,90,123} on ionization and derivatization efficiency was confirmed by an examination of the slopes of the calibration curves that were constructed in five different lots of human control serum. The high precision of these slopes (1.3, 2.0, and 4.2% C.V.) in methods A, B, and C, respectively, confirms the absence of a "relative" matrix effect in all methods developed.

4.3.6 Analyte Stability

Standard solution stability was confirmed for a period of 20 days when refrigerated. QC samples (n = 5 at each concentration) were subjected to three freeze-thaw cycles consisting of a thaw to reach room temperature, then refreezing at -20 °C. These samples, together with a set (n = 5 at each concentration) of human QC samples

that were not subjected to additional freeze-thaw cycles, were then defrosted and analyzed. In all cases, the results for the samples that were subjected to additional freeze-thaw cycles were within $\pm 9\%$ of the nominal value (Table 4-3).

Table 4-2 Extraction Recovery and Assessment of the “Absolute” Matrix Effects During the Determination of Vitamin D₃ and Internal Standard in Human Control Serum Using Methods A, B and C^a

Vitamin D ₃ Concentration (ng/mL)	%Extraction Recovery ^b			% Matrix Effect ^c		
<i>Method</i>	A	B	C	A	B	C
0.5	81	68		92	142	
5.0			26			121
10.0	78	68		89	141	
25.0	88	70		89	145	
100			31			114
250			30			110
Mean:	82	69	29	90	143	115
D ₆ -I						
10.0 (II)	84	63		92	140	
100 (II)			24			116

^a Determined in three different lots of control human serum

^b Extraction recovery was calculated by dividing the mean peak height of analyte spiked before extraction by the respective mean peak height of analyte spiked after extraction and multiplying by 100.

^c Matrix effect was calculated by dividing the mean peak height of an analyte spiked after extraction by the mean peak height of the neat analyte standard and multiplying by 100.

Table 4-3 Intra-day Analysis of Serum QC Samples Containing Vitamin D₃

In-tube LLE (Method A)	Low QC	Middle QC	High QC
Nominal Conc. (ng/mL)	1.5	12.5	22.0
Mean Calculated Conc. (ng/mL), n=5	1.4	11.5	21.3
Accuracy ^a (%)	96.0	92.0	96.8
%C.V. ^b	9.6	2.5	2.8
96-well Plate LLE (Method B)	Low QC	Middle QC	High QC
Nominal Conc. (ng/mL)	1.5	12.5	22.0
Mean Calculated Conc. (ng/mL), n=5	1.6	12.8	23.9
Accuracy ^a (%)	103.6	102.0	108.4
%C.V. ^b	3.0	6.0	3.3
In-tip SPME (Method C)	Low QC	Middle QC	High QC
Nominal Conc. (ng/mL)	15	125	220
Mean Calculated Conc. (ng/mL), n=5	16.0	132.0	237.0
Accuracy ^a (%)	106.7	105.6	107.7
%C.V. ^b	4.4	7.0	5.8

^a Expressed as [(mean calculated concentration)/(nominal concentration)] × 100%.

^b Expressed as coefficient of variation (%C.V.).

4.3.7 Methods Comparison

The LLOQ of 0.5 ng/mL was achieved using both in-tube and 96-well plate LLE methods, however, in the 96-well plate LLE method, only 0.4 mL of human serum needed to be processed instead of 1 mL required in the in-tube LLE method. Both procedures were validated in the same concentration range of 0.5 - 25 ng/mL. In the in-tip SPME method, the LLOQ was 5 ng/mL but only 0.1 mL of serum was required. The method C was validated in the concentration range of 5 to 250 ng/mL. The linearity of the calibration curves, intraday precision, and accuracy were satisfactory in all methods. Recoveries of analytes using in-tube LLE were above 80% compared to about 70% in the 96-well plate LLE. The recoveries in both LLE methods were much higher than those from the in-tip SPME (~30%). However, the overall sample preparation time was decreased from 9 hours per 96 samples required in method A to about 3 hours in Method B and 2 hours in method C with much less labor efforts involved. The matrix effects in different human serum samples from the three methods were also examined. Since a stable isotope-labeled internal standard was used in all methods, a potential “relative” matrix effect on ionization was shown not to have any adverse effect on the quantitation of Vitamin D₃ in different serum lots. Based on the excellent intra-day precision and accuracy results obtained in all methods that were obtained using five different lots of control human serum, any differences in the “absolute” matrix effect on ionization or any difference in derivatization efficiency did not have any significant effect on the precision, accuracy, and the reliability of the analysis. This was confirmed by the analysis of subjects’ samples using methods A and B. Both methods generated very similar concentrations data (Figure 4-8).

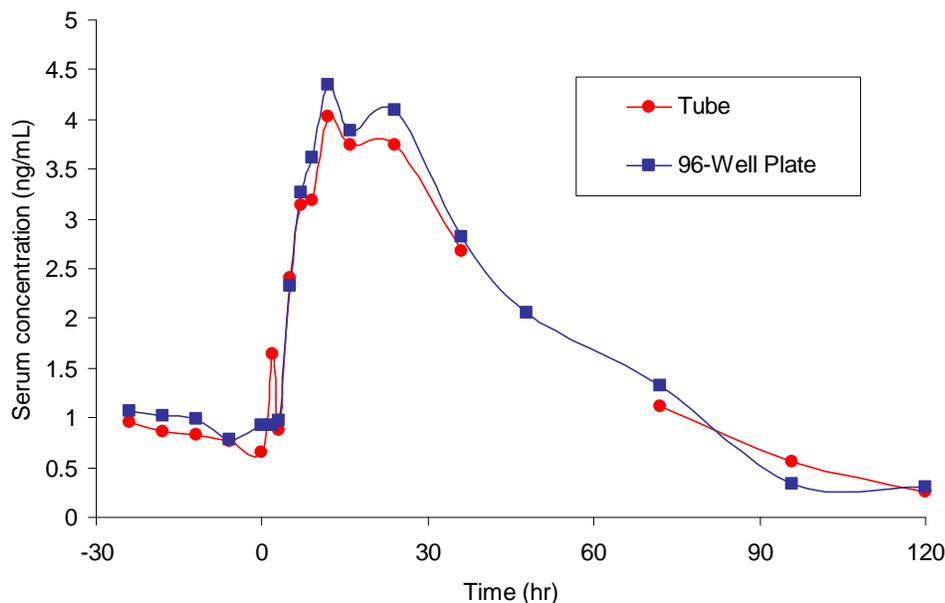


Figure 4-8 Concentration-time profile of vitamin D₃ in serum of a healthy subject (A) after single-dose administration of 70 µg of vitamin D₃

Matthews *et al*¹⁴⁵ used standard models of noise to develop a method that evaluates ion-current ratio noise (i) that varies with the signal intensity and (ii) that is signal dependent. An empirical equation to predict the standard deviations of sample measurements using stable isotope-labeled internal standard was derived as follows:

$$\sigma_T = \left[R^2 \alpha t_{tl} \left(\frac{1}{A_a t_a} + \frac{1}{A_b t_b} \right) + \sigma_C^2 \right]^{1/2} \quad (\text{equation 1})$$

where σ_T is the total variance of a mass spectrometric ion-current measurement and σ_C is the constant noise. R ($R = A_a/A_b$) is the measured ratio of two species, a and b. A_a and A_b are integrated peak areas for a and b, respectively, usually in arbitrary unit; t_{tl} , t_a , and t_b are the total cycle times, dwell times for ion beam a and b, respectively. α is a

normalization factor that accounts for the relationship between ions collected and the instrument-reported peak area. Among these terms in the above equation, σ_C and α are constants and can be determined from two or more points that satisfy the equation and the rest of the terms are either known or can be measured. Because σ_C and α are related to the noise contribution from signal dependent and constant noise sources, respectively, these constants can be applied to define the precision of an ion-current ratio measurement for the instrument and method used. These constants can also be used to predict the precision for other ratio measurements to be measured by the same system or to compare the performance of two different systems.

In order to compare the effect of signal intensity, ion-current ratio magnitude, and internal standard on the measurement precision in Methods A and B, a reference compound **V** and its deuterated internal standard (D_8 -**V**) were used to determine σ_C and α . The ion-current integrated areas (A_a , A_b) and the ion-current ratio ($R = A_a/A_b$) were measured for the reference compound and its internal standard using the same Sciex API 3000 mass spectrometer as used for the Vitamin D₃ determination. Using equation 1, the following results were obtained, $\alpha = 1.02 \times 10^4$ and $\sigma_C = 2.88 \times 10^{-2}$. Both five calibration curves from validation runs in methods A and B were used to evaluate the effect of sample size (ion-current intensity, A) and ion-current ratio (R) on the precision of ion-current measurements. Representative plots from the in-tube LLE method A are shown in Figures 4-9 and 4-10. The values σ_C and α obtained from the reference compound were used to calculate σ_T as a function of the ion-current ratio from the above equation. Contour lines for $1\sigma_T$ and $2\sigma_T$ were then inserted into the figures as broken

and solid lines, respectively. If equation 1 accurately models the noise of the ion-current ratio measurement, then 68% of the points in Figures 6a and 6b should fall within $1\sigma_T$ and 95% within $2\sigma_T$. Experimentally, in terms of the effect of ion-current ratio on the ion-current ratio precision, for method A, 71% of the data points lie within the $1\sigma_T$ contours and 93% lie within the $2\sigma_T$ contours; while in method B, the corresponding numbers are 64% and 87%, respectively. In terms of the effect of ion-current intensity on the ion-current ratio precision for method A, 67% of the data points lie within the $1\sigma_T$ contours and 90% lie within the $2\sigma_T$ contours while in method B, the similar numbers are 63% and 84%, respectively. The standard deviations predicted by the empirical equation are very close to standard deviations of samples measured experimentally in both methods during methods validation. However, in method A, relative error increases when $R < 0.1$ or $R > 2.5$, while in method B, the relative error increases when $R < 0.25$ or $R > 2$, which indicates that statistically, the possibilities of measurement errors are greater in the method B in comparison with method A. The lower recoveries of analytes and larger "absolute" matrix effect are due to relatively dirty extracts that could all make quantitation more prone to errors in method B than in method A.

Although method C did not meet the assay sensitivity requirement for clinical sample analysis after dosing with 70 μg of **I**, the advantages of using SPME to support PK studies at higher doses of **I** were also evident. First of all, the procedure is simple and organic solvent consumption is far less than that of the two LLE, methods A and B. Less than 500 μL of solvent was used during the extraction and desorption process in method C, compared with more than 17 mL in method A and 2.56 mL in method B. Secondly, the sample preparation time was significantly reduced because of the use of a simple and

automated SPME process. Our studies further demonstrated that as an alternative approach for routine analysis of **I**, SPME is more suitable for PK studies when dose levels are relatively high and the concept of coupling the in-tip SPME with automated liquid handling system has proven to be a suitable direction for SPME automation in liquid chromatography both in terms of automation of fiber coating procedure and sample analysis.

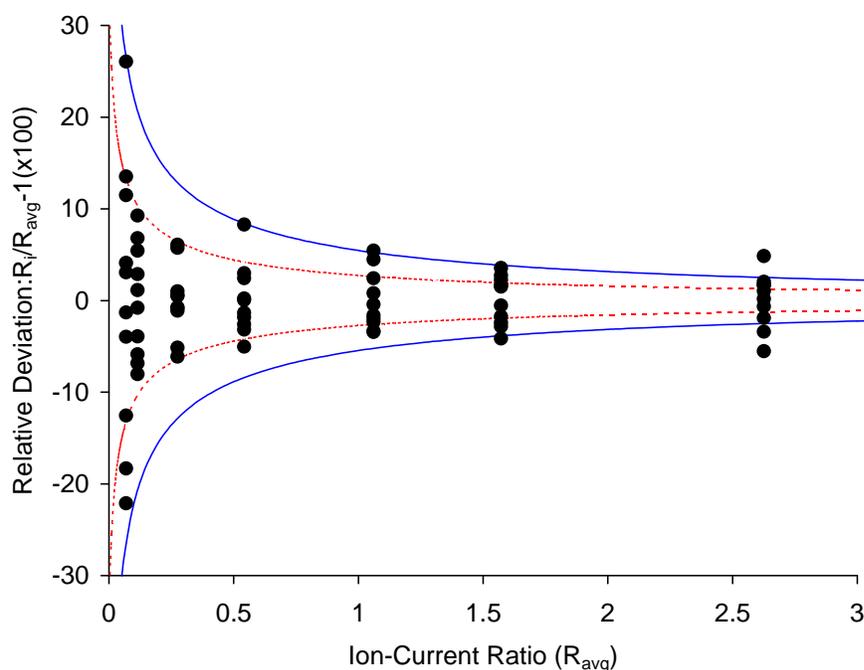


Figure 4-9 Effect of ion-current ratio (R) on the ion-current ratio precision. The mean ratio for each sample was determined (R_{avg} , values displayed on the x-axis). The y-axis plots the relative deviation of each measurement from the mean value of each sample. The broken and solid lines are the theoretical $1 \sigma_T$ and $2 \sigma_T$ envelopes, respectively, calculated using the empirical equation 1

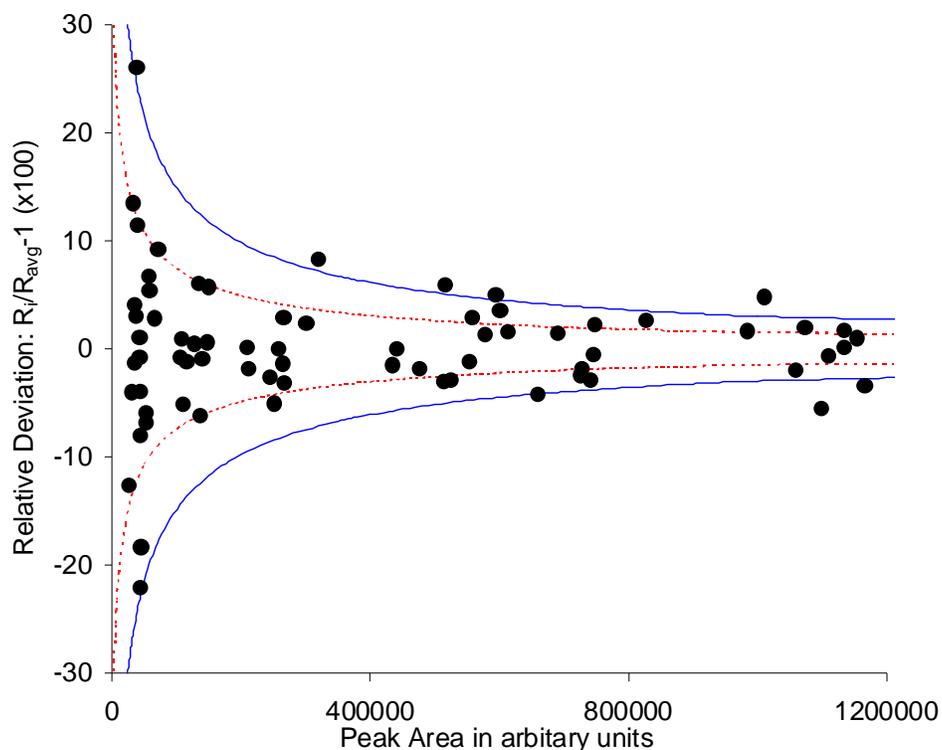


Figure 4-10 Effect of sample size (ion-current intensity) on the ion-current ratio precision. The x-axis shows the peak area of each sample. The y-axis plots the relative deviation of each measurement from the mean value of each sample. The broken and solid lines are the theoretical $1\sigma_T$ and $2\sigma_T$ envelopes, respectively, calculated using the empirical equation

4.3.8 Clinical Sample Analysis

Both methods A and B were applied for the determination of **I** in more than 400 serum samples from a clinical study in which healthy subject received single oral dose of 70 μg of **I**. Inter-day precision and accuracy of the method for the clinical samples analysis were determined by analyzing QC samples at low, medium, and high concentrations. Table 4-4 demonstrates the mean values, method precision and accuracy

for QC samples prepared before the analysis of the study samples, and for QC samples analyzed in replicate with the daily runs of clinical samples. The precision for daily runs (%C.V.) was less than 8.7% with accuracy ranging from 98.9-99.9%.

Table 4-4 Inter-day Analysis of Serum QC Samples from a Clinical Study

	Low QC (ng/mL)	Middle QC (ng/mL)	High QC (ng/mL)
Daily Results:			
Run 1	1.38	11.8	20.8
	1.46	11.6	22.1
Run 2	1.59	13.1	22.1
	1.66	13.0	22.7
Run 3	1.40	12.3	21.8
	1.63	12.9	22.6
Run 4	1.45	12.3	21.5
	1.30	12.9	21.3
Initial Mean (n=8)	1.48	12.5	21.9
Accuracy ^a (%)	98.9	99.9	99.4
%C.V. ^b	8.7	4.7	3.0

^a Expressed as [(mean calculated concentration)/(nominal concentration)] × 100%.

^b Coefficient of variation.

4.4 Conclusions

Selective and sensitive HPLC-MS/MS methods using in-tube LLE, 96-well plate LLE, and in-tip SPME with derivatization were developed and validated for the determination of vitamin D₃ in human serum. The use of chemical derivatization was necessary to improve analyte ionization efficiency, detection selectivity, and assay sensitivity in the presence of biological matrix and in the presence of metabolites. Among the three validated methods, the in-tube LLE method A, although tedious and time

consuming, provided better accuracy and precision than the 96-well plate LLE and in-tip SPME, and was chosen for the determination of vitamin D₃ in human serum after dosing human subjects with low oral doses of 70 µg of vitamin D₃. The 96-well plate LLE method B increased sample throughput and provided comparable assay accuracy, precision, and the same LLOQ (0.5 ng/mL) as Method A. Method B required lower sample volume (0.4 mL) in comparison with method A (1 mL). The in-tip SPME coupled with automated liquid handling system provided a new alternative approach for high throughput routine drug analysis. For the first time, a simple, fast, and high throughput method was developed for preparing monolithic in-tip SPME fibers using photopolymerization. These disposable fiber tips completely eliminated the carryover effect and unnecessary pre-conditioning steps associated with the use of non-disposable fibers. The feasibility of using the automated SPME-HPLC-MSMS as an alternative approach in bioanalysis was demonstrated for supporting PK studies at higher doses of analytes.

Chapter 5

Applications of In-tip SPME (Part II): HILIC-MS/MS for the Determination of Three Polar Compounds, IMP, CIL and BLI (MK-4698) in Biological Fluids

5.1 Introduction

One of the major recent fundamental advances in bioanalytical applications of SPME is the development of high throughput SPME using multi-well plate technology.⁶⁴ Traditionally, in bioanalytical analysis where LC is selected as the main separation technique due to the non-volatile and/or polar properties of the drug compounds, interfacing of LC and SPME has included such strategies as manual injection interface tees, in-tube SPME, and off-line desorption followed by a conventional liquid injection. Neither manual desorption interface or off-line desorption allows automated sample preparation as all of the SPME steps are performed manually with single or multiple fibers making it impractical to simultaneously process a large number of samples. In-tube SPME permits a high degree of automation using commercially available HPLC autosamplers, however, each sample is still processed serially resulting in low overall throughput. An automated SPME system has been developed recently by PAS Technology, which consists of a three-arm robotic autosampler that is fully controlled with Concept software and two orbital agitators. The system is capable of performing all sample preparation automatically including the addition of internal standard, multi-fiber SPME extraction and desorption with controlled agitation, solvent evaporation and reconstitution, as well as final sample injection. Some very promising results have been obtained using this instrument such as the determination of ochratoxin A in human urine

with method precision less than 14% and accuracy ranged from 91 to 114%;¹⁴⁶ and more recently, an automated SPME-LC-MS/MS method for the analysis of four benzodiazepines in whole blood has been fully validated with a low limit of quantification (LLOQ) of 4 ng/mL.⁷⁸ However, there are some major drawbacks of the system, large sample volume and desorption solvent (800-1000 μ L) is required in order to achieve acceptable precision and accuracy due to the geometric design of the SPME multi-fiber device, the biggest advantage of solvent-less over other sample preparation methods is abandoned. In addition, the claimed high throughput is questionable when multiple plates are processed as sample extraction, desorption, and injection could not be performed simultaneously. A new concept of in-tip SPME has been introduced recently in the development of automated high throughput SPME methods in bioanalysis.¹⁴² The in-tip SPME technique takes the advantage of widely used commercially available automated liquid handling systems and couples the fiber SPME with the system in a unique configuration. In-tip SPME maintains the simplicity and advantages of traditional SPME approaches and is easily adopted for automation without introducing additional devices. More importantly, the approach is amenable to all fiber types possessing a wide range of different coating materials, which will overcome the drawback of limited selections of commercial available fibers and broaden its use with HPLC.

Primaxin®, a combination of the carbapenem IMP and the renal dehydropeptidase inhibitor, CIL, has for many years been used as a potent antibacterial agent for the treatment of serious infections because of its broad spectrum of antimicrobial activity.¹⁴⁷⁻

¹⁵⁰ Unfortunately, Gram-negative pathogens that are resistant to all the beta-lactam agents, including Primaxin®, as well as to all other classes of antimicrobial agents, have

emerged as an urgent problem in the hospital. One of the resistance mechanisms is the high-level expression of β -lactamase that inactivates β -lactam antibiotics by catalyzing the hydrolysis of the β -lactam amide bond to produce a ring-opened structure. MK-4698 (BLI) is being developed as a novel, non- β -lactam, phosphonate-based β -lactamase inhibitor¹⁵¹ for combination with Primaxin to restore the activity of imipenem and increase coverage and efficacy against multi-drug resistant Gram-negative pathogens. The historical methods to analyze Primaxin® were reported as separate assays for the two analytes IMP and CIL, respectively. For IMP, several methods that have been reported are based on either microbiological assays^{152,153} which cannot differentiate IMP from other coadministered antibiotics, or HPLC with UV detection after either ultrafiltration^{154,155} using relatively large volume of samples, or, most recently, protein precipitation^{156,157} with limited dynamic ranges (≤ 100 fold). For CIL, HPLC methods that involved solid phase extraction using C18 cartridge, and then reversed-phase chromatography followed by either post-column derivatization for fluorescence detection¹⁵⁸ or direct UV detection,¹⁵⁹ have been described in determination of the analyte in biological fluid. The methods involved complex procedures or provided narrow dynamic ranges (≤ 100 -fold) with ≥ 0.75 $\mu\text{g/mL}$ limit of detection.

In support of the β -lactamase inhibitor program, one of our objectives is to develop a fast and sensitive simultaneous assay for all three analytes – IMP, CIL, and BLI. Clearly, such a method is more desirable than the individual determination of each substance because of the low sample amount available in preclinical studies and timesaving analysis for fast data turn around. This paper describes a newly developed HILIC-MS/MS method using in-tip SPME for simultaneous determination of three polar,

non-structurally related compounds – IMP, CIL, and BLI in biological fluids. In the meantime, the in-tip SPME assay is compared to a different sample preparation method using PPT in terms of assay sensitivity and selectivity, recovery and matrix effects, and the advantages and limitations of in-tip SPME for high throughput drug analysis in drug discovery environment are discussed.

5.2 Experimental

5.2.1 Chemicals and Materials

IMP, CIL, MK-4698, and internal standards (ISa and Isb, Figure 5-1) were received from the Merck Research Laboratories, Merck & Co. (West Point, PA). HPLC grade ACN, laboratory grade formic acid (90%), and ammonium acetate were purchased from Fisher Scientific (Pittsburgh, PA, USA). Ethylene glycol, 2-(N-morpholino) ethanesulfonic (MES) acid, and MES sodium salt were obtained from Sigma-Aldrich (Milwaukee, WI, USA). Ethyl alcohol was purchased from Quantum Chemical Corporation (Tuscola, IL, USA). Control plasma (EDTA as anticoagulant) from Sprague Dawley rats was purchased from Bioreclamation Inc. (NY, USA). Mouse control blood was obtained from Merck Research laboratories. Water was purified by a Milli-Q ultra-pure water system from Millipore (Bedford, MA, USA). PDMS-DVB fibers (60 μ m) were purchased from Supelco (Bellefonte, PA, USA).

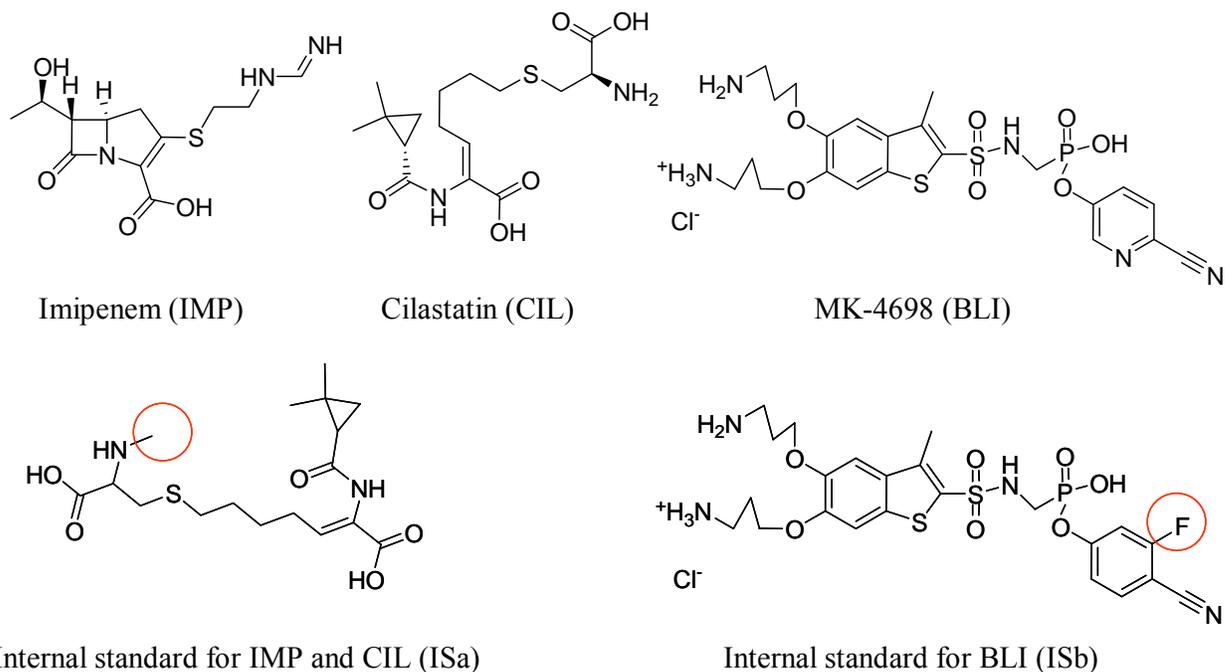


Figure 5-1 Chemical Structures of IMP, CIL, BLI (MK-4698) and the internal standards ISa (for IMP and CIL) and ISb (for BLI).

5.2.2 LC-MS/MS

A HPLC micro pump (Series 200 from Perkin Elmer, Ontario, Canada) coupled with a 96-Well Plate Autosampler (HTS PAL System from Leap Technology, Carrboro, NC, USA) was used to perform the HPLC separation. A PE Sciex API 4000 triple-quadrupole mass spectrometer (Sciex, Toronto, Canada) with a TIS interface ionization source operated in a positive ion mode was used to quantitate all analytes. The samples were analyzed on a Waters Atlantis HILIC Silica (50mm x 2.1mm, 3 μ) column with a mobile phase composed of 15 mM ammonium formate (pH 3) in 80% ACN at a flow rate of 0.4 mL/min. Analytical run time was 4 min/run. A divert valve was used to control the

acquisition window which was open between 0.6 and 4 minutes after injection. The column was maintained at room temperature and the autosampler was set at 5°C. The instrument settings were adjusted to maximize the response for the analytes and their internal standards, respectively, using analyte infusion in the presence of mobile phase. The ion pairs (precursor ion → product ion) selected for MRM, optimized declustering potential (DP), collision energy (CE), collision-cell exit potential (CXP), and entrance potential (EP) for each analyte are summarized in Table 5-1. A voltage of 4.5 kv was applied to the sprayer. The turbo gas temperature was 550°C and the auxiliary gas flow was 60 L/min. The flow settings of nebulizing gas (nitrogen), collision gas (nitrogen), and curtain gas (nitrogen) at the instrument were 40, 7 (CGT = 2×10^{15} molecules per cm^2), and 45 L/min, respectively. The dwell time was 100 msec for each analyte and internal standard. Both Q1 and Q3 quadrupoles were set at unit resolution. Peak area ratios were calculated using Analyst software version 1.4. Calibration curves were obtained by weighed ($1/x^2$) least squares linear regression of the peak area ratio of the analyte to the internal standard versus the nominal concentration (x) of analyte. The internal standard ISa was used for qualification of IMP and CIL and ISb was used for BLI. In-tip SPME extraction, desorption, and liquid transfer in PPT sample preparation was performed using a Tomtec Quadra 96 workstation (Hamden, CT, USA).

Table 5-1 Mass Spectrometry Conditions for IMP, CIL and BLI, Respectively

Analytes	Ion Transition, <i>m/z</i> (precursor → product)	DP (V)	CE (V)	CXP (V)	EP (V)
IMP	300.2 → 142.0	53	36	12	10
CIL	359.3 → 202.4	55	21	4	10
BLI	570.1 → 236.2	91	55	6	10
Internal Standard					
Isa	373.3 → 233.1	55	26	14	10
ISb	587.2 → 236.0	86	55	6	10

5.2.3 Preparation of Stabilizing Solution

The stabilizing solution (traditional stabilizer) was prepared by combining 1M MES (pH 6.0) buffer with 50% ethylene glycol at 1:1 (v/v) ratio, while 1M MES was prepared by dissolving 10.88g MES acid (molecular weight = 195.2) and 9.62g MES sodium salt (molecular weight = 217.2) in a 100 mL mill-Q water, without pH adjustment.

All study samples were treated with stabilizer and stored at -70°C before analysis. For mouse blood samples, the stabilizer was added at 1:1 (v/v) ratio to the unknown samples. For rat plasma samples, since an additional component, 50% acetonitrile, was necessary to stabilize BLI, the final treatment of sample was in the format of rat plasma sample: stabilizer: 50% acetonitrile at 1:1:1 (v/v/v) ratio.

5.2.4 Preparation of Standards and QC Samples

Two stock solutions in ACN/water (50/50, v/v) for each of the analytes, IMP, CIL, and BLI, at 2 mg/mL were prepared from two separate weighing. One set of analyte stock solutions was used to prepare calibration standards, and the other set was used to make QC samples. The secondary standard stock, a solution containing 500 µg/mL of each

analyte, was prepared by mixing 250 μL from each primary standard stock of IMP, CIL, and BLI with 250 μL of Milli-Q water. Working standards were prepared by serial dilutions of the secondary standard stock with ACN/water (50/50, v/v), and stored in amber glass vials at 4°C. All solutions were used within three days after preparation, except for the primary stocks of MK-4698 and CIL which could be used for up to two weeks. An internal standard stock solution at 1 mg/mL in ACN/water (50/50, v/v) was prepared for ISa and Isb, respectively. The IS working solution including both ISa and Isb, was prepared either at 1 $\mu\text{g}/\text{mL}$ in 50% ACN for in-tip SPME or at 250 ng/mL in 100% ACN served as the protein precipitation solvent for PPT method. Due to stability issue, each control matrix (rat plasma and mouse blood) was mixed with an equal volume of stabilizer – 1 M MES (pH 6): 50% ethylene glycol = 1:1.

Rat plasma calibration standards were prepared daily by adding 25 μL of working standard and 50 μL of stabilizer-containing control plasma (equivalent to 25 μL control plasma and 25 μL stabilizer) to provide the final concentrations of IMP, CIL, and BLI at concentrations ranged from 0.5 to 100 $\mu\text{g}/\text{mL}$ for in-tip SPME and 0.1 to 100 $\mu\text{g}/\text{mL}$ for PPT. Mouse blood calibration standards were prepared daily by mixing 40 μL stabilizer-containing control blood with 20 μL working standard solutions to provide the final concentrations of IMP, CIL, and BLI at concentrations ranging from 0.5 to 100 $\mu\text{g}/\text{mL}$ for in-tip SPME and 0.1 to 100 $\mu\text{g}/\text{mL}$ for PPT.

The QC samples for rat plasma or mouse blood were prepared and tested at each of the five concentrations: lowest, second lowest, mid-range, second highest, and the highest standards of IMP, CIL, and BLI in their corresponding matrixes containing appropriate stabilizer (as specified in Section 2.3). Stability QCs were prepared at 0.5, 50,

and 1000 [10x upper limit of quantification (ULOQ) with <2% organic solvent in the final matrix] $\mu\text{g/mL}$, stored in a -70°C freezer for at least overnight, and then placed at room temperature for at least 4 hours before conducting stability tests.

5.2.5 In-tip SPME

Different types of coatings were used and compared including PDMS-DVB, C18 and C30 phase silica-based coatings, and Oasis HLB-coated polymer monoliths. In-tip SPME extraction and desorption process for all experiments was fully automated using a Tomtec Quadra 96 workstation. The preparation of these in-tip SPME fibers and the detailed sample process procedures were described previously.¹⁴²

For rat plasma samples, since there is a special need for an extra stabilizer – 50% ACN right after sample collection, an aliquot of 75 μL stabilizer-treated rat sample (equivalent to 25 μL plasma with 25 μL stabilizer and 25 μL 50% acetonitrile) was transferred into a plate, without the addition of make-up solvent, (because the extra volume of 50% ACN in the sample matched the solvent volume for working standards in the calibration curve). The sample mixture was then vortex-mixed for about 1 minute and placed on the deck of a Tomtec Quadra 96 workstation for SPME preparation. The Tomtec Quadra 96 workstation was programmed as such that the whole process was running in a sequence of tip loading, extractions, washing, and desorption. The sample extraction and desorption process was accomplished through repeated aspiration and dispensing of the sample solution and desorption solvent (100% ACN), respectively. The total extraction time was about 40 minutes with 5 minutes desorption time. 15 μL of desorption solution was directly injected to the HPLC-MS/MS system for analysis.

Mouse blood samples were prepared in a similar way as those of rat plasma samples except that 40 μL of samples (equivalent to 20 μL blood with 20 μL stabilizer) were transferred, due to the limited sample volume available. The blood samples were mixed with 20 μL of 50% acetonitrile (make-up volume to match standards) before SPME preparation.

5.2.6 PPT

In PPT, for rat plasma samples, an aliquot of 75 μL stabilizer-treated rat samples was transferred into a plate, protein crashed with 250 μL of the IS working solution without making-up solvent, vortexed for ~ 3 minutes, and centrifuged at 10°C , 3500 rpm (2000 RCF, relative centrifugal force) for 5 minutes. A 10 μL supernatant was injected into LC-MS/MS system for analysis. For mouse blood, an aliquot of 40 μL stabilizer-treated mouse samples was transferred and mixed with 20 μL ethanol and 20 μL 50% ACN (make-up volume to match standards) in each micro sample tube to allow for the complete dialysis of blood cells. A 250 μL of the IS working solution was then added to each sample mix for protein precipitation, followed by the same procedures for centrifugation and injection as indicated for rat plasma samples above.

5.2.7 Post-column Infusion

Post-column infusion experiments were conducted in this study to investigate matrix effects of phospholipids in rat plasma. The plasma supernatant solution was prepared by mixing pooled blank rat plasma with ACN in a ratio of 1:3 following centrifuged for 5 min at 3500 rpm. The supernatant was removed and diluted with water

in a 1:1 ratio. The analyte solution, prepared at a concentration of 1 µg/mL of IMP, CIL, and BLI in 50% ACN, was post-column infused from the syringe pump to the LC effluent at a flow rate of 60 µL/min. After the signals of the analyte MRM transitions were stable, the plasma supernatant solution (10 µL) containing phospholipids was injected onto the column at the same chromatographic conditions of the analytes and the analytes post-column infusion spectra were acquired. Phospholipids were detected with a positive precursor ion scan of m/z 184 which resulted in total ion chromatograms to qualitatively monitor all phospholipids in rat plasma.

5.2.8 Method Validation (Precision, Accuracy, Stability, Recovery, and Matrix Effects)

The precisions of in-tip SPME and PPT methods were determined by using replicate analysis (n=5) of drug compounds in five different sources of rat plasma at all concentrations utilized for the construction of calibration curves. Due to limited sources of mouse blood, triplicate analysis (n=3) was performed in three pooled lots of mouse blood. The linearity of each calibration curve was confirmed by plotting the peak area ratio of the drug to internal standard *versus* drug concentration. The unknown sample concentrations were calculated from the equation $y = slope * x + intercept$, as determined by weighted ($1/x^2$) linear regression of the standard curve. The accuracies of the methods were determined as the percentage between the mean concentrations observed and the nominal concentrations. The precision and accuracy of the methods as measured by the coefficient of variation (%CV) were required to be <20% at the concentrations determined. The intra-day accuracy and precision was determined by analyzing six replicates of QC samples at each of the five concentrations: lowest, second lowest, mid-

range, second highest, and the highest standards. F-T and room temperature stabilities were evaluated jointly using QC samples (at low, high, and 10x high concentrations) that went through a freezing-and-thawing cycle with at least one overnight storage at -70°C and at least 4 h at room temperature.

Extraction recovery and matrix effects were evaluated for IMP, CIL, and BLI in rat plasma. Recovery was determined by comparing the mean absolute peak areas of standards obtained from pre-spiked plasma samples prepared from different plasma sources to those from post-spiked plasma samples prepared in plasma from the same different sources. Matrix enhancement/suppression of ionization or "absolute" matrix effect was evaluated by comparing the absolute peak areas of the standards in post-spiked extraction samples to neat standards injected in the mobile phase. "Relative" matrix effect on ionization was evaluated with an examination of the slopes of five standard curves in five different lots of plasma.

5.3 Results and Discussion

5.3.1 Mass Spectrometry for Simultaneous Determination of Three Zwitterions

All three analytes, IMP, CIL, and BLI, are zwitterions that could be detected under either positive or negative ionization mode, while BLI and CIL have the potential to be doubly charged under the appropriate conditions. Establishing mass spectrometry conditions for the simultaneous determination of these compounds was a challenge. Since the charge state correlates with the buffer pH relative to the pKa of the functional groups in a compound structure, MS response was evaluated by infusion of each compound under different mobile phases, including MP1 (50% ACN in water), MP2 (50% ACN in 5

mM ammonium formate buffer, pH 3), and MP3 (50% ACN in 5 mM ammonium acetate, pH 5), to assess the charge state and signal intensity of the corresponding precursor → product mass transition under different ionization modes. The results showed that IMP and CIL strongly preferred positive ionization mode regardless of the mobile phase choices as defined above; while BLI gave similar MS response under both singly charged positive and negative ionization modes regardless of the pH of mobile phases. The doubly charged positive ionization of BLI was extremely sensitive to the change of mobile phase, causing dramatic signal loss when the mobile phase was switched from MP1 to MP2 or MP3. Considering the sensitivity and stability of ionization for all compounds, positive ionization mode was chosen to detect all analytes. The relatively stable MS response under different mobile phases (pH 3 vs. pH 5) provided flexibility for selecting HPLC conditions.

The product ion scan spectra of the MH^+ ions of the analytes – IMP, CIL, BIL, ISa, and ISa – are presented in Figure 5-2 and the final MRM transitions used for the three analytes and two internal standards are summarized in Table 5-1.

Potential interferences among the five compounds due to impurities in standard materials and cross talk among ion channels were examined by injecting a neat solution containing one compound at its highest working concentration and monitoring all five MRM channels. Based on this assessment, no cross-talk or interference was observed.

5.3.2 HILIC Chromatography Conditions

IMP, CIL, and BLI are highly polar compounds. Most of the previously published HPLC methods for IMP and CIL were performed on C8 or C18 reversed phase material

using mobile phases containing borate or phosphate buffers.¹⁵⁴⁻¹⁵⁹ Even at as low as 10% acetonitrile or methanol in mobile phase, no sufficient retention was observed, especially for IMP. Those conditions could not be applied to a LC-MS assay because the mobile phases were not compatible with a mass spectrometer. Column evaluation on Thermo Scientific FluroPhase PFP and Phenomenex Luna CN under different mobile phase pHs (3 or 5) indicated that the tested conditions worked for one or two of the three analytes, but not for all.

HILIC has become a powerful technique for the retention of polar analytes because of its excellent mobile phase compatibility and complementary selectivity to RP chromatography.¹⁶⁰⁻¹⁶⁴ Evaluation of a Waters Atlantis HILIC Silica column (50mm x 2.1 mm, 3 μ) for IMP, CIL, and BLI resulted in favorable retentions. The elution conditions were subsequently optimized by evaluating organic content, pH, and salt concentration in mobile phase. Higher percentages of ACN caused longer retention; pH 5 gave more retention, but a broader peak and less sensitivity in comparison to pH 3 in the presence of same ACN content; higher salt concentration resulted in a shaper peak, but more ion suppression on the mass spectrometer. To balance the retention, peak shape, and sensitivity among all three analytes and their corresponding internal standards, an isocratic elution using 15 mM ammonium formate (pH 3) in 80% ACN at 0.4 mL/minute flow rate was selected, and the total run time under this isocratic condition was 4 minutes per injection. Figure 5-3 shows a typical chromatogram of a sample containing IMP, CIL, and BLI with capacity factors (k') of 4.2, 2.3, and 8.6, respectively.

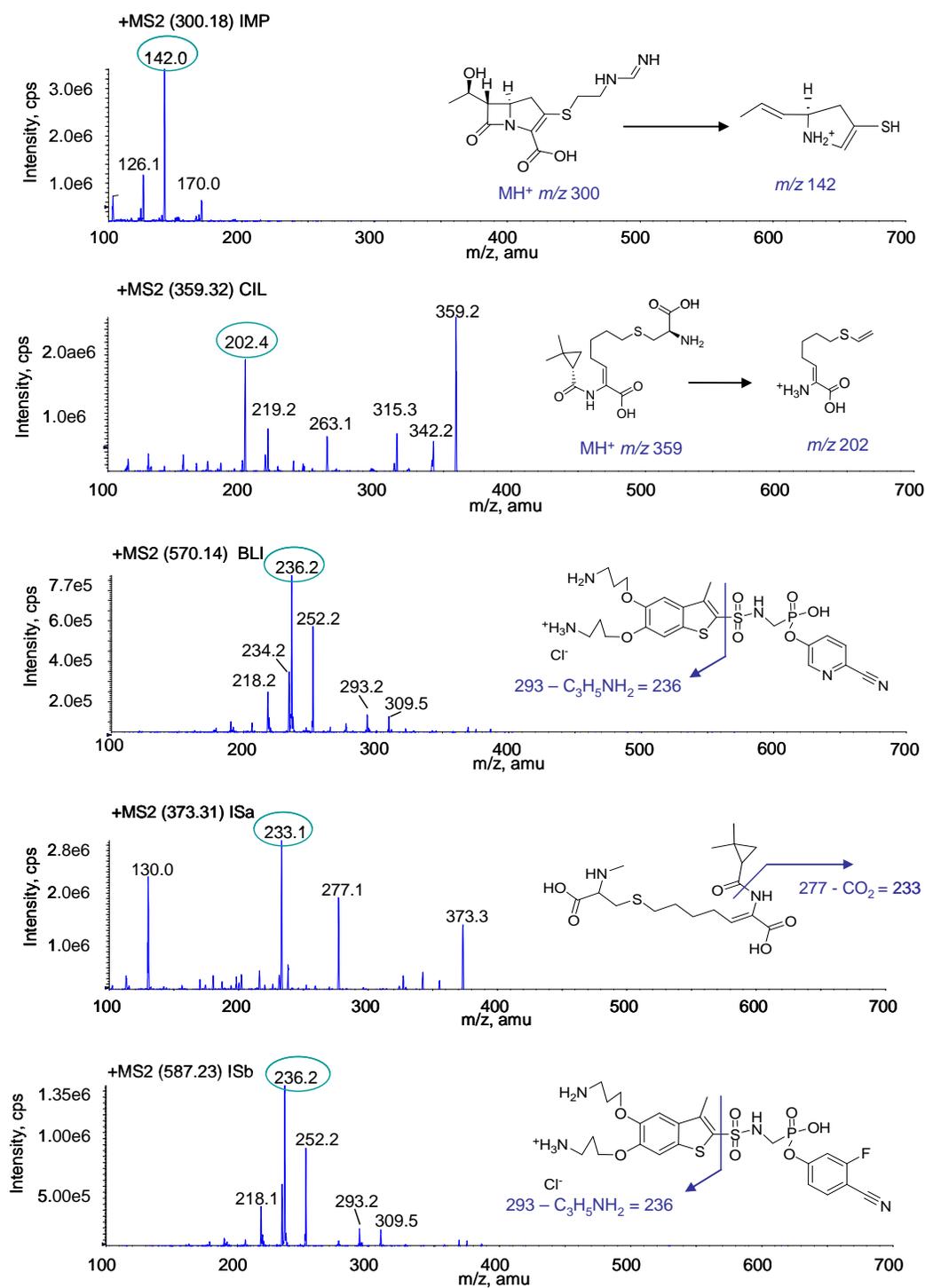


Figure 5-2 Product ion spectra of MH^+ ions of IMP, CIL, BLI, ISa and ISb

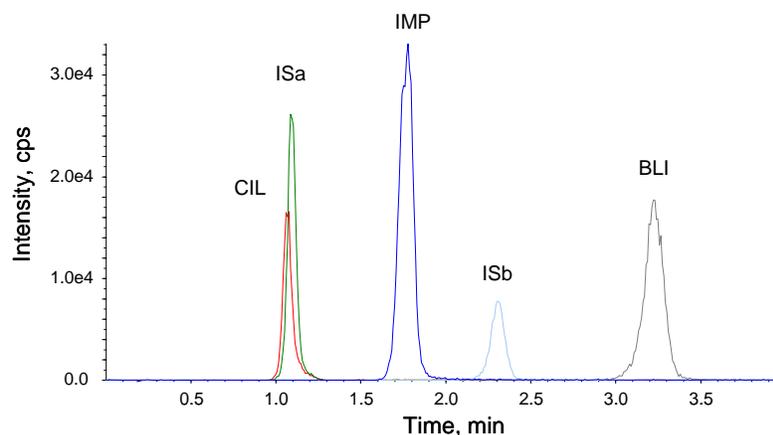


Figure 5-3 Representative chromatograms on Waters Atlantis HILIC column using isocratic mobile phase for the separation of IMP, CIL, BLI and their corresponding internal standards.

5.3.3 Stability of IMP, CLI and BLI in Biological Fluids

As previously described, all samples have to be stabilized immediately to avoid analyte loss by hydrolysis. IMP is most stable at neutral pH; the most effective stabilizing buffers for storage of IMP in plasma samples were the zwitterionic buffers, such as 2-(N-morpholino)ethanesulfonic (MES) at pH 6.0, 3-morpholino-propanesulfonic acid (MOPS) at pH 7.2, or 4-(2-hydroxyethyl) piperazine-ethanesulfonic acid (HEPES) at pH 7.0. Ethylene glycol is essential to the stability of imipenem for long-term storage because it disrupts the organized structure that leads to breakdown of β -lactams in the frozen state. Stabilizers using 0.5 to 1 M buffer (MES or MOPS or HEPES) in combination with 50% ethylene glycol at 1:1 (v/v) ratio were reported.¹⁵⁵⁻¹⁵⁸

In this work, the traditional stabilizer for IMP plasma assay – 1 M MES (pH 6.0): 50% ethylene glycol (1:1, v/v) – was adapted and used by mixing with equal volume of biologic samples. This stabilizer worked well for IMP and CIL in all tested biological

fluids, including rat plasma and mouse blood under -70°C . However, the data for BLI in rat plasma indicated an analyte loss at the low BLI concentration, while BLI in mouse blood was stable after storage in a -70°C freezer. To minimize analyte loss for BLI in rat plasma, more stabilizing conditions were investigated. Frozen QCs in rat plasma in the presence of the following stabilizers were compared after a F-T cycle in a -70°C freezer overnight: *Stabilizer #1* (the traditional stabilizer) – 1 M MES (pH6.0) :50% ethylene glycol in water (1:1, v/v); *Stabilizer #2* – stabilizer #1:10% formic acid (1:1, v/v); *Stabilizer #3* – 1 M MES (pH6.0) :50% ethylene glycol in ACN (1:1, v/v); *Stabilizer #4* – premixed stabilizer including stabilizer #1 :50% ACN (1:1, v/v); and *Stabilizer #5* – stepwise addition of stabilizer #1 and 50% ACN to rat plasma sample at 1:1:1 (v/v/v) ratio. The results showed that ~25 – 50% BLI loss in rat plasma with Stabilizer #1, total IMP loss and ~25-50 % BLI loss with Stabilizer #2, ~22% IMP loss with Stabilizer #3, ~15% BLI loss with Stabilizer #4, while Stabilizer #5 gave the best QC accuracy and precision for all three analytes, IMP, CIL, and BLI in rat plasma. Based on these results, stabilizer #5 was selected for the treatment of rat plasma samples.

5.3.4 Comparison of Overall Performance of SPME Coatings

Figure 5-4 demonstrated the comparison of absolute extraction recoveries obtained using different types of SPME coating fibers for the extraction of IMP, CIL, and BLI in rat plasma. The extraction and desorption conditions were kept the same and the recoveries were evaluated at 100 $\mu\text{g}/\text{mL}$ of each analyte. It was found that Oasis HLB polymer monoliths provided much better recoveries from 9 to 16% compared with other tested SPME coating fibers with recoveries of less than 2% in all three analytes.

Although it was impossible to make direct comparisons of fiber coatings between polymer monoliths and other tested fibers with coating dimensions of 1 cm of length and 10-100 μm of thickness, it was believed that the total surface area of polymer monoliths was much larger due to the inner surface area of the flow channel as well as outer surface area of the polymer monoliths. In addition, the homogeneous distribution of Oasis HLB particles in polymer monolithic materials enhanced the weak intermolecular interactions and hydrophobic interactions between analytes and extraction sorbent, which improved extraction efficiency of polymer monoliths over other type of SPME fibers test in the study.

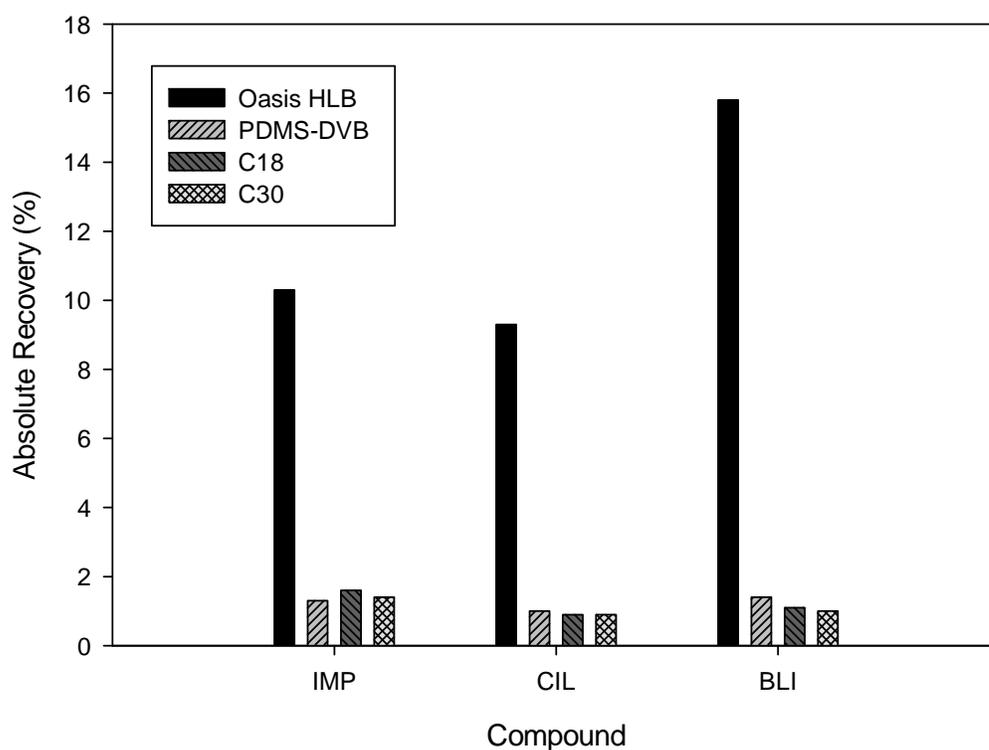


Figure 5-4 Comparison of absolute extraction recoveries obtained using different types of SPME coatings for the extraction of IMP, CIL and BLI in rat plasma.

5.3.5 Methods Validation

Both in-tip SPME and PPT assays were validated according to Merck internal SOPs for non-clinical validation of bioanalytical methods based on LC-MS/MS for non-GLP studies of Preclinical Candidate Compounds (PCCs). Because the independent sources of the same matrix are not always available, assessment of the intraday variability of calibration curve is normally conducted in triplicate with minimum of six standards in pooled or individual sources. However, in order to evaluate matrix effects in both assays, five independent calibration curves were constructed in five different sources of rat plasma, a practice that is commonly used in clinical assay validation. The assay specificity was examined for interferences from endogenous components in control rat plasma and mouse blood as well as predose samples. In all the MRM channels, no interferences were observed within the retention time windows of all analytes.

5.3.5.1 Intraday precision and accuracy

In-tip SPME assay using Oasis HLB-coated polymer monoliths was validated in rat plasma and mouse blood over the concentration range of 0.5 – 100 µg/mL for each analyte (IMP, CIL, and BLI). The resulting method precision and accuracy data is presented in Tables 5-2 and 5-3. In rat plasma, the intraday precision (%CV) of the method was equal to or lower than 12% for all concentrations for each of the analytes, and the method accuracy was found to be 89.9-108.4% of the nominal concentration for the entire standards evaluated. The correlation coefficient for the mean calibration curves constructed from five different sources of rat plasma was 0.9975, 0.9982, and 0.9972 for IMP, CIL, and BLI, respectively. In mouse blood, triplicate calibration curves were

prepared in single pooled blood matrix; method precision was found to be no more than 8.0% with accuracy within $\pm 8.4\%$ of nominal concentration.

The PPT assay was found linear within the range of 0.1-100 $\mu\text{g/mL}$ using the same amount of rat plasma or mouse blood samples as those of in-tip SPME assay. Assay accuracy was found to be 86.6-112.1% of nominal values for IMP, CIL, and BLI across the tested matrices and the intraday precision (%CV) of the method was equal to or lower than 10.2% for all concentrations (Tables 2 and 3). The corresponding LLOQ at 0.1 $\mu\text{g/mL}$ was established based on the criteria that back calculated values ranged within 20% of their nominal values and $<20\%$ precision (%CV) evaluated with different standard curves. The correlation coefficient for the mean calibration curves constructed from five different sources of rat plasma was 0.9992, 0.9982, and 0.9964 for IMP, CIL, and BLI, respectively.

Representative extracted ion chromatograms of LLOQ standard for the three analytes using in-tip SPME are displayed in Figure 5-5.

5.3.5.2 Analytes Stability

For in-tip SPME, QC samples, containing all three analytes, IMP, CIL, and BLI, were prepared at 0.5, 1, 10, 80, and 100 $\mu\text{g/mL}$ in rat plasma and mouse blood, respectively, with their corresponding stabilizers; while for PPT, QC samples were prepared at 0.1, 0.2, 5, 80, and 100 $\mu\text{g/mL}$ in rat plasma and mouse blood, respectively. The initial QC results showed below 20% precision and 80-120% accuracy for all compounds in tested biological matrix for both methods.

Table 5-2 Calibration Curves for the Determination of IMP, CLI and BLI in Rat Plasma Using In-tip SPME and PPT

Nominal Conc. ($\mu\text{g/mL}$)	Accuracy % ^a (n= 5, rat plasma)					
	In-tip SPME			PPT		
	IMP	CIL	BLI	IMP	CIL	BLI
0.1	--	--	--	99.3 [2.5]	95.1 [2.4]	99.0 [3.1]
0.2	--	--	--	99.2 [4.0]	105.6 [4.3]	100.9 [4.7]
0.5	99.5 [4.3]	108.4 [10.9]	103.4 [8.8]	105.0 [5.9]	102.9 [2.3]	99.6 [10.2]
1	97.9 [6.5]	105.6 [4.0]	102.1 [4.3]	100.1 [4.5]	112.1 [4.0]	103.9 [9.8]
2	98.9 [4.3]	102.2 [4.4]	100.6 [4.7]	100.1 [3.8]	105.4 [5.8]	105.2 [5.7]
5	100.7 [6.6]	105.4 [5.2]	104.2 [6.2]	99.9 [1.7]	105.4 [2.7]	104.0 [5.9]
10	99.0 [4.3]	100.4 [3.0]	98.7 [3.6]	98.8 [1.3]	101.6 [4.8]	104.8 [1.8]
20	99.0 [2.5]	99.9 [3.0]	97.8 [2.3]	94.0 [1.7]	98.6 [2.7]	97.7 [3.7]
50	106.1 [4.3]	96.2 [5.8]	96.7 [2.7]	100.6 [5.2]	93.9 [4.1]	101.7 [8.5]
80	102.0 [9.8]	93.1 [7.3]	98.5 [8.2]	100.1 [4.3]	86.6 [5.0]	89.7 [5.4]
100	97.9 [10.1]	89.9 [9.3]	96.9 [12.0]	102.8 [2.4]	89.2 [5.1]	93.7 [6.3]
r ^b	0.9975	0.9982	0.9972	0.9992	0.9982	0.9964
%CV ^c	10.1	8.8	10.4	3.1	5.6	6.0

^a Expressed as [(back calculated concentration)/(nominal concentration)] x 100 (%)

^b Linear regression of peak area ratio of analyte/internal standard vs. concentration (x), $y = \text{intercept} + \text{slope} * x$, using $1/x^2$ weighing factor, with correlation of coefficient (r).

Table 5-3 Calibration Curves for the Determination of IMP, CLI and BLI in Mouse Blood Using In-tip SPME and PPT

Nominal Conc. ($\mu\text{g/mL}$)	Accuracy % ^a (n= 3, mouse blood)					
	In-tip SPME			PPT		
	IMP	CIL	BLI	IMP	CIL	BLI
0.1	--	--	--	99.6 [0.6]	96.8 [1.0]	101.1 [5.6]
0.2	--	--	--	101.1 [2.4]	102.7 [3.0]	99.2 [8.6]
0.5	99.9 [2.5]	105.4 [3.1]	97.1 [7.3]	102.4 [2.6]	104.5 [6.4]	106.0 [3.0]
1	100.4 [1.3]	106.7 [4.6]	103.0 [1.9]	100.7 [1.6]	107.9 [3.7]	109.3 [0.5]
2	96.2 [5.0]	101.3 [3.3]	93.5 [2.9]	99.8 [2.8]	103.2 [5.7]	103.7 [1.6]
5	100.5 [3.3]	101.5 4.9	96.7 [6.3]	102.6 [4.9]	105.1 [2.1]	104.2 [0.6]
10	100.5 [7.1]	101.2 [5.2]	92.7 [6.0]	103.9 [2.8]	106.2 [0.9]	106.1 [3.5]
20	99.7 [6.9]	100.5 [4.5]	103.4 2.8	97.2 [3.2]	99.6 [3.0]	97.6 [1.3]
50	102.9 [0.8]	100.0 [8.0]	104.9 [7.1]	101.9 [2.4]	96.2 4.3	95.4 [0.7]
80	99.2 [2.1]	91.6 [2.1]	101.2 [6.7]	96.8 [2.3]	91.1 [6.2]	90.4 [1.3]
100	100.0 [6.0]	92.4 [4.1]	107.1 [6.8]	96.4 [6.3]	87.4 [4.6]	89.7 [1.5]

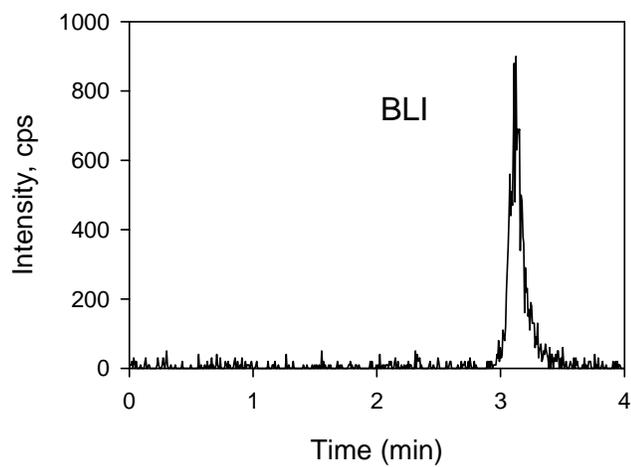
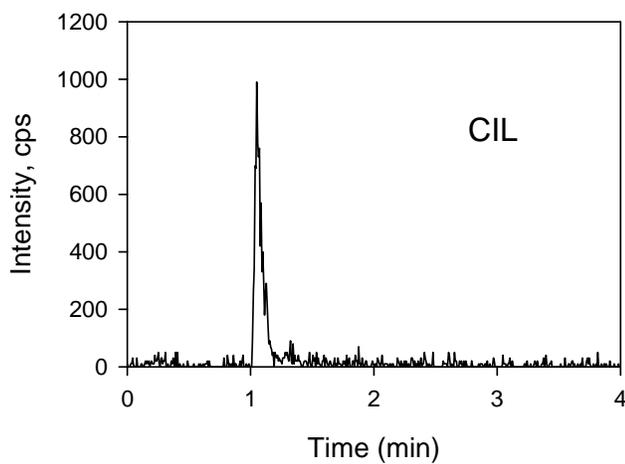
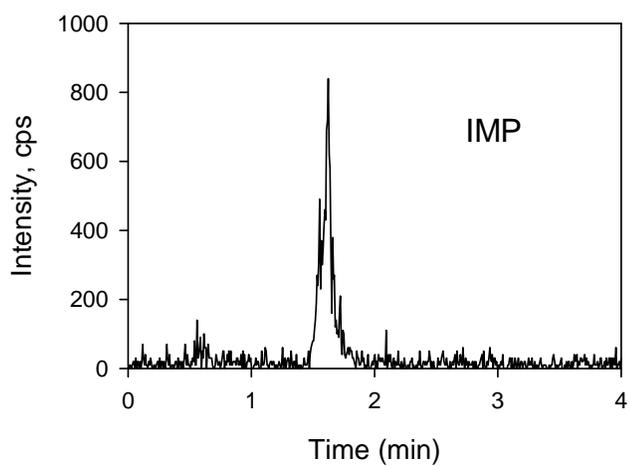


Figure 5-5 Representative extracted ion chromatograms from rat plasma for IMP, CIL, and BLI at 0.5 $\mu\text{g/mL}$ (LLOQ) using in-tip SPME.

QC freeze-and-thaw and room temperature stabilities were tested at the concentrations of 0.5, 50, and 1000 (10 times of ULOQ) for IMP, CIL, and BLI in rat plasma and mouse blood. The stability QC samples were tested after storage at -70°C for at least overnight and then at room temperature for at least 4 h. The results (Table 5-4) indicated that there was no stability issue for the assays under the specified storage conditions. The QC samples at 1000 µg/mL not only showed the stability at 10x ULOQ, but also demonstrated the ability to dilute samples above the upper limit of the standard curve.

Table 5-4 Stability^a QCs Containing IMP, CIL and BLI in Rat Plasma and Mouse Blood Using In-tip SPME and PPT

Matrix	Nominal Conc. (nM)	Accuracy ^b (%) [%CV ^c] (n=3)		
		IMP	CIL	BLI
In-tip SPME				
Rat Plasma	0.5	104.0 [4.2]	105.7 [9.0]	102.5 [6.3]
	50	102.3 [9.7]	103.6 [10.3]	100.9 [10.2]
	1000 (10xULOQ)	99.2 [3.0]	101.7 [1.0]	103.3 [0.3]
Mouse Blood	0.5	112.1 [11.1]	113.6 [6.4]	118.0 [9.0]
	50	92.6 [6.2]	104.6 [3.2]	100.8 [1.8]
	1000 (10xULOQ)	86.9 [4.1]	104.5 [1.7]	95.5 [1.2]
PPT				
Rat Plasma	0.5	110.1 [1.8]	107.2 [5.1]	96.4 [1.2]
	50	107.2 [2.2]	100.9 [1.3]	92.1 [1.8]
	1000 (10xULOQ)	109.4 [3.0]	94.5 [2.3]	90.1 [1.2]
Mouse Blood	0.5	110.8 [11.9]	116.6 [2.5]	105.2 [8.8]
	50	104.5 [6.4]	114.6 [3.4]	100.2 [3.4]
	1000 (10xULOQ)	105.0 [1.3]	118.9 [1.4]	101.7 [2.6]

- ^a Stability covered freeze-and-thaw after storing under -70°C for at least overnight and room-temperature for at least 4 hours
- ^b Expressed as [(mean calculated concentration)/(nominal concentration)]x100 (%)
- ^c Precision expressed as coefficient of variation (%CV)

5.3.5.3 Recovery and Matrix Effects

Extraction recovery and the matrix effects were evaluated for IMP, CIL, and BLI with standards spiked at concentrations of 1, 10, and 100 µg/mL using in-tip SPME; and 0.5, 5, and 50 µg/mL using PPT in five different sources of rat plasma. Mouse blood was not investigated due to lack of enough different sources since only one pooled control blood was used in the study.

For in-tip SPME, the mean recoveries of IMP, CIL, and BLI over the concentration range were 10.3, 9.3, and 15.8%, respectively (Table 5-5); while for PPT, the corresponding values were 98.7, 98.8, and 99.2%, respectively. Recovery was found consistently over the entire range of the standard curve in each method which indicated that extraction of the analytes in each method was conducted consistently. However, large variations were observed in both methods for "absolute" matrix effects with %CV from 12.8 to 26.7% for in-tip SPME, and from 9.3 to 25.9% for PPT, which indicated that matrix effects on ionization at different concentrations might not be the same. Significant ionization suppression with more than 25% was observed for CIL in both methods. The "relative" matrix effects on ionization were evaluated by examination the slope data that was obtained using five different sources of rat plasma, with a precision of 10.1, 8.8, and 10.4% C.V. for IMP, CIL, and BLI, respectively for in-tip SPME; and 3.1, 5.6, and 6.0% for IMP, CIL, and BLI, respectively for PPT. Based on the generally accepted criteria

that the relative standard deviation should not exceed 3-4% limit for the method to be considered reliable and free from the relative matrix effects, further experiments should be conducted to investigate the causes of these matrix effects.

Table 5-5 Calculated Mean Recovery and Matrix Effects for IMP, CIL and BLI Using In-tip SPME and PPT

Analyte	Recovery (%)		Matrix effects (%)	
	PPT ^a	In-tip SPME ^b	PPT ^a	In-tip SPME ^b
IMP	98.7 [2.4]	10.3 [8.4]	117.3 [17.1]	101.2 [16.6]
CIL	98.8 [2.7]	9.3 [7.5]	72.0 [25.9]	65.1 [26.7]
BLI	99.2 [1.9]	15.8 [3.6]	126.0 [9.3]	111.9 [12.8]

^a For PPT, recovery and matrix effects were evaluated at 0.5, 5, and 50 µg/mL using five different lots of rat plasma

^b For in-tip SPME, recovery and matrix effects were evaluated at 1, 10, and 100 µg/mL using five different lots of rat plasma

Among many matrix interferences, phospholipids have been identified as the major source of matrix effects.^{165,166} Phospholipids are extremely abundant in biological membranes and the glycerphosphocholines (GPCho's) constitute the major phospholipids in plasma. The post-column infusion was performed and the post-column spectra for IMP, CIL, and BLI after injection of phospholipids obtained from rat plasma extract were captured to clearly illustrate the phospholipids suppression effects to the analytes at the regions where phospholipids eluted from the columns under the same chromatographic conditions as the analytes. Figure 5-6 displayed the post-column spectra for IMP, CIL, and BLI after injection of mobile phase and phospholipids extract, as well as total ion chromatogram of phospholipids using precursor ion scan of m/z 184 to monitor all

phospholipids. Since stabilizer was used in this study, the potential interference from the stabilizer solutions was also investigated. Significant ion suppression was found for CIL at the elution window from 0.5 to 1.5 minute from both phospholipids and stabilizer solutions; and slightly ion enhancement for IMP and BLI at 1.5-2.0 minute and 3.0-3.5 minute window from the stabilizer solutions, respectively. The quantification results from "absolute" matrix effects measurements and the graphic illustration from post-column infusion were in excellent agreements. The relatively large standard deviation from the five curves slope data in both methods demonstrated that analytes should be separated from the endogenous interference as much as possible and the analog internal standard might not be able to compensate matrix effects even if it was co-elute with the analyte. Based on previous studies,¹²³ the utilization of stable isotope-labeled internal standards could effectively eliminate relative matrix effect liability, the precision of standard line slopes in five different lots of a biological fluid was less than 2.4%, which clearly indicated that stable isotope-labeled internal standard should be used when available, especially in SPME bioanalytical analysis considering the fact of its lower recovery compared with other extraction methods.

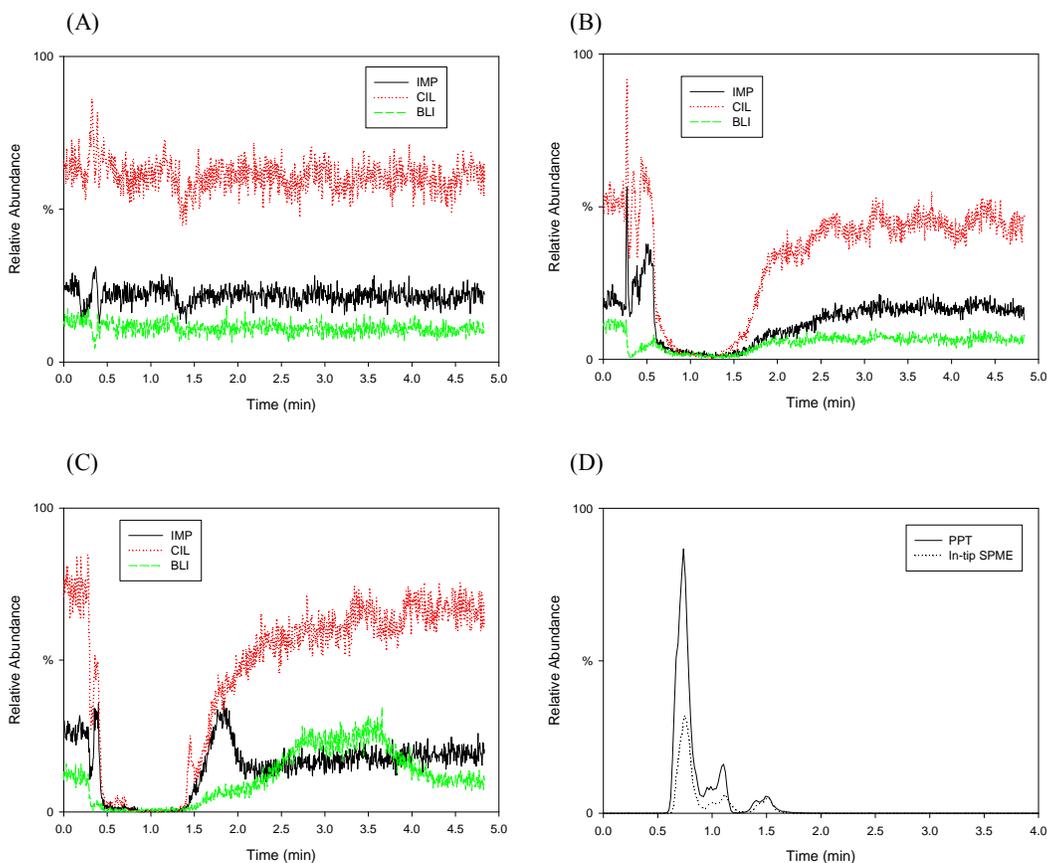


Figure 5-6 Post-column infusion spectra of a solution containing imipenem (IMP), cilastatin (CIL), and MK-4698 (BLI) at a concentration of 1 $\mu\text{g}/\text{mL}$ for each analyte after injection of (A) mobile phase; (B) phospholipids extracts; and (C) stabilizing solution under an isocratic elution of acetonitrile (ACN):15 mM ammonium formate (pH 3, 80:20, v/v) with a HILIC Silica column. (D) Precursor ion scan of m/z 184 monitoring all phospholipids using 25 μL of rat plasma prepared by PPT and in-tip SPME, respectively.

5.3.6 Comparison between In-tip SPME and PPT

Both in-tip SPME and PPT methods were validated in rat plasma and mouse blood with small sample volume, the linearity of the calibration curves, the intraday precision and accuracy were satisfactory in both methods. Recovery and matrix effects were also evaluated for IMP, CIL, and BLI using five different sources of rat plasma at three concentrations for in-tip SPME and PPT, respectively. Both methods worked well

for handling multiple analytes simultaneously regardless of their polarities, in addition, since a HILIC column with 80% acetonitrile in mobile phase was used, the final sample mix after in-tip SPME and PPT matched the mobile phase perfectly, so that the time-consuming solvent evaporation and reconstitution steps were avoided. In the reported study, PPT clearly demonstrated big advantages over in-tip SPME in terms of LLOQ, recovery, and sample preparation time. Compared with LLOQ of 0.5 $\mu\text{g/mL}$ for in-tip SPME, PPT was able to achieve a LLOQ of 0.1 $\mu\text{g/mL}$ using the same amount of sample volume with less amount of sample injection. The recoveries for three compounds were about 100%, which was expected in PPT, while for in-tip SPME, the mean calculated recoveries were generally below 15%. Although "relative" matrix effects were observed in both methods, the relative standard deviations of the calibration curve slopes for the three compounds from PPT were slightly better than those obtained from in-tip SPME, despite the relative larger "absolute" matrix effects observed in PPT, especially for CIL. In terms of sample preparation time, PPT was much faster than in-tip SPME mainly due to the long extraction time which was the time limiting step for overall sample preparation time in the latter. The advantages and limitations of SPME methods in general were evidently shown in this study. First, the process was relatively simple with automated in-tip SPME approach and the organic solvent consumption was much less than that in PPT, which is the biggest advantage of SPME technique compared with all other conventional sample preparation methods. Secondly, SPME demonstrated its advantage of dealing with blood samples over PPT and other methods as well such as LLE and SPE. In PPT, one problem associated with the direct protein precipitation for the treatment of blood samples was protein aggregation without sufficient lysis of red blood

cells. The commonly used method – addition of formic acid to help cell lysis was not suitable for IMP because its β -lactam ring could be hydrolyzed much faster under acidic conditions. Therefore, 20 μ L of ethanol was added to lysis the red blood cells in PPT, however, with in-tip SPME, no additional sample treatment was necessary and blood sample extraction was performed directly with in-tip SPME. In addition, since sample volume was limited to 10-20 μ L of mouse blood, practically, it was impossible to repeat any samples in PPT. This was not an issue using in-tip SPME as samples could be extracted multiple times.

In general, if sensitivity is an issue, SPME will be more troublesome than PPT because of the relatively lower recovery and, as it is often the case, a large amount of sample is introduced to the LC-MS/MS system in order to achieve the required sensitivity with a risk of encountering potential matrix effects. This statement is further supported by the validation results of smaller calibration range and larger "relative" matrix effects using in-tip SPME in this particular study. Moreover, it seemed that in-tip SPME was more sensitive to internal standards than that of PPT and the precision and accuracy of in-tip SPME method largely depended on the selections of appropriate internal standards.

5.3.7 Applications of In-tip SPME and PPT in Preclinical Sample Analysis

In reality, the validated PPT method has been used successfully to analyze more than 600 samples in several clinical studies, including single administration or co-administration of IMP, CIL, and BLI in different animal species through either intravenous or subcutaneous dosing regimens. In order to compare the results of pharmacokinetic data obtained from two widely different sample preparation methods,

plasma samples from rat subjects co-administration of 80 mg/kg IMP, 80 mg/kg CIL, and 80 mg/kg BLI through 1-h IV infusion were analyzed simultaneously after sample extraction using in-tip SPME and PPT, respectively. For all three compounds, it was found that the differences between $AUC_{0-\infty}$, C_{max} , clearance (CL), and volume of distribution (V_{ss}) obtained from two methods were within all 20%, except that for CIL, the variation was about 30% (Table 5-6). In addition, sparse mouse blood samples from a mouse PK/PD model study with co-administration of 10 mg/kg of three compounds through 30-min IV infusion were also analyzed using both approaches. The linear relationship of concentrations obtained from PPT versus those from in-tip SPME clearly demonstrated the excellent agreement of the two methods (Figure 5-7).

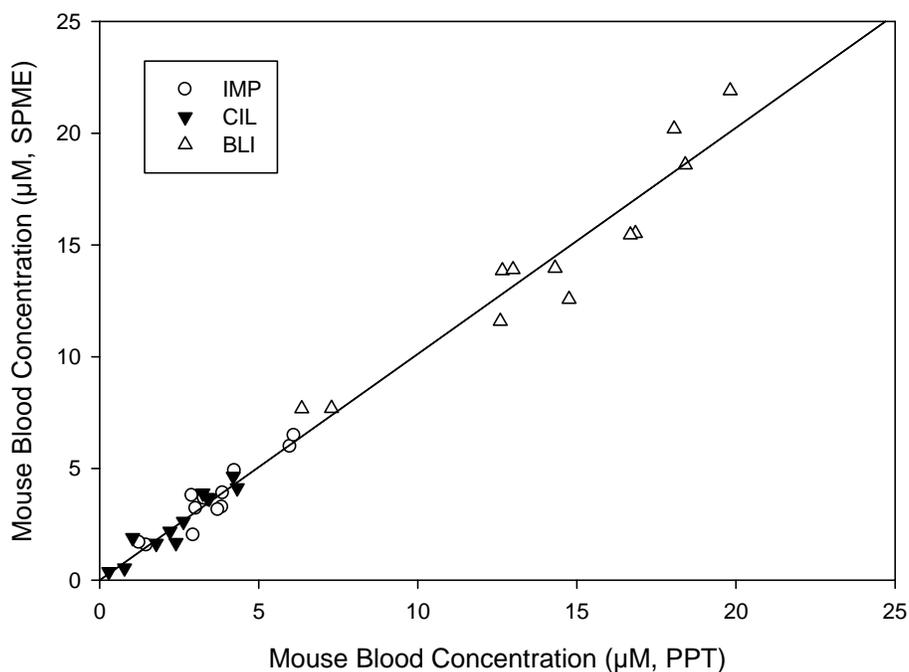


Figure 5-7 Comparison of IMP, CIL and BLI concentrations obtained using PPT and in-tip SPME extraction methods in mouse blood samples co-administration of 10 mg/kg of the three compounds through 30-min IV infusion from a mouse PK/PD model study.

5.4 Conclusions

A LC-MS/MS method coupled with in-tip SPME has been established and validated to determine IMP, CIL, and BLI simultaneously in rat plasma and mouse blood. The overall data demonstrated that automated in-tip SPME was comparable to the PPT method in terms of assay accuracy and precision. Despite its lower recovery, in-tip SPME clearly showed big advantages over PPT in mouse blood sample preparation. Matrix effects should be thoroughly investigated, especially the impact of relative matrix effects to the performance of the SPME assay. The results of this study also indicated that a high degree of automation is not a limiting factor of utilizing SPME technique in routine preclinical sample analysis, developing selective and high extraction capacity of SPME coatings is essential in any SPME methods for drug analysis. Oasis HLB-coated polymer monoliths provided much better extraction recovery than other traditional fiber based SPME coatings, and have great potential for high throughput bioanalysis.

Table 5-6 Mean Plasma Pharmacokinetic Parameters Following Coadministration of 80 mg/kg IMP, CIL and BLI Through 1-h IV Infusion in Rats

Compound	Assay	AUC _{0-∞} (hr*μM)	C _{max} (μM)	CL (mL/min/kg)	V _{ss} (L/kg)
IMP	PPT	191.1 (20.0)	184.3 (10.2)	0.422 (0.043)	0.080 (0.003)
	SPME	194.1 (4.4)	195.7 (9.4)	0.412 (0.009)	0.080 (0.005)
	Ratio (PPT/SPME)	0.98 (4.55)	0.94 (1.09)	1.02 (4.66)	0.99 (0.60)
CIL	PPT	171.3 (62.9)	142.4 (46.0)	0.521 (0.224)	0.201 (0.030)
	SPME	143.5 (47.8)	127.5 (21.8)	0.596 (0.172)	0.152 (0.037)
	Ratio (PPT/SPME)	1.19 (1.32)	1.12 (2.11)	0.88 (1.31)	1.32 (0.80)
BLI	PPT	282.0 (18.0)	228.1 (55.8)	0.284 (0.019)	0.128 (0.075)
	SPME	334.6 (10.8)	241.9 (63.5)	0.239 (0.008)	0.132 (0.007)
	Ratio (PPT/SPME)	0.84 (1.67)	0.94 (0.88)	1.19 (2.42)	0.97 (1.07)

Chapter 6

Evaluation of Matrix Effects in Bioanalysis using Automated In-tip SPME and Liquid Chromatography with Tandem Mass Spectrometry

6.1 Introduction

Matrix effects that are defined as interference from matrix components that are unrelated to the analyte,¹⁶⁷ have received more and more attention recently in bioanalytical analysis using LC-MS/MS detection. In many cases, even though the assays are claimed to be fully validated, large signal variations are observed in real clinical sample analysis, mainly due to the matrix components that are not detected in the MS/MS spectra, but co-elute with the analyte, and therefore adversely affects the analyte ionization process and results in either ion enhancement or ion suppression.¹⁶⁸⁻¹⁷³ Matrix effects could cause significant errors in precision and accuracy and, therefore, invalidate the assessment of pharmacokinetic results based on the HPLC-MS/MS assays.¹⁷⁴

In order to better understand matrix effects in bioanalysis using HPLC-MS/MS, extensive studies have been performed to explore the possible mechanism of matrix effects, qualitative and quantitative assessment and measurement of matrix effects, and on the elimination and reduction of matrix effects. A number of papers, reviews, and book chapters have been published on the topics.¹⁷⁵⁻⁻¹⁷⁸ Although the mechanism of matrix induced ion enhancement or ion suppression is still not fully understood, several different mechanisms have been proposed based on the ionization technique that was used. The two most common ionization techniques are APCI and ESI. APCI is known to be less prone to exhibiting matrix effects when compared to ESI because, in ESI, most ions are generated in the solution phase followed by transfer to the gas phase while in

APCI, neutral molecules are first vaporized into the gas phase followed by ionization *via* the corona discharge process. Therefore, ionization efficiency can be affected by matrix components in both solution and gas phase for ESI and only in gas phase for APCI. The post-column infusion method developed by Bonfiglio et al.¹⁷⁹ provides a qualitative assessment of matrix effects, in which blank sample extracts are injected on the HPLC column under conditions chosen for the assay while a constant amount of analyte is infused into the HPLC stream before it enters the ion source of the mass spectrometer. Ion enhancement or ion suppression caused by matrix effects is shown as a change of MS response of the infused analyte following injection of blank sample extract. This post-column infusion graphically indicates potential regions in the chromatograms prone to matrix effects. As a result, chromatographic conditions can be adjusted to minimize matrix effects. However, since post-column infusion is usually performed at relatively high concentrations, matrix effects are not investigated for samples at low concentrations. In addition, when a large number of compounds are analyzed in one method, each compound should be infused individually to evaluate matrix effects which would make the approach less practical. Also, a “relative” matrix effect, a difference in the matrix effect between biological fluids originating from different sources or subjects, a case commonly encountered in PK studies, cannot be assessed using this qualitative, post-infusion experiments. A quantitative method to assess matrix effects was proposed by Matuszewski et al.⁸⁹ using a matrix factor defined as a ratio of the analyte peak response in the presence of matrix ions (post-spike sample solution) to the analyte peak response in the absence of matrix ions (neat sample solution). A matrix factor larger or less than one indicates ion enhancement or ion suppression, respectively. The authors suggested that

this so called "absolute" matrix effect is not critical for a reliable bioanalytical assay. However, what is important is to evaluate the "relative" matrix effect, the matrix effect values in biofluids (for example human plasma) originating from at least five different sources (subjects). A good indicator of the relative matrix effect is to use the precision of standard line slopes constructed in at least five different sources of the biofluid and calculate the relative standard deviation of these slopes.¹²³ It was suggested, that the precision value for these slopes should be less than 3-4% for the method to be considered free from "relative" matrix effect.

Strategies to eliminate or reduce matrix effects include choosing suitable instruments and ionization modes, improving chromatographic conditions to have a better separation between endogenous components and analytes by modifying mobile phase composition, using ultra-performance liquid chromatography (UPLC), using a stable-isotope labeled internal standard of analytes, selecting appropriate analogs as internal standards, preparing standards and quality control samples in pre-dose samples to eliminate the matrix difference between the calibration standards and the samples, and introducing a minimum amount of sample to the HPLC-MS/MS system. Above all, the most efficient way of reducing matrix effects is to separate analytes from endogenous biofluid components/impurities by an effective sample preparation method.

PPT, LLE, and SPE are the most commonly used sample preparation techniques in bioanalytical analysis. As the simplest and fastest method for sample preparation, PPT does not result in a very clean extract and is most likely to cause ion suppression in ESI as this method fails to sufficiently remove endogenous components such as lipids, phospholipids, and fatty acids. In comparison with PPT, LLE and SPE provide cleaner

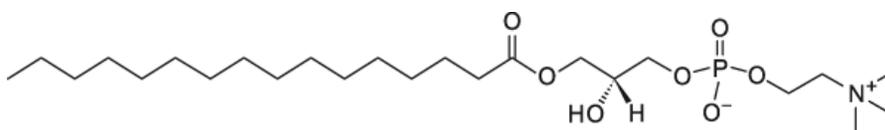
extracts; however, analyte recovery and matrix effects should be taken into consideration. In recent years, SPME, a solvent-free extraction technique that combines sampling, sample clean-up, and pre-concentration into a single step, has been widely used in many areas of analytical chemistry.^{180,181} The technique involves exposure of the sample matrix to a small amount of extracting phase dispersed on a solid support. The SPME process involves the performance of two basic steps: (i) partitioning of analytes between the extraction phase and the sample matrix and (ii) desorption of concentrated extracts into an analytical instrument. SPME has been used routinely in combination with gas chromatography (GC) and gas chromatography–mass spectrometry (GC-MS) and was also introduced for direct coupling with LC and LC-MS in order to analyze weakly volatile or thermally labile compounds not amenable to GC or GC-MS. Theoretically, SPME could provide sample clean-up that is as effective as or better than that obtained by SPE since a small volume of sorbent is used and the absolute amount of analytes of interest and potential interferences extracted by SPME are much smaller and depend on the magnitude of their distribution constant. However, in comparison with headspace SPME in most GC or GC-MS applications, direct SPME in HPLC or HPLC-MS is relatively "dirty" in bioanalysis as the SPME fiber is directly immersed in the complex matrix, or biological fluid (blood, plasma, urine, etc). Because SPME is a non-exhaustive extraction method, it cannot equally compensate for changes of the composition of the matrix as in the case of LLE, therefore, quantification is more prone to errors due to changes of matrix. Hence, it is essential and important that matrix effects be extensively investigated during SPME methods development and validation.

Among many matrix interferences, phospholipids have been identified as the major source of matrix effects.^{165,166} Phospholipids are extremely abundant in biological membranes and the GPCho's constitute the major phospholipids in plasma. In this work, six representative phospholipids from SPME extracts were monitored and measured using commercially available and tailor-made SPME fibers and the selectivity and extraction efficiency of phospholipids using different SPME fibers was investigated. The pH and salt effects on phospholipids recovery with different fiber coatings were also evaluated. Overall cleanliness of sample extracts from SPME and other sample preparation methods such as PPT, LLE, and SPE was compared, and the matrix effects and recovery of various compounds using different methods were quantitatively compared. Based on the experimental results, general approaches of evaluating matrix effects and strategies of reducing or eliminating matrix effects are proposed in SPME methods development and validation. It should be pointed out that SPME is performed using in-tip SPME format to further demonstrate the feasibility of SPME automation and the great advantages of in-tip SPME for high throughput quantitative determination of drugs in the pharmaceutical industry. This approach is simple and easy for automation without introducing additional devices. More importantly, in-tip SPME format is very flexible and amenable to all fibers types possessing a wide range of different coating materials, which will overcome the drawback of limited selection of commercial available fibers and broaden its use with HPLC-MS. To the best of our knowledge, this is the first comprehensive study on the evaluation of matrix effects in SPME. The experimental approaches and results could be used as guide and reference for future methods development and validation in bioanalytical analysis using SPME.

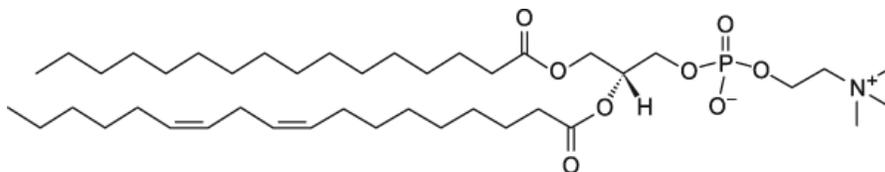
6.2 Experimental

6.2.1 Chemicals and Materials

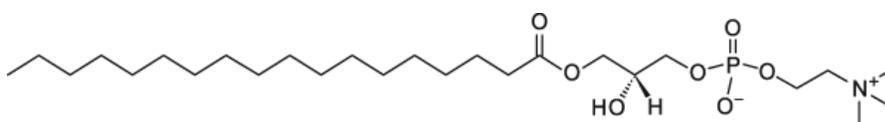
Diazepam, nordiazepam, oxazepam, and diazepam-*d*₅ were purchased from Cerilliant (Round Rock, TX, USA) as 1 mg/mL methanolic solutions, while lorazepam was purchased as a 1 mg/mL solution in acetonitrile. IMP and CIL were purchased from Sigma Chemical Company (St. Louis, MO, USA). MK-4698 and all the analog or isotopic labeled internal standards used in this work were synthesized at Merck Research Laboratories (Rahway, NJ, USA). Glycerophospholipids were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Structures of selected compounds are shown in Figure 6-1. All other chemicals such as triacontyldimethylchlorosilane, ethylene glycol dimethacrylate, n-octadecyldimethylorosilane, dimethoxy- α -phenylacetophenone, ethylene glycol, 2-(N-morpholino)ethanesulfonic (MES) acid, MES sodium salt, ammonium acetate, formic acid (95%), and 1-decanol were purchased from Sigma-Aldrich (Milwaukee, WI, USA), and all HPLC grade solvents were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Deionized water was obtained by passing in-house water through a Millipore Milli-Q plus system (Bedford, MA, USA). Different lots of drug free human and animal plasma were obtained from Biological Specialties Corp. (Lansdale, PA, USA) and stored at -20 °C before use. PDMS-DVB fibers (60 μ m) were purchased from Supelco (Bellefonte, PA USA) and Oasis HLB (5 mg) μ Elution SPE plates were from Waters Corp. (Milford, MA USA). 96-Well collection plates (1.2 and 2.4 mL) and mats were purchased from Marsh Biomedical (Rochester, NY, USA).



1-hexadecanoyl-*sn*-glycero-3-phosphocholine (16:0 Lyso PC)



1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (16:0-18:2 PC)



1-stearoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (18:0 LysoPC)

Figure 6-1 Structures of selected phospholipids.

6.2.2 HPLC-MS/MS

Tandem mass spectrometry was performed using Sciex API 3000/4000 triple quadrupole mass spectrometers (Foster City, CA, USA) equipped with a turbo ion spray (TIS) source. The HPLC system included a Perkin-Elmer (Norwalk, CT, USA) LC-200 micro pump with a CTC PAL Leap autosampler (Carrboro, NC, USA) for 96-well plates, and a TLX2 system from Thermo Scientific (Franklin, MA, USA). Different columns were used in chromatographic separation of various analytes, such as a Restek (Bellefonte, PA, USA) BDS Hypersil C18 column (50×2.1 mm, $3 \mu\text{m}$) and a Waters (Milford, MA, USA) Atlantis HILIC Silica (50×2.1 mm, $3 \mu\text{m}$). Mobile phase consisted of (1) ACN:15 mM ammonium formate (pH 3, 80:20, v/v), flow rate of 0.4 mL/min (isocratic); (2) ACN (0.1% formic acid): H₂O (0.1% formic acid), flow rate of 0.4 mL/min, gradient separations performed from 10% to 95% ACN; and (3) ACN (0.1% formic acid): H₂O (0.1% formic acid) (50:50, v/v), flow rate of 0.4 mL/min (isocratic). A 1:1 (v/v) "cocktail" of ACN/acetone/IPA (40:40:20, v/v/v) and ACN (0.1 % formic acid) were used as washing solvents for the autosampler. Injection volume was from 5 to 15 μL depending on the sample loop installed and assay sensitivity. The mass spectrometer sources and compounds acquisition parameters were optimized by infusing a neat solution of a compound prepared in 50% ACN in water at a flow rate of 20 $\mu\text{L}/\text{min}$ into a mobile phase pumped at 0.2 mL/min through the turbo ion spray interface. A summary of instrumental conditions used for each compound in the study is given in Table 6-1. A Packard MultiPROBE II liquid handling system (Meriden, CT, USA) was used for sample and solution pipetting.

Table 6-1 Summary of HPLC-MS/MS Conditions

Analyte	pKa	Log P	Ion Transition, <i>m/z</i> (precursor → product)	DP (V)	CE (V)	CXP (V)	EP (V)	HPLC-MS/MS
Diazepam	3.4	2.82	285.0 → 154.2	26	39	10	10	ACN (0.1% formic acid): H ₂ O (0.1% formic acid), flow rate of 0.4 mL/min, gradient separations performed from 10% to 95% ACN; Restek BDS Hypersil C18 column (5 × 2.1 mm, 3 μm), API 3000, TIS
Lorazepam	13	2.39	321.1 → 275.1	21	31	14	10	
Oxazepam	12.4	2.24	287.2 → 241.1	26	31	18	10	
Nordiazepam	11.8	2.93	271.0 → 140.2	31	39	8	10	
Diazepam-d ₅	--	--	290.1 → 154.2	31	39	10	10	
IMP	4.29	2.78	300.2 → 142.0	53	36	12	10	20% (15 mM ammonium formate, pH 3) : 80% ACN, isocratic, flow rate of 0.4 mL/min; Atlantis HILIC Silica (50×2.1mm, 3μm), API 4000, TIS
CIL	2.09	2.41	359.3 → 202.4	55	21	4	10	
BLI (MK-4698)	1.26	1.11	570.1 → 236.2	91	55	6	10	
ISa for IMP, CIL	2.11	2.65	373.3 → 233.1	55	26	14	10	
ISb for BLI	1.23	2.24	587.2 → 236.0	86	55	6	10	

6.2.3 In-tip SPME Fibers Preparation

Different types of SPME fibers were prepared and compared to evaluate the matrix effects: (1) PDMS-DVB, (2) C18 and C30 phase silica-based coatings, and (3) Oasis HLB-coated polymer monoliths.

In-tip PDMS-DVB fibers and Oasis HLB-coated polymer monoliths were prepared as described previously.¹⁴² C18 and C30 phase silica-based coatings were prepared based on a procedure of entrapment of porous silica particles in a network of polymerized silicate, followed by *in situ* derivatization to attach the desired extraction phase. Briefly, stainless steel wire of 0.02" diameter was cut into 10 cm pieces. The wires were chemically etched for 30 min with hydrochloric acid and rinsed thoroughly with purified water and dried in an oven at 130°C for 1 h. The dried and cooled wires were dipped in potassium silicate solution such that a length of 1.5 cm was covered and then carefully rolled over 5 µm porous silica particles. The resulting silicate-silica coating was exposed to fumes of concentrated nitric acid for 10 s and allowed to dry at ambient temperature for at least 12 h. The coated wires were placed in vials containing 10 mL of the derivatization solutions consisted of 10% either n-octadecyldimethylchlorosilane for C18, or triacontyldimethylchlorosilane for C30 in anhydrous toluene and the vials were immersed in a silicone oil bath at 70°C for 24 h. Following derivatization, the fibers were rinsed successively for 15 min with toluene, tetrahydrofuran, MeOH, 50:50 (v:v) MeOH:water and water, and were allowed to dry overnight before initial use. The tailor-made C18 and C30 fibers were prepared the same way as PDMS-DVB fibers for in-tip SPME automation.

6.2.4 Sample Preparation

6.2.4.1 Monitoring of Phospholipids

Two MS/MS methods were used to monitor phospholipids in positive ESI mode; one is based on a positive precursor ion scan of m/z 184, which results in total ion chromatograms to qualitatively monitor all phospholipids in plasma, and the other is the quantitative determination of specific phospholipids using MRM. The selected phospholipids monitored were: 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (16:0 LysoPC), 1-oleoyl-2-hydroxy-sn-glycero-3-phosphocholine (18:1 LysoPC), 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine (18:0 LysoPC), 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine(16:0-18:2 PC), 1-dodecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine (12:0-18:1, PC), and 1-(9Z,12Z-octadecadienoyl)-2-(5Z,8Z,11Z,14Z-eicosatetraenoyl)-sn-glycero-3-phosphocholine (18:2-22:4 PC).

Phospholipid standard solutions (16:0 LysoPC) from 1 to 500 μM were prepared by transferring an appropriate volume of each lipid stock solution of about 2 mg/mL and diluting with MeOH:water (50:50, v/v), and were used to evaluate the effect of phospholipids on analyte recovery in SPME. To investigate the pH effect on phospholipids recovery, 100 μL of plasma samples was extracted using different types of fibers under acidic, neutral, and basic conditions by spiking with 100 μL of 0.1 N HCl, PBS buffer (pH = 7), and 0.1 N NaOH, respectively. Salt effect with mixing of 100 μL of water containing 10% NaCl (w/v), 1% NaCl (w/v), and 0% NaCl, respectively was also investigated.

Phospholipids extracted from plasma using SPME were compared with those from other extraction methods, such as PPT, LLE, and SPE. In these comparison studies, the same amount of blank plasma was applied for different extraction methods and the extracts were dried down and reconstituted in the same amount of 50% ACN. The same volumes of the extract were injected for analysis.

6.2.4.2 Post-column Infusion

The plasma supernatant solution was obtained by mixing pooled blank plasma with ACN in a ratio of 1:3 following centrifugation for 5 min at 4000 rpm. The analyte solution, prepared at a concentration of 1 µg/mL of each studied analyte in 50% ACN, was post-column infused from the syringe pump to the HPLC effluent at a flow rate of 60 µL/min. After the signals of the analyte MRM transitions were stable, the plasma supernatant solution (10 µL) containing various phospholipids was injected onto the column under the same chromatographic conditions as the analytes and analytes post-column infusion spectra were acquired. Different chromatographic conditions were evaluated for studying matrix effects with different analytes. For benzodiazepines, including diazepam, nordiazepam, oxazepam, lorazepam, and diazepam-*d*₅, a gradient elution started at 10% B (0.1% formic acid in ACN) for the first 0.5 min, and was linearly ramped to 95% B in 2 min, held for 1 min and returned to 10% B in 1.5 min. For IMP, CIL, and BLI post-column infusion experiments, an isocratic elution of 20% A (15 mM ammonium formate, pH 3) and 80% B (ACN) was used.

6.2.4.3 In-tip SPME Conditions

In-tip SPME extraction and desorption process for all experiments was fully automated using a Tomtec Quadra 96 workstation. The detailed sample preparation procedures were described previously.²² In summary, 96-well sample extraction and desorption plates, as well as tip washing plate, waste plate, and reservoirs containing desorption solvent were placed on the deck of the Tomtec Quadra 96 workstation. The Tomtec Quadra 96 workstation was programmed as such that the whole process ran in a sequence of tip loading, extractions, washing, desorption, and tip cleaning. The sample extraction and desorption process was accomplished through repeated aspirating and dispensing of sample solution and desorption solvent, respectively. After sample extraction and desorption, the extracts on the plate were either directly injected to the HPLC-MS/MS system or went through an evaporation/reconstitution step if enhanced sensitivity was required. PDMS-DVB, C18, and C30 fibers were preconditioned for 30 minutes using 50% methanol before initial use, and in-tip polymer monoliths fibers were preconditioned with 2×50 μL of 50% ACN followed by 2×50 μL of water before sample extraction.

6.2.4.4 PPT, LLE, and SPE Conditions

In PPT sample preparation, blank plasma samples or plasma samples along with standards were mixed well and protein precipitated with 100% ACN in a ratio of 1:3 or greater depending on the experiment. Samples were vortex-mixed for about 3 min, and centrifuged at 10°C, 3500 rpm for 5 min. The supernatant was removed, dried down and reconstituted in 150 μL of 50% ACN and injected into the HPLC-MS/MS system for analysis. In some cases, an aliquot of supernatant from PPT was directly injected to the

system for analysis. The PPT experiments were conducted using either tube or 96-well plate based on the number of samples.

LLE was performed in 96-well plate format where organic solvent such as MTBE, hexane, or ethyl acetate was added in 4:1 ratio to plasma. In some cases, the organic solvent was acidified or basified to increase analyte recovery. The sample plate was sealed with mat made of molded PTFE/silicone liner and was rotor-mixed 20 min for LLE. The plate was then centrifuged at 10°C, 3500 rpm for 10 min and the top organic layer was removed, dried down, and reconstituted in 150 µL of 50% ACN in water and injected into the HPLC-MS/MS system for analysis.

In SPE extraction, the Oasis HLB (5 mg) µElution SPE plate was conditioned with 2×50 µL of ACN followed by equilibration with 2×50 µL of 0.1 M acetic acid. Blank plasma (250 µL) was diluted (1:1, v/v) with 0.1 M acetic acid and loaded onto the plate. The plate was washed with 200 µL of 0.1 M acetic acid followed by 200 µL of 10% ACN in water, and eluted with 2×100 µL of ACN. The eluate was dried down and reconstituted in 150 µL of 50% ACN in water and injected into the HPLC-MS/MS system for analysis.

6.2.5 Recovery and Matrix Effects

Three sets of standard samples were prepared to evaluate recovery and matrix effects, as recommended in reference.⁹⁰ The first set of samples were prepared to evaluate MS/MS response for neat standards injected in the mobile phase. The second set was prepared in plasma extracts originating from different plasma sources and spiked after extraction. The third set was prepared in plasma from the same sources as in second set,

but plasma samples were spiked here before extraction. Recovery was determined by comparing the mean absolute peak areas of standards obtained from the third set to those from the second set. Matrix enhancement/suppression of ionization or "absolute" matrix effect was evaluated by comparing the absolute peak areas of the standards in post spiked extraction samples (second set), to neat standards (first set). In general, a concentration from the standard curve was selected and prepared in five different lots of plasma for recovery and matrix effects assessment. "Relative" matrix effect on ionization was evaluated by an examination of the slopes of five standard curves constructed in five different lots of plasma.¹²³

Human control plasma was used in most of the experiments except where animal plasma is indicated.

6.3 Results and Discussion

6.3.1 Monitoring of Phospholipids

Phospholipids are composed of ester or amide derivatives of glycerol or sphingosine with fatty acids and phosphoric acid and, therefore, are classified into two different classes, GPCCho's and sphingomyelins. Phospholipids are abundant in plasma with total concentrations of about 1.6-3.0 mg/mL, and glycerophosphocholines are considered the major phospholipids in plasma that could cause significant matrix effects during HPLC-MS/MS analysis. Several techniques are used to monitor phospholipids in HPLC-MS/MS; each technique has its advantages and limitations and could be used for different experimental purposes. Little et al.¹⁶⁶ developed an "in-source multiple reaction monitoring" (IS-MRM) method to detect GPCCho's during HPLC-MS/MS method

development. The approach uses high energy in-source collision induced dissociation (CID) to yield characteristic product ions (m/z 184) in positive ion electrospray mode, which corresponds to trimethylammonium-ethyl phosphate ions that are formed from mono- and di-substituted GPCho's. This simple approach could simultaneously monitor all GPCho's using only one channel in an MRM HPLC-MS/MS experiment, however, the fragmentation pathway to form the m/z 184 ion reveals little information about different classes of GPCho's, such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylserines, etc, when specific classes need to be monitored and eliminated during sample preparation. Another technique to monitor phospholipids is to use precursor ion and neutral loss scans. Positive precursor ion scan of m/z 184, positive neutral loss scan of 141 and 185 Da, and negative precursor ion scan of m/z 153 have been used to monitor different classes of phospholipids. The phospholipid profiles are, in general, very similar to those obtained from IS-MRM, however, as pointed out by Jemal et al from a recent comprehensive study on phospholipids in HPLC-MS/MS bioanalysis,¹⁸² a very important advantage of using precursor ion scan of m/z 184 over IS-MRM m/z 184 \rightarrow m/z 184 is that the former not only detects all the phospholipids that have the choline polar head, but it also identifies the precursor ions corresponding to each chromatography peak, which is not the case with the latter. Compared with qualitative assessment of all classes of phospholipids from both IS-MRM and precursor ion scan, quantitative determination of specific phospholipids could only be obtained using individual multiple reaction monitoring (MRM) transitions.

Both precursor ion scan and individual MRM methods were applied in this work. It was found that signal intensity of phospholipids profiles obtained from positive

precursor ion scan of m/z 184 was significantly higher than that from positive neutral loss or negative precursor ion scan in both human and animal plasma under the same chromatographic conditions, which is in agreement with that observed by Jemal et al.,¹⁸² when supernatant of ACN-precipitated human plasma was injected to HPLC-MS/MS in the detection of phospholipids. In addition, more chromatographic peaks of phospholipids were observed from positive precursor ion scan of m/z 184 at the same retention window. Based on these observations, the positive precursor ion scan of m/z 184 seems to be sufficient to monitor overall phospholipids mass spectra for the evaluation of matrix effects in bioanalytical method development. This assumption was further verified from MRM monitoring of 16:0 Lyso PC (m/z 496 \rightarrow 184), 18:1 Lyso PC (m/z 522 \rightarrow 184), 18:0 Lyso PC (m/z 524 \rightarrow 184), 12:0-18:1 PC (m/z 703 \rightarrow 184), 16:0-18:2 PC (m/z 758 \rightarrow 184), and 18:2-22:4 PC (m/z 806 \rightarrow 184), representative classes of GPCho's that were most frequently evaluated during matrix effects assessment. All mass spectra of these individual lipids were captured in positive precursor ion scan.

Due to the unique structures of GPCho's, for example, phosphatidylcholine consisting of both a polar head group including a negatively charged phosphate group and a positively charged quaternary amine group with one or two long alkyl chains, these long alkyl chains could make these lipids extremely hydrophobic and they could be retained in reversed phase columns if the percentage of organic solvent is not high enough for their elution. In this study, it was found that the majority of the selected phospholipids would not elute from the column if the mobile phase was 50% ACN in water under isocratic conditions, but all phospholipids were eluted with 95% ACN (0.1% formic acid) at a flow rate of 0.4 ml/min in less than 5 minutes. In addition, all the

phospholipids were eluted with ACN:15 mM ammonium formate (pH 3, 80:20, v/v) using a HILIC column at a retention window of 0.5 to 2 minutes. No carry-over was observed from previous injections in double blank samples.

6.3.2 SPME Fiber Selection on Phospholipids Extraction

In order to reduce or eliminate matrix effects in bioanalysis using SPME technique, it is very important to evaluate the SPME fiber and extraction efficiency on phospholipids in plasma samples. Since SPME extraction and desorption processes for all experiments were conducted in multiple fiber extraction format using automation, SPME fibers from different coating procedures were used for comparison. Two of the major factors affecting analyte extraction in SPME method development, the pH and salt effects on phospholipids extraction recovery with different types of coatings, were investigated in this study.

Compared with other exhaustive extraction techniques, such as LLE and SPE, SPME is an equilibrium-based sample preparation technique. The extraction efficiency of SPME is determined by the partitioning of analyte between the sample matrix and the extraction phase. The higher the affinity the analyte for the extraction phase relative to the sample matrix, the greater amount of analyte is extracted. Partitioning is controlled by the physicochemical properties of the analyte, the sample matrix, and the extraction phase. In most SPME methods development, the determination of extraction time profile and equilibrium time is a prerequisite for method optimization. To minimize the errors caused by different sampling time and to achieve the maximum sensitivity, the extraction time should be equal to or longer than the equilibrium time. However, in bioanalysis, the

equilibrium time could be very long which is impractical for high throughput applications. Therefore, SPME is often used with pre-equilibrium extraction times, provided the experimental conditions, such as agitation and temperature, are well controlled. To determine the extraction time profiles of various phospholipids in human plasma with different SPME fibers, 5, 10, 40, 80, 240, and 480 aspiration/dispense cycles were evaluated while other experimental conditions were kept constant such as aspiration/dispense volume and speed. Five replicates of different SPME fibers were simultaneously placed in a 96-well plate for extraction at each time point. The plasma samples were used directly without any pH or ionic strength modifications, and all the experiments were conducted at room temperature. Representative extraction time profiles of phospholipids are shown in Figure 6-2.

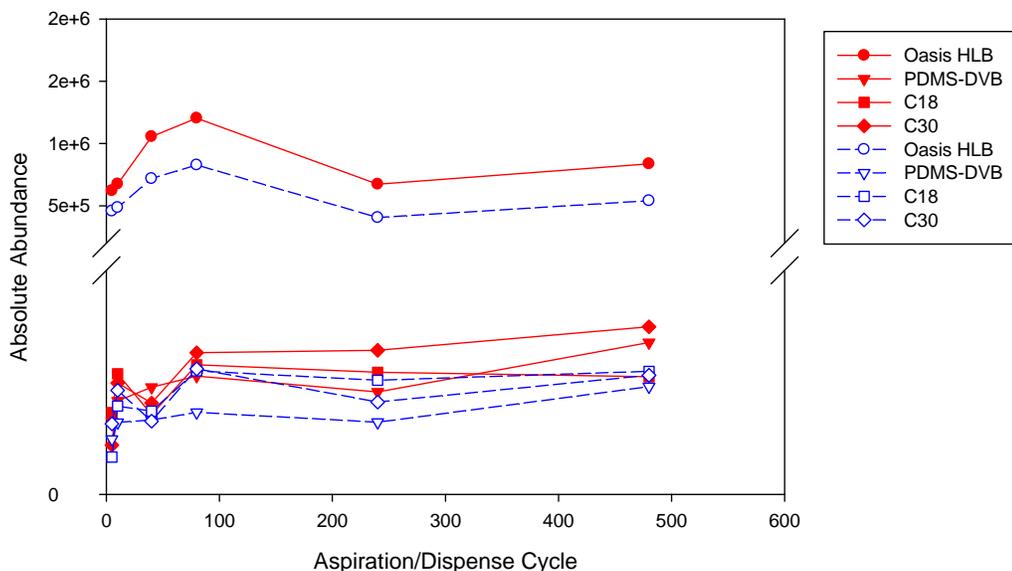


Figure 6-2 Extraction time profiles for selected phospholipids (16:0 Lyso PC, open symbols, and 18:0 Lyso PC, solid symbols) using four different types of SPME fibers in 250 μ L human control plasma under SPME conditions as described in the experimental section. Extraction time profiles for other monitored phospholipids are omitted for clarity.

It was found that equilibrium was achieved fairly rapidly, in less than 10 minutes, for all the phospholipids monitored regardless of the different fiber coatings. Overall, based on absolute peak areas, Oasis HLB-coated polymer monoliths exhibited higher extraction efficiency than other types of coatings, while PDMS-DVB, C18 and C30 phase silica-based coatings showed similar extraction efficiency. The absolute amount extracted can be calculated if calibration curves were constructed for each phospholipid monitored prepared in PBS. For porous polymer SPME fibers (DVB), extraction of analytes is based on adsorption rather than absorption, and weak intermolecular interactions and hydrophobic interactions play the most important role in analytes extraction. According to the Langmuir isothermal model, which describes equilibrium analyte extraction by porous polymer SPME coatings, the number of surface sites where adsorption can take place is limited and, therefore, porous polymer coatings of large surface area with more active sites will have higher extraction capacities. A 65 μm PDMS/DVB commercial fiber with coating length of 1 cm leads to an extract volume of 0.357 mm³ and surface area approximately of 8 mm²; the corresponding values of volume and surface area for tailor-made 10 μm C18 and C30 phase silica-based coatings are 0.256 mm³ and 25 mm², respectively. Although it is difficult to accurately calculate the extraction volume and surface area of Oasis HLB-coated polymer monoliths since the polymer is physically attached to the wall of the tip and a capillary is used to create a main channel through the monolithic polymer, it is believed that the surface area is much larger than that of a conventional SPME fiber, because both inside and outside surface areas of the polymer are used for extraction. In general, the recovery using Oasis HLB-coated polymer is about 5 to 10 times higher than that obtained from PDMS-DVB and C18 or C30 phase

silica-based coatings. In terms of selectivity, Figure 6-3 shows all four coatings provide better extraction recoveries for PC lipids than Lyso PC lipids with 16:0-18:2 PC as the predominant phospholipids detected in human plasma. The relatively large amount of PC lipids in human plasma compared with LysoPC lipids and the more hydrophobic characteristic of PC lipids could both contribute to the differences of selectivity. However, it should be pointed out that the extraction recovery was relatively constant for 12:0-18:1 PC in all four different fiber coatings.

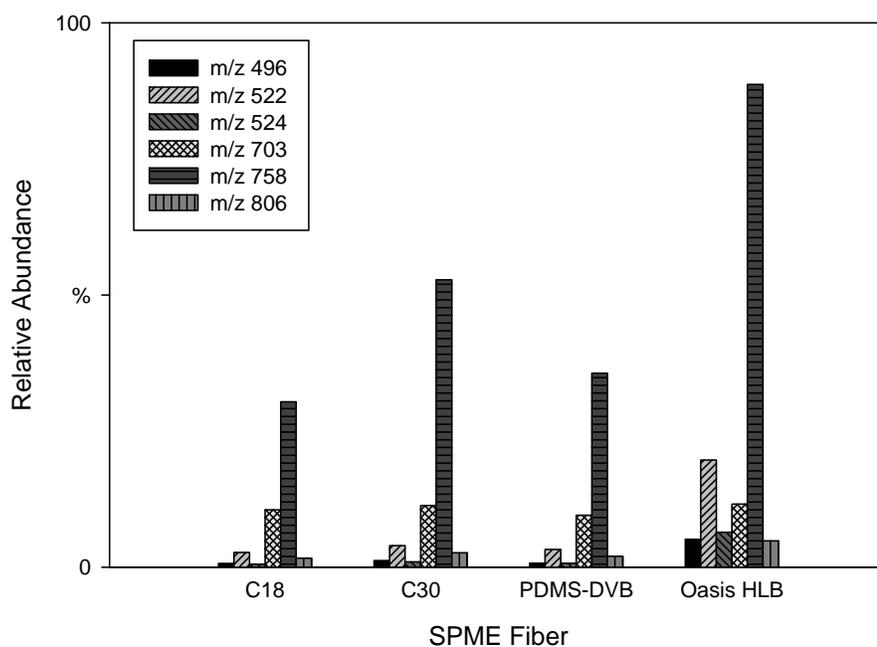


Figure 6-3 Comparison of fiber extraction selectivity for monitored phospholipids using four different types of SPME fibers under same SPME conditions as described in the experimental section. The monitored phospholipids including: 16:0 Lyso PC (m/z 496), 18:1 Lyso PC (m/z 522), 18:0 Lyso PC (m/z 524), 12:0-18:1 PC (m/z 703), 16:0-18:2 PC (m/z 758), and 18:2-22:4 PC (m/z 806).

By using four different fibers of each kind (PDMS-DVB, C18, C30, and Oasis HLB-coated polymer monoliths) for extraction, the fiber-to-fiber coating reproducibility could be evaluated. Good inter-fiber reproducibility is very important for high throughput

sample analysis since multiple fibers will be used simultaneously during extraction. The reproducibility of all types of fibers in phospholipids extraction in human plasma was found to be very good as indicated by R.S.D. values ranging from 3 to 20% shown in Figure 6-4. Many factors could affect the performance of the fiber in biological fluids extraction, and, therefore, the results of fiber-to-fiber reproducibility, such as uniform fiber fabrication during preparation, effect of extraction speed, and carry-over of analyte in the extraction phase, etc. are all important. It is expected that in high throughput multiple fibers SPME analysis, the automation of the coating procedure and sample extraction and desorption will improve inter-fiber reproducibility and consequently system performance. Disposable fibers, with their simplicity of use and low-cost, should be used to completely eliminate carry-over effects.

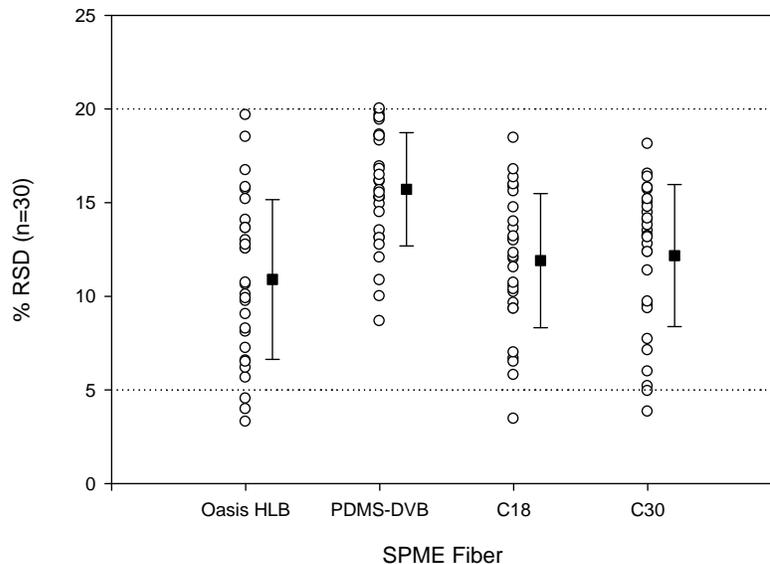


Figure 6-4 Dependence of %RSD (n=30) of five monitored phospholipids extracted using five different fibers at each aspiration/dispense cycle time point (5, 10, 40, 80, 240, and 480) with PDMS-DVB, C18 and C30 phase silica-based coatings, and Oasis HLB-coated polymer monoliths.

The pH-dependency of phospholipids recoveries from human plasma samples has been investigated at different pH conditions using different types of coatings. On average, the extraction recovery for all phospholipids monitored was about 25% higher under basic conditions than that of neutral conditions, while in acidic conditions, the extraction recovery was about 20% lower. The pH effects on recoveries of different phospholipids varied from 9 to 37% in acidic conditions, and from 10 to 47% in basic conditions; LysoPC lipids seemed more sensitive to pH changes than PC lipids. These observations are in agreement with literature that phospholipids could be significantly removed from plasma at acidic conditions during LLE as they are more ionized and, therefore, would favor less non-polar phase during extraction.¹⁸³ Salt effects on phospholipids recoveries were also investigated with different amounts of NaCl in plasma samples. It was found that the extraction recovery of all phospholipids increased as NaCl amount increased. This increase was about 24% in 1% NaCl and 37% in 10% NaCl. Contrary to the pH effects, it was interesting to note that the salt effects were very similar for all phospholipids monitored with relative standard deviation of 6% and 3%, respectively, under two salts conditions for the mean extraction recovery of six phospholipids.

6.3.3 Comparison of SPME with PPT, LLE, and SPE

Since PPT, LLE, and SPE are the most widely used sample preparation methods in bioanalysis, it was interesting to compare the extract cleanness of phospholipids in plasma samples in order to evaluate the matrix effects of using SPME technique compared with other approaches. Two experiments were designed to compare SPME with PPT and SPE, and SPME with PPT and LLE, respectively, and the liquid

chromatographic conditions selected were commonly used in bioanalysis for drug discovery and development.

In the first scenario, Oasis HLB-coated polymer monoliths SPME fiber and Oasis HLB (5 mg) μ Elution SPE plate were chosen for sample preparation along with PPT using 250 μ L human control plasma. SPME was performed at equilibrium to make sure that maximum phospholipids were extracted. To ensure a valid and accurate comparison of the results, some variables were strictly controlled such as the same dry-down, reconstitution step, and final solvent composition of reconstituted extracts and volume of plasma. A gradient separation was performed from 10% to 95% ACN (0.1% formic acid) on a regular C18 column to fully elute the phospholipids monitored with precursor ion scan of m/z 184. Figure 6-5(A) contains the representative TICs of the phospholipids extracted from PPT, SPE, and SPME, respectively. It is very clear that all chromatograms have very similar patterns in the elution window between 2 to 5 minutes; both SPE and SPME methods provide a significantly cleaner extract than PPT, and SPE removes more hydrophobic PC lipids than LysoPC lipids compared with SPME method. MeOH and ACN are often selected as the desorption solvent in SPME. It was found that many of the phospholipids were more soluble in MeOH than ACN and, therefore, using MeOH as desorption solvent in SPME could end up with more phospholipids interferences. In the second scenario, experiments were conducted on a Waters Atlantis HILIC Silica column with mobile phase consisting of ACN:15 mM ammonium formate (pH 3, 80:20, v/v), since more and more hydrophilic interaction chromatography (HILIC)-MS/MS methods have been developed in drug analysis for many polar compounds. A total of 25 μ L of rat plasma was used for sample preparation with the same fibers used for SPME as in

scenario one. LLE was performed by using MTBE as extraction solvent for comparison. Figure 6-5(B) shows that the phospholipids monitored were coming out of the HILIC column very early at a retention window from 0.5 to 2 minutes, and the shape of the chromatograms obtained from PPT, LLE, and SPME, respectively, was almost identical with total peak areas decreasing from PPT to SPME to LLE. This indicated that the total phospholipids extracted from different sample preparation methods were very similar under the current experimental conditions.

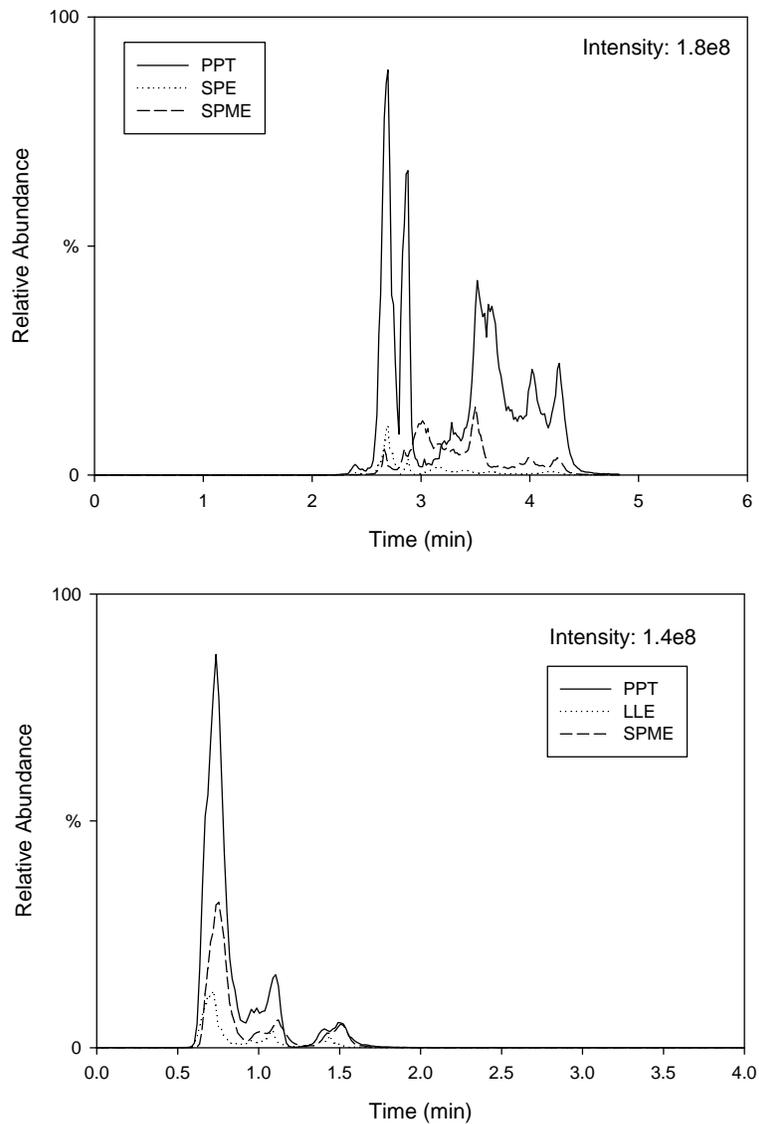


Figure 6-5 Precursor ion scan of m/z 184 monitoring all phospholipids using various sample extraction methods under different chromatographic conditions: (A) 250 μ L human plasma prepared by PPT, SPE and SPME, respectively, with a gradient separation from 10% to 95% ACN (0.1% formic acid) on a regular C18 column; (B) 25 μ L of rat plasma prepared by PPT, LLE, and SPME, respectively, on a HILIC Silica column with mobile phase consisted of acetonitrile (ACN):15 mM ammonium formate (pH 3, 80:20, v/v).

In both scenarios, PPT was identified as the least effective method in terms of sample clean-up followed by SPME, then SPE or LLE. It is believed that in SPE or LLE, the analyte to be extracted is partitioned between sorbent or solvent and a plasma matrix in a buffer with weak eluting strength. The intermolecular forces involved for the retention of analyte with the sorbent or solvent are hydrogen bonding, dipole-dipole forces, ionic interactions, and Van der Waals forces and, therefore, the endogenous interferences could be selectively removed by adjusting eluting solvent strength. Chambers et al.¹⁸⁴ compared various sample preparation techniques including PPT, LLE, and SPE for plasma samples with respect to extract cleanliness, matrix effects, and recovery, and pointed out that both reversed-phase and cation exchange SPE resulted in significantly lower phospholipids levels, and that the most effective sample preparation technique was mix-mode strong cation exchange SPE which combined the retention mechanisms of reversed-phase and ion exchange. SPME is a technique that combines sampling, sample clean-up, and pre-concentration into a single step and on a single device. In contrast to SPE, SPME is also a non-exhaustive extraction method, uses a much smaller volume of sorbent and, therefore, the absolute amount of analytes of interests as well as potential interferences extracted by SPME are much smaller. Theoretically, SPME should provide sample clean-up as effective as or better than SPE with no or minimal matrix effects. Although SPME did not provide cleaner final extracts

compared to SPE or LLE, it should be pointed out that in SPME experiment, no sample pre-treatment was performed, and the elution solvent was either 100% MeOH or ACN. The current study data indicated that matrix effects should be carefully investigated especially in those cases where SPME is used directly without any sample treatment. Since sample preparation is accomplished in one-step, endogenous interfering compounds co-extracted with the analytes will not be removed during sample preparation and could potentially cause matrix effects in bioanalysis.

“Absolute” matrix effects from different biological fluids could be different. A complete validation is required when analytical matrix is changed, for example, from rat plasma to human plasma, or plasma to urine. In the current study, experiments were also performed to compare the relative amount of phospholipids extracted using SPME from different biological matrices including rat, monkey, and dog plasma, as well as human plasma with different anticoagulants, such as Na-heparin or EDTA. Figure 6-6 clearly illustrates the differences of phospholipid contents in different matrices which further demonstrates the necessity of performing a full validation if biological matrix is changed.

6.3.4 Recovery and Matrix Effects

In order to qualitatively assess matrix effects in bioanalytical analysis using SPME, post-column infusion experiments were conducted to graphically illustrate the potential regions of ion enhancement or ion suppression due to the effects of phospholipids on analyte ionization. Recoveries and "absolute" as well as "relative" matrix effects from analytes with a wide range of chemical properties were also measured

and calculated under various chromatographic conditions using different sample preparation methods to further compare SPME with other conventional approaches.

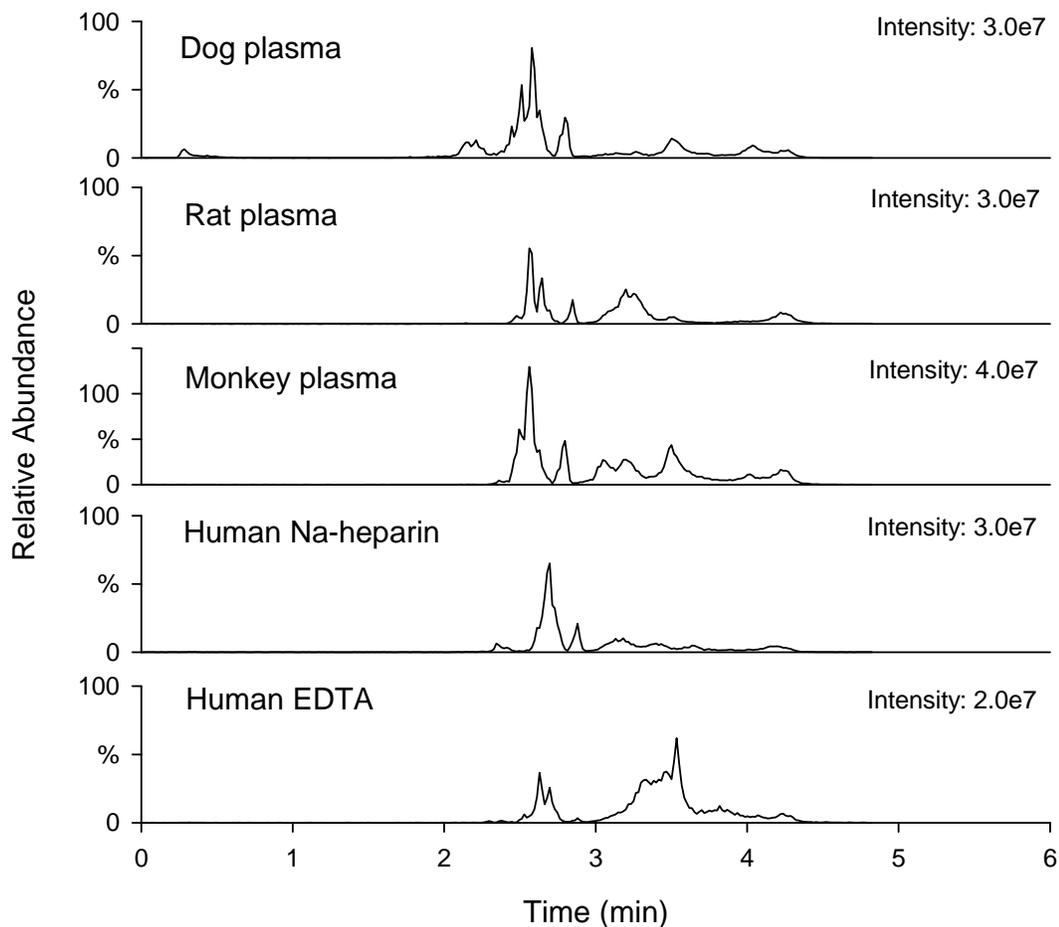


Figure 6-6 Precursor ion scan of m/z 184 monitoring all phospholipids using Oasis HLB-coated polymer monoliths in-tip SPME extracted from 250 μ L dog, rat, monkey, human Na-heparin, and human EDTA plasma, respectively, with a gradient separation from 10% to 95% ACN (0.1% formic acid) on a regular C18 column.

The post-column infusion was performed under two chromatographic conditions as described in detail in the experimental section. The post-column spectra for different analytes after injection of phospholipids were illustrated in Figures 6-7(A) and 6-8(A),

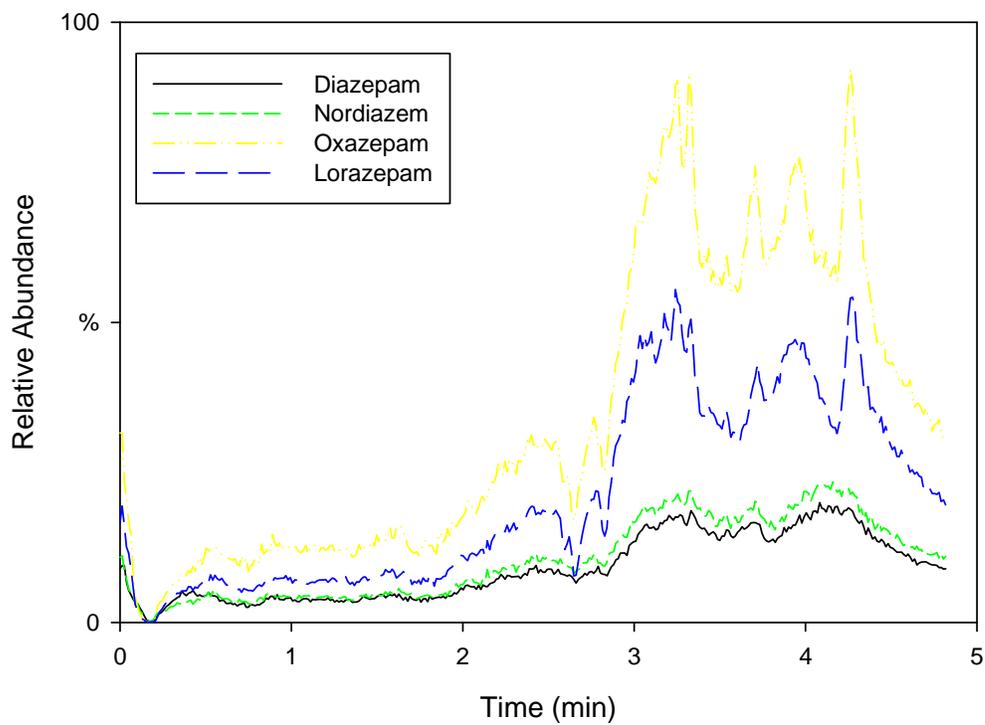
respectively. The spectra clearly indicated the phospholipids suppression effects to the analytes at the regions where phospholipids eluted from the columns under corresponding chromatographic conditions. The matrix effects were compound dependent. In Figure 6-7(A), the effects of endogenous phospholipids on lorazepam and oxazepam were significantly larger than those on diazepam and nordiazepam; while in Figure 6-8(A), significant ion suppression was observed for CIL and the effects of phospholipids on IMP and BLI were relatively small. The recoveries and "absolute" matrix effects for diazepam, lorazepam, nordiazepam, and oxazepam from SPE and SPME using Oasis HLB polymer monoliths and PDMS-DVB are listed in Table 6-2, as well as the corresponding results for BLI, IMP, and CIL obtained from PPT and SPME using Oasis HLB polymer monoliths and 30 phase coated fibers. In order to increase benzodiazepine recoveries, plasma samples were diluted with an equal amount of 25 mM ammonium formate at pH 7 during SPE and SPME sample preparation. Overall, SPE and PPT methods provided higher recoveries (more than 80% for all the compounds) compared with the SPME method. This was not surprising as both methods were exhaustive extraction methods. However, it was interesting to see that Oasis HLB polymer monoliths gave much better recoveries from 4.4 to 23.1% compared with conventional SPME fiber geometry recoveries of 0.9 to 3.9 %. In terms of "absolute" matrix effects, most of the compounds did not show severe decrease or increase from phospholipids regardless of extraction methods, except that oxazepam signal was decreased (71.4%) using Oasis HLB polymer monoliths, and CIL signal was 74.2% and 73.2% using Oasis HLB polymer monoliths and C30 phase coated fibers, respectively. However, significant matrix effects were observed for CIL with 56% ion suppression in PPT extraction method. It was interesting

to note that the slope of the calibration curve constructed in PBS buffer was very similar to that in plasma for the four different types of coatings, which indicated that phospholipids in plasma samples did not alter the interactions between analytes and adsorption-type coatings. The "relative" matrix effects of phospholipids on analyte quantification were measured by determining the precision of the slopes of five standard curves in five different lots of plasma. It is generally accepted that the relative standard deviation should not exceed 3-4% for the method to be considered reliable and free from "relative" matrix effects issues. The results for diazepam, lorazepam, nordiazepam, and oxazepam quantifications using Oasis HLB polymer monoliths are summarized in Table 6-3. The relative standard deviation of standard line slopes were less than 4% for all four compounds which indicated that the SPME method was free from matrix interferences in the calibration range from 5 to 1000 ng/mL. By scrutinizing the individual accuracy data, it was noticed that, in general, the relative standard deviations at each concentration were larger for lorazepam and oxazepam than those for diazepam and nordiazepam. The quantitative results agreed very well with experimental observations when endogenous phospholipids were monitored simultaneously for four compounds with MRM transitions. The chromatograms shown in Figure 6-7(B) exhibited that the four compounds were completely separated from the phospholipids with representative 16:0 LysoPC and 18:0 LysoPC that were eluted earlier than other monitored lipids. It was anticipated that the analytes should not be affected by lipids interferences; this was verified from their more precise quantitative results. The larger inter subject variations for lorazepam and oxazepam might be due to the higher sensitivity of these two compounds to the lipid interference, which is shown in Figure 6-7(A). Similar correlations between slope

variations and the interference from phospholipids are shown in Table 6-4 from the quantitative results of BLI, IMP, and CIL determination using Oasis HLB polymer monoliths in rat plasma, and illustrated in Figures 6-8(A) and 6-8(B) with chromatographic separations. The slope standard deviations for BLI, IMP, and CIL using SPME method were 10.4, 10.1 and 8.8%, respectively, with a calibration range from 0.5 to 100 µg/mL for all three compounds. The relatively large variation in the five curves for CIL appeared to be due to the matrix interferences despite the fact that the analog internal standard almost co-eluted with the analyte. It was very interesting to compare the %CV for absolute peak areas for different compounds and their corresponding internal standards to understand the behavior of internal standards and their compensation of absolute matrix effects. In Figure 6-9, absolute %CV differences were obtained for each compound by calculating the differences between %CV of absolute peak areas at each concentration point from five calibration curves and %CV of absolute peak areas of internal standards. An isotope-labeled internal standard diazepam-*d*₅ was used for diazepam, nordiazepam, lorazepam, and oxazepam; while for BLI, IMP, and CIL, two analog internal standards were applied. It was evident that the labeled internal standard compensated for matrix effects as demonstrated by the high precision of slopes in five different lots of plasma for benzodiazepines; on the other hand, the analog internal standards did not behave the same way as the drug compounds during sample extraction for BLI, IMP, and CIL. It is generally accepted that in most cases, the utilization of stable isotope-labeled internal standards effectively eliminates relative matrix effect liability. In the case of the four benzodiazepines studied here, the absence of a “relative” matrix effect for diazepam is not surprising since a labeled internal standard (diazepam-*d*₅) was

utilized. For three other diazepam, the absence of a “relative” matrix effect using as internal standard diazepam- d_5 is clear, but probably due to chance and not the design since the diazepam- d_5 served as any other internal standard analog. As it was clearly concluded in earlier bioanalytical studies¹²³ using PPT, SPE, and LLE extraction methods, it is highly recommended that in SPME bioanalysis isotope-labeled internal standard be used for each analyte considering low recovery of analytes and potential co-extraction of matrix components.

(A)



(B)

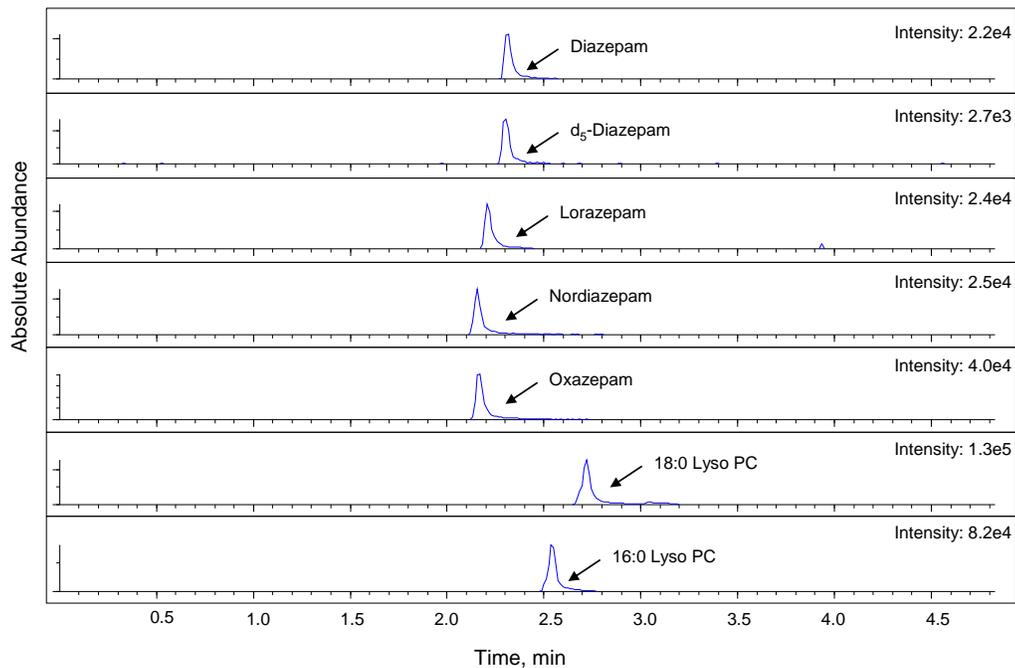
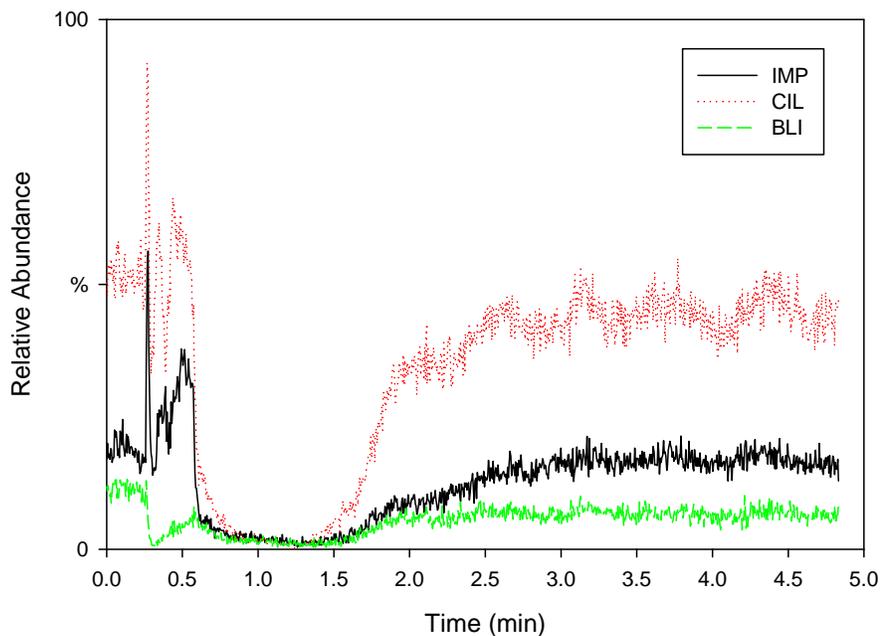


Figure 6-7 (A) Post-column infusion spectra of a solution containing diazepam, nordiazepam, oxazepam, and lorazepam at a concentration of 1 $\mu\text{g}/\text{mL}$ for each analyte after injection of plasma supernatant under a gradient elution from 10% to 95% ACN (0.1% formic acid) with a C18 column; (B) Chromatograms of diazepam, nordiazepam, oxazepam, and lorazepam, as well as phospholipids, 16:0 Lyso PC and 18:0 Lyso PC, under the same chromatographic separation conditions

(A)



(B)

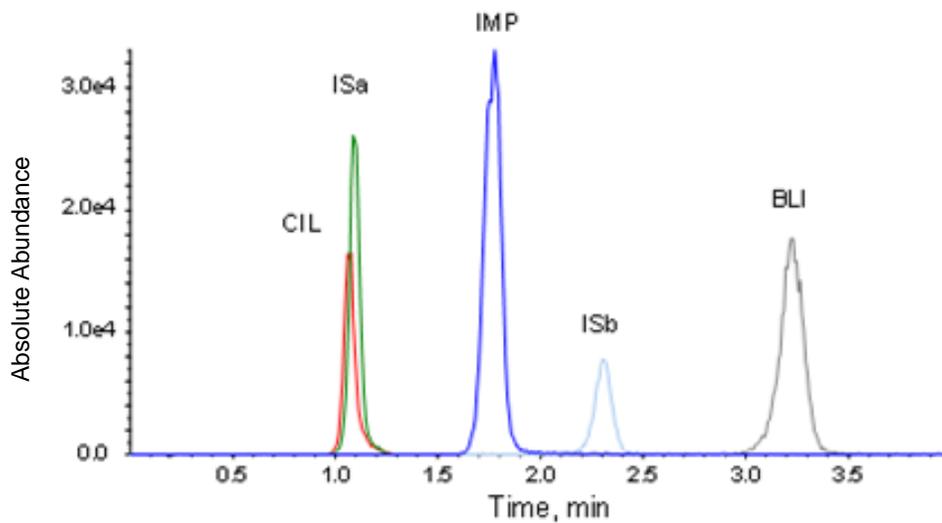


Figure 6-8 (A) Post-column infusion spectra of a solution containing imipenem (IMP), cilastatin (CIL), and MK-4698 (BLI) at a concentration of 1 $\mu\text{g}/\text{mL}$ for each analyte after injection of plasma supernatant under an isocratic elution of acetonitrile (ACN):15 mM ammonium formate (pH 3, 80:20, v/v) with a HILIC Silica column; (B) Chromatograms of IMP, CIL, and BLI under the same chromatographic separation conditions

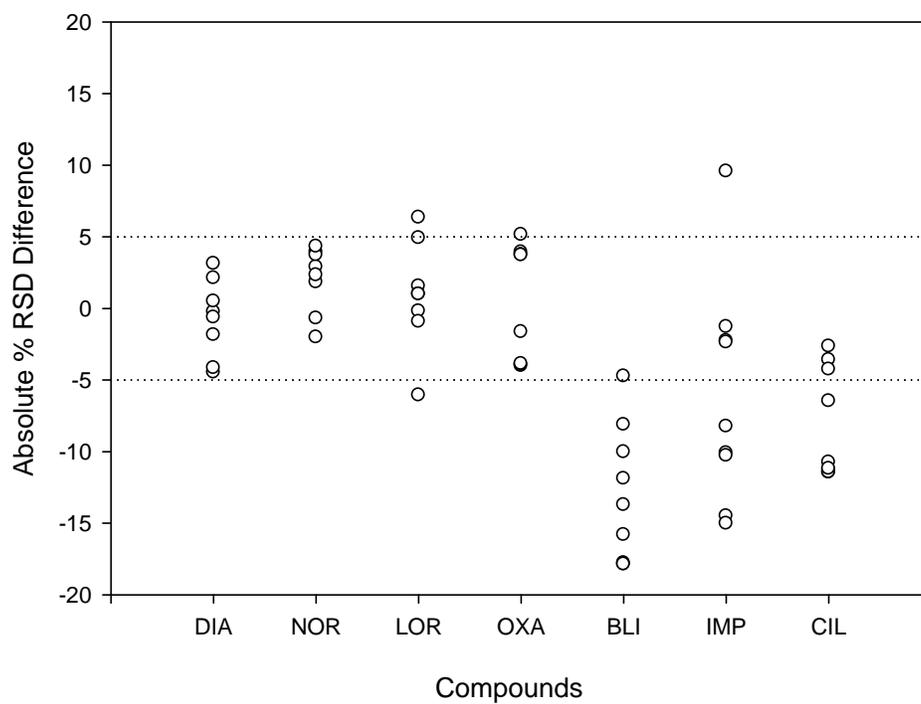


Figure 6-9 Absolute %RSD differences between %RSD of absolute peak areas for drug compounds at each concentration in the calibration curve and %RSD of absolute peak areas of internal standard in five curves validation. DIA (diazepam), NOR (nordiazepam) LOR (lorazepam), OXA (oxazepam), BLI (MK-4698), IMP (imipenem), and CIL (cilastatin)

Table 6-2 Calculated Recovery and “Absolute” Matrix Effects for Various Compounds using Different Sample Preparation Methods

Analyte ^a	Recovery (%)		Matrix effects (%)	
	Oasis HLB (SPME)	PDMS-DVB (SPME)	Oasis HLB (SPME)	PDMS-DVB (SPME)
Diazepam	46.5 [9.7]	12.3 [9.2]	96.2 [7.1]	91.6 [6.8]
Lorazepam	18.6 [3.2]	3.7 [24.0]	88.1 [13.9]	111.2 [18.2]
Nordiazepam	22.2 [7.0]	5.8 [15.5]	91.7 [13.6]	108.9 [13.6]
Oxazepam	13.7 [8.7]	3.3 [27.0]	71.4 [11.7]	98.1 [21.7]
Analyte ^b	PPT	Oasis HLB (SPME)	PPT	Oasis HLB (SPME)
Imipenem (IMP)	98.7 [2.4]	10.3 [8.4]	117.3 [17.1]	101.2 [16.6]
Cilastatin (CIL)	98.8 [2.7]	9.3 [7.5]	72.0 [25.9]	65.1 [26.7]
MK-4698 (BLI)	99.2 [1.9]	15.8 [3.6]	126.0 [9.3]	111.9 [12.8]

^a Recovery and “absolute” matrix effects were evaluated at 0.1 µg/mL for benzodiazepines, and three concentrations for BLI, IMP, and CIL in five different lots of plasma (0.5, 5 and 50 µg/mL for PPT; 1, 10, and 100 µg/mL for SPME)

^b To avoid analyte loss because of hydrolysis in biological matrices, rat plasma samples were treated with a stabilizing solution prepared by combining 1 M MES (pH 6.0) buffer with 50% ethylene glycol at 1:1 (v:v) ratio for BLI, IMP, and CIL

Table 6-3 Calibration Curves for the Determination of Diazepam, Lorazepam, Nordiazepam, and Oxazepam in Five Different Lots of Human Plasma using SPME

Nominal Conc. (ng/mL)	Accuracy (%) ^a [%CV] (n= 5)			
	Diazepam	Lorazepam	Nordiazepam	Oxazepam
5	101.1 [11.7]	99.1 [12.5]	103.2 [2.4]	101.5 [14.5]
10	99.2 [3.8]	98.1 [5.2]	92.5 [1.9]	95.6 [10.8]
20	95.9 [1.5]	102.7 [5.2]	98.6 [2.8]	99.4 [2.1]
50	105.2 [3.0]	112.9 [11.5]	109.6 [5.5]	111.5 [10.1]
100	99.9 [3.7]	98.4 [4.5]	100.4 [6.5]	96.3 [7.6]
200	96.1 [0.6]	96.7 [7.7]	95.4 [6.3]	99.2 [10.1]
500	102.4 [1.9]	99.8 [6.5]	101.9 [5.4]	100.8 [11.4]
1000	100.3 [2.1]	92.4 [2.4]	98.4 [3.8]	95.6 [5.4]
r ² ^b	0.9998	0.9986	0.9996	0.9993
%CV ^c	0.8	1.6	1.3	4.0

^a Expressed as [(mean calculated concentration)/(nominal concentration)] × 100%

^b Linear regression of peak area ratio of analyte/internal standard vs. concentration (x), y= intercept + slope * x, using 1/x² weighing factor, with correlation of coefficient (r²)

^c Coefficient of variation (%CV) for slopes of five standard curves

Table 6-4 Calibration Curves for the Determination of BLI, IMP, and CIL in Five Different Lots of Rat Plasma using SPME

Nominal Conc. (µg/mL)	Accuracy (%) ^a [%CV] (n= 5)		
	Imipenem (IMP)	Cilastatin (CIL)	MK-4698 (BLI)
0.5	99.5 [4.3]	108.4 [10.9]	103.4 [8.8]
1	97.9 [6.5]	105.6 [4.0]	102.1 [4.3]
2	98.9 [4.3]	102.2 [4.4]	100.6 [4.7]
5	100.7 [6.6]	105.4 [5.2]	104.2 [6.2]
10	99.0 [4.3]	100.4 [3.0]	98.7 [3.6]
20	99.0 [2.5]	99.9 [3.0]	97.8 [2.3]
50	106.1 [4.3]	96.2 [5.8]	96.7 [2.7]
80	102.0 [9.8]	93.1 [7.3]	98.5 [8.2]
100	97.9 [10.1]	89.9 [9.3]	96.9 [12.0]
r ² ^b	0.9975	0.9982	0.9972
%CV ^c	10.1	8.8	10.4

^a Expressed as [(mean calculated concentration)/(nominal concentration)] × 100%

^b Linear regression of peak area ratio of analyte/internal standard vs. concentration (x), y= intercept + slope * x, using 1/x² weighing factor, with correlation of coefficient (r²)

^c Coefficient of variation (%CV) for slopes of five standard curves

6.3.5 Matrix Effects Evaluation in SPME Methods Validation

For MS based bioanalytical assays, matrix effects should be investigated to ensure that precision, selectivity, and sensitivity will not be compromised.¹⁸⁵ Although there are no specific guidelines from the FDA on how matrix effects should be evaluated during method validation, a common practice to assess matrix effects is to use post-column infusion with post-extraction spiked samples according to a comprehensive literature overview of validated HPLC-MS/MS methods for analysis of drugs in biological fluids.¹⁷⁵ However, there are only a few papers using SPME in drug analysis in which matrix effects were evaluated.^{105,110} During a new SPME method development, researchers focus on factors such as fiber coating, extraction mode, and agitation method selections; extraction and desorption conditions optimization; distribution constants calculations and extraction time profile determinations, as well as calibration method selection, etc. In fact, in most cases, SPME method validation is based on a single lot of matrix with analysis of multiple independent standard curves.⁷⁸ In recent years, one of the major fundamental advances in bioanalytical applications of SPME is the development of *in vivo* SPME which allows for direct immersion of the SPME fiber into a living system for the direct extraction of the analyte, thereby eliminating the requirement to remove a representative sample of biofluid or tissue from the living system. Despite the great potential applications of *in vivo* SPME in determination of drug pharmacokinetic properties in biological species, this very promising technique, since its first application was published in 2003, has not been widely accepted and all the related research work is limited to one laboratory.¹⁸⁶⁻¹⁸⁸ One of the reasons is due to the complexity of applying this technique in routine analysis. On the other hand, it has to be pointed out that the lack

of solid and comprehensive validation of the assay is another major reason of preventing its wide utilization, especially in the pharmaceutical industry. *In vivo* SPME assay validation has to take into consideration inter- and intra-species matrix variability as in most preclinical and clinical studies different animal species and/or human subjects are involved. Last but not least, development of an *in vivo* SPME assay using one single fiber without any process automation is practically impossible.

Based on the results of this study, it has been demonstrated that SPME did not provide the cleanest sample extraction method in terms of extracting endogenous components such as phospholipids from biological fluids. Thus, matrix effects cannot be neglected in SPME assay validation. Matrix effects should be thoroughly investigated during assay development and evaluated throughout sample analysis by repetitive analysis of incurred samples. Strategies to reduce or eliminate matrix effects from validation of conventional sample preparation methods, such as PPT, LLE, and SPE should be applied in SPME methods validation. To evaluate matrix effects, post-column infusion experiment should be conducted initially to directly observe their impact on the chromatographic separation used in a MS based SPME method. Direct comparison using pre-spiking and post-spiking approaches provide the most efficient and straightforward ways to detect and determine the extent of matrix effects since the same amount of analyte and internal standard are prepared in matrix free solvent and different sources of biological fluids. Both recovery and matrix effects can be calculated from the comparison of analyte response between pre-spiking and post-spiking samples, and between post-spiking and matrix-free solvent samples, respectively. It is highly recommended that matrix effects be evaluated not only in five different sources of biological matrix, but also

at all concentrations within the calibration curve range, as matrix effects at low concentrations might be quite different compared with those at higher concentrations. In order to reduce or eliminate matrix effects in SPME, besides approaches such as separation of endogenous components from analytes, preparation of standards and QC samples using pre-dose samples, and selection of different MS ionization modes, efforts should focus on optimization of extraction efficiency to increase SPME assay sensitivity so that a minimum amount of extracts will be introduced into the system. More importantly, a stable isotope-labeled internal standard should be used for each analyte, and when isotope-labeled internal standard is not available, careful studies need to be carried out to select a suitable unlabeled analog internal standard with a matched pKa and logP values and an appropriate concentration level. Parallel SPME sample preparation using multi-well plate technology with automation will generate large amounts of data in SPME method development with significant reduction in sample preparation time and great improvement in method reproducibility and precision compared with the traditional single fiber approach.

6.4 Conclusions

Matrix effects in bioanalysis using SPME should be thoroughly investigated in method development and validation, as the assumption that SPME should provide sample clean-up as effective or better than SPE with no or minimal matrix effects due to its non-exhaustive, equilibrium extraction characteristics is really dependent different experimental conditions. Generally, SPME is less effective than LLE and SPE in terms of cleanliness of removing phospholipids from sample preparation extracts when no sample

pre-treatment is performed and the elution solution is 100% organic solvent, but it is better than the least effective method, PPT. "Absolute" and "relative" matrix effects have been observed in a validated SPME assay and have had direct impact on the precision and accuracy of the method. It has been found that extraction equilibrium was rapidly achieved for phospholipids monitored in all test fibers including PDMS-DVB, C18 and C30 phase silica-based coatings and Oasis HLB-coated polymer monoliths, and phospholipids extraction recovery decreased at lower pH and higher salt concentrations. Automated in-tip SPME provides an efficient way to evaluate matrix effects in bioanalysis over traditional approaches where SPME steps are normally performed manually. Using manual SPME, evaluation of matrix effects would be laborious making this important evaluation impractical and, thus, ignored in pharmaceutical and bioanalytical applications. Strategies to reduce or eliminate matrix effects such as introducing minimum amount of sample and separation of endogenous components should be applied in SPME methods development. The importance of selecting an appropriate internal standard is critical to the success of establishing reliable quantitative SPME-HPLC-MS/MS methods for supporting pharmacokinetic studies.

Chapter 7

Automated In-tip SPME-LC/MSMS for High Throughput Drug Analysis

7.1 Introduction

The limited selection of commercially available SPME coatings are always listed as one of the main reasons that prevent the widespread application of SPME in biological sample analysis.^{189,190} Actually, in the past decades, the development of new coatings is one of the most active areas of SPME studies, many fiber coating techniques¹⁹¹ are now available in the literature including sol-gel coating technology,¹⁹² electrochemical^{193,194} and chemical procedures,¹⁹⁵ and physical deposition of biocompatible materials.⁹⁸ However, compared with the rapid growth of SPE sorbents in the market, the progress of developing commercial available new SPME coatings is not substantial. The initially developed single fiber-SPME format, which remains the most widely used form of SPME technique, apparently, could not meet the needs for high throughput biological sample analysis. The lack of instrumentation to perform SPME process in automated fashion could be a factor for manufactures not willing to produce new SPME coatings because of small market return values. Recently, the automation of SPME in a 96-well plate format was successfully achieved with the design and development of a new SPME robotic station, which is capable of preparing up to 96 samples in an automated way. Some applications of such automated SPME technique include high throughout analysis of drugs in complex biofluids such as whole blood,⁷⁸ automated drug-protein binding studies,¹⁹⁶ and high throughput toxicological screening studies to monitor exposure to various mycotoxins such as Ochratoxin A.¹⁹⁷ In spite of the promise of this automated

approach, some of the limitations of the robotic system such as using a large amount of biological samples make SPME less attractive in routine bioanalysis for drug discovery and development studies. In addition, many advantages of the unique SPME technique including fast sample preparation, solvent-less, and extremes of sample volumes, etc. no longer exist; on the contrary, some principle disadvantages such as relative low recoveries and carry-over effects become more notable. Thus, it is necessary to explore other automation approaches so that SPME can be widely accepted as an alternative method in bioanalytical sample analysis in the pharmaceutical industry.

Automated in-tip SPME technique has been proposed and developed recently and some reviews and studies have been published on this topic.^{142,198} The in-tip SPME technique takes advantage of widely used commercially available automated liquid handling systems, and has coupled the fiber SPME with the system in a unique configuration. In-tip SPME is simple and easy for automation without introducing additional devices, which could be easily adopted by chemists for bioanalytical analysis. More importantly, in-tip SPME maintains the simplicity and advantages of conventional fiber SPME technique, and the approach is emendable to all fibers types possessing a wide range of different coating materials, which will overcome the drawback of limited selections of commercial available fibers and broaden its use with HPLC-MS/MS.

The objective of this study was to systematically evaluate the performance of the in-tip SPME automation using tailor-made Oasis HLB polymer monoliths in-tip fibers. Parameters including uniformity and speed of aspiration and dispense to extraction efficiency, effects of in-tip SPME fibers positions, cross-contamination and carryover, fiber-to-fiber and multiple-extraction reproducibility, and selection of optimal calibration

methods were extensively investigated. Oasis polymer monolith fibers along with commercially available SPME fibers PDMS-DVB were compared to Varian OMIX™ C18 μ -SPE tips to illustrate the advantages and limitations of SPME versus SPE in drug analysis using benzodiazepines as model compounds. Head-to-head comparisons between automation in-tip SPME and other configurations of automated SPME approaches such as blade and thin-film geometries demonstrated that the automation system did not play a critical role in quantitative determination of analytes in biological fluids, and in-tip SPME is apparently simple to use, easy for automation, flexible in fiber coating selections, and cost effective. Strategies for in-tip SPME method development and validation were proposed which would dramatically decrease the method development time than that of any other manual or automated SPME approach. A comprehensive summary to compare SPME with other traditional sample preparation methods including PPT, LLE, and SPE were tried to describe the status and future prospects of SPME in bioanalysis. Some potential applications of in-tip SPME in bioanalysis and directions for SPME automation development were also discussed.

7.2 Experimental

7.2.1 Chemicals and Materials

Diazepam, nordiazepam, oxazepam, and diazepam-*d*₅ were purchased from Cerilliant (Round Rock, TX, USA) as 1 mg/mL methanolic solutions, while lorazepam was purchased as a 1 mg/mL solution in ACN. The initial stock solutions were stored at 4°C in a refrigerator. All HPLC grade solvents, including ACN (0.1% formic acid), H₂O (0.1% formic acid), and 50% ACN (0.1% formic acid), were purchased from Fisher

Scientific (Fair Lawn, NJ, USA). Phosphate-buffered saline solution (PBS, pH 7.4) was prepared by dissolving 8.0 g of sodium chloride, 0.2 g of potassium chloride, 0.2 g of potassium phosphate, and 1.44 g of sodium phosphate in 1 L of deionized water and adjusting the pH to 7.4, if necessary. Deionized water was obtained by passing in-house water through a Millipore Milli-Q plus system (Bedford, MA, USA). Different lots of drug free human plasma were obtained from Biological Specialties Corp. (Lansdale, PA, USA) and stored at -20 °C before use. PDMS-DVB fibers (60 µm) were purchased from Supelco (Bellefonte, PA USA) and OMIX™ C18 µ-SPE tips were obtained from Varian Corp. (Walnut Creek, CA USA). Oasis HLB-coated polymer monoliths in-tip SPME fibers were prepared based on photo-polymerization and the detailed procedures were described previously.¹⁹⁹ 96-Well deep plates (1.2 and 2.4 mL) and mats were purchased from Marsh Biomedical (Rochester, NY, USA). PE frits (25 µM, 6.3 mm in diameter) purchased from Innovative Microplate (Chicopee MA, USA), and non-sterilized polypropylene pipette tips purchased from Tomtec Inc (Hamden, CT, USA) were used to prepare PDMS-DVB in-tip SPME fibers.¹⁴²

7.2.2 HPLC-MS/MS

Tandem mass spectrometry was performed using a Sciex API 3000 triple quadrupole mass spectrometers (Foster City, CA, USA) equipped with a TIS source. The HPLC system included a Perkin-Elmer (Norwalk, CT, USA) LC-200 micro pump with a CTC PAL Leap autosampler (Carrboro, NC, USA) for 96-well plates, and a TLX2 system from Thermo Scientific (Franklin, MA, USA). Chromatographic separation was achieved with gradient elution on a Restek (Bellefonte, PA, USA) BDS Hypersil C18

column (50×2.1 mm, 3 μ m) using mobile phase consisted of ACN (0.1% formic acid): H₂O (0.1% formic acid) at a flow rate of 0.4 mL/min. A "cocktail" of ACN/acetone/IPA (40:40:20, v/v/v) and 50% of ACN (0.1 % formic acid) were used as washing solvents for the autosampler, and the injection volume was 15 μ L. The mass spectrometer sources and compounds acquisition parameters were optimized by infusing a neat solution of a compound prepared in 50% ACN at a flow rate of 20 μ L/min into a mobile phase pumped at 0.2 mL/min through the turbo ion spray interface in the positive mode under MRM conditions. The transitions monitored were 285.0 \rightarrow 154.2 for diazepam, 321.1 \rightarrow 275.1 for lorazepam, 271.0 \rightarrow 140.2 for nordiazepam, 287.2 \rightarrow 241.1 for oxazepam, and 290.1 \rightarrow 154.2 for diazepam-d₅, respectively. The source temperature and ion-spray voltage were set at 450°C and 5000 V, respectively. The curtain, nebulizer, and collision gases were set at 7, 10, and 10, respectively. A Packard MultiPROBE II liquid handling system (Meriden, CT, USA) was used for sample and solution pipetting. In-tip SPME extraction and desorption were performed using a Tomtec Quadra 96 workstation (Hamden, CT, USA).

7.2.3 In-tip SPME Procedures

The automated in-tip SPME procedures have been described in previous chapters. In summary, the Tomtec Quadra 96 workstation was programmed as such that the whole process ran in a sequence: After the tip plate was loaded, 100 μ L of plasma sample was repeatedly aspirated and dispensed from the sample plate for about 40 minutes (320 aspiration/dispense cycles). When extraction was completed, in-tip SPME fibers were washed once with 100 μ L water at the wash reservoir, then 200 μ L of desorption solvent

(ACN with 0.1% formic acid) was aspirated from the desorption solvent reservoir with 50 μL air gap and dispensed into an empty sample collection plate, followed by 100 μL of desorption solvent repeatedly aspirated and dispensed in the same plate for about 5 minutes (40 aspiration/dispense cycles). Finally, the in-tip SPME fibers were moved to the cleaning plate for fiber cleaning. The sample collection plate was evaporated to dryness under heated N_2 stream and reconstituted in 150 μL of 50% ACN, and 15 μL aliquots were injected into the HPLC-MS/MS system.

7.2.4 Sample Preparation

A stock solution (100 $\mu\text{g}/\text{mL}$) of the four analytes, diazepam, lorazepam, nordiazepam, and oxazepam was prepared in 50% ACN from the initial stock solutions originally kept in a refrigerator at 4°C. This stock solution was further diluted with 50% ACN to give a series of working standards with concentrations of 25, 50, 100, 250, 500, 1000, 2500, and 5000 ng/mL . The internal standard (IS, diazepam- d_5) was also prepared as a stock solution (100 $\mu\text{g}/\text{mL}$) in 50% ACN. A working standard solution of 1000 ng/mL of IS was used for plasma samples analyses. All standard solutions were stored at 4°C. Plasma standards were prepared by adding 50 μL of each working standard to 250 μL of human control plasma. The resulting plasma standard concentrations ranged from 5 to 1000 ng/mL . No sample pre-treatment, such as pH adjustment or ionic strength modification, was performed. Working standards preparation, internal standard addition, as well as plasma sample transfer was all performed by a Packard MultiPROBE II liquid handling system to minimize labor intensive. The sample plate should be vortex-mixed thoroughly before being loaded onto the Tomtec workstation.

Three sets of standard samples were prepared to evaluate recovery and matrix effects. The first set of samples was prepared to evaluate MS/MS response for neat standards injected in the mobile phase. The second set was prepared in plasma extracts originating from different plasma sources and spiked after extraction, and the third set was prepared in plasma from the same sources as in second set. The plasma samples were spiked before extraction. Recovery was determined by comparing the mean absolute peak areas of standards obtained from the third set to those from the second set. Matrix enhancement/suppression of ionization or "absolute" matrix effect was evaluated by comparing the absolute peak areas of the standards in post-spiked extraction samples to neat standards.

The precision of the in-tip SPME method using different type of fiber coatings was determined by using replicate analysis (n=5) of benzodiazepines in five different lots of human plasma at all concentrations utilized for construction of the calibration curves. The accuracy of the method was determined as the percentage between the mean concentrations observed and the nominal concentrations.

7.3 Results and Discussion

7.3.1 Performance of In-tip SPME Automation

The evaluations of the performance of the in-tip SPME automation included the uniformity of aspiration and dispense in all wells and impact of the aspiration/dispense speed to extraction efficiency, effect of position of in-tip SPME fibers within wells, potential cross-contamination across wells during automation processes, fiber-to-fiber reproducibility, carry-over effect, multiple-extraction reproducibility, and selection of

optimal calibration methods. The performance of different SPME fiber coatings was also compared with commercially available Varian C18 μ -SPE tips in terms of standard curve validation, recovery, and matrix effects.

7.3.1.1 Uniformity and Speed of Aspiration and Dispense

The uniformity of agitation is critical to SPME method precision especially when pre-equilibrium extraction is applied. Unlike other different agitation methods such as magnetic stirring, sonication, orbital shaking, etc., agitation in in-tip SPME automation is achieved by simply utilizing aspiration and dispense functions of the automation system. Therefore, the uniformity of agitation largely depends on the pipetting precision of the system. It has been reported that for Tomtec Workstation, a %CV of 2 to 3% could be achieved when aspiration and dispense volumes range from 10 to 450 μ L.²⁰⁰ Other factors, such as viscosity of the biological sample and/or fiber to fiber reproducibility, could also contribute to the overall performance of agitation. To evaluate the uniformity of aspiration and dispense, 12 individual Oasis HLB-coated polymer monoliths and PDMS-DVB fibers, as well as Varian C18 μ -SPE tips were placed randomly in a 96-tip plate for sample extraction. Experimental conditions including analyte and internal standard concentrations in each well, sample extraction time, and desorption solvent and time were all kept the same to avoid any differences that could contribute to the precision of the uniformity. 200 ng/mL of diazepam in plasma with diazepam-*d*₅ as internal standard was used in the experiment, and 80 aspiration/dispense cycles were applied with desorption conditions described in the experimental section. It was anticipated that the uniformity of aspiration and dispense should be independent of various tips and the positions of sample wells. This was confirmed by the consistence of %CV values from 12

independent extractions using different types of tips with 2.5%, 2.8%, and 2.4% for Oasis HLB-coated polymer monoliths, PDMS-DVB and Varian C18 μ -SPE tips, respectively, despite that the amount of diazepam extracted was different with different types of coatings/tips.

The speed of aspiration and dispense is another important parameter for in-tip SPME process, faster aspiration allows rapid transfer of analytes from the sample solution to fiber coatings. The faster the speed, the shorter the equilibrium time and the higher the amount of analyte extracted under pre-equilibrium conditions. There are three types of aspiration and dispense speed available, from low, medium, to high in a typical Tomtec Quadra 96 workstation. It was found that there were no significant differences among three different types of speed in terms of the uniformity of aspiration with CV% values between 5.4-9.1% from five individual extractions at each speed using Oasis HLB polymer monoliths. High speed is recommended for in-tip SPME extraction using a Tomtec Quadra 96 workstation to a shorten extraction and desorption time

7.3.1.2 Position of In-tip SPME Fibers

During in-tip SPME sample extraction, 96 tips could be loaded simultaneously by a Tomtec workstation and placed at any position within the wells of a 96-well plate. To investigate the effect of position of in-tip SPME fibers to the extraction variations of intra and inter-wells, three different positions inside the wells were tested: the tips positioned immediately below the sample surface, the tips positioned in the middle of the sample solution, and the tips positioned near the bottom of the well. Since the analyte extraction was accomplished through aspiration and dispense cycles, no differences were observed

on the amount of analyte extracted as long as other parameter were kept the same such as aspiration/dispense cycles, aspiration and dispense volume, as well as aspiration and dispense speed.

7.3.1.3 Cross-contamination and Carry-over

Cross-contamination could be a potential issue for any high throughput methods using an automated liquid handling system. Since sample extraction and desorption processes with in-tip SPME approach are fully automated, it is important to evaluate cross-contamination in each step. According to the sample process procedures described in the experimental section, the main steps of automated in-tip SPME includes tip loading and conditioning, sample extraction, washing, desorption, and tip cleaning. Because sample extraction and desorption steps are accomplished through repeated aspiration and dispense cycles within the wells of the 96-well plate, cross-contamination is unlikely to happen in these steps. However, as the probe with 96 tips is moving from one place to another on the deck of the Tomtec workstation during the whole process, tip dripping from plasma residues or desorption solvent could occur which may cause cross-contamination in certain wells. To eliminate these possibilities, there are two functions of the Tomtec workstation that could be utilized. One is to shake the tip rack to the sidewall of the sample plate after sample extraction to avoid dripping from plasma residues; the other is to apply air gap at the end of each tip during desorption solvent transfer. Experiments were conducted by testing random blank well locations during assay validation with different types of coatings, and no cross-contaminations were observed in any of these blank well samples when these two functions were applied.

In fiber SPME, carryover is inevitable due to the repeated use of a single fiber. Carryover is caused by the incomplete desorption of analyte from the SPME coating, therefore, great effort has to be put in to minimize carryover by developing additional steps in the method to clean the fiber between each extraction. This will definitely affect the overall efficiency of sample throughput even in automated SPME method. Carryover was investigated with different fiber coatings using automated in-tip SPME approach, and the highest concentration diazepam standard in plasma assay validation (1000 ng/mL) was used. It was found that carryover was ranged from 4 to 11% using 100 μ L of ACN (0.1% formic acid) as desorption solvent with 20 times of aspiration and dispense cycles. The degree of the carryover effects was in agreement with our previous observations²¹ which indicated that carryover could not be neglected. Currently, the possible strategies to minimize or eliminate carryover are to explore optimized desorption conditions such as using different solvent combinations and increasing fiber cleaning time with large volume of desorption solvent, or to use disposable fibers, which will be more feasible to apply SPME assay for routine bioanalytical analysis. In this study, carryover was minimized with additional 40 times of aspiration and dispense cycles using 80% MeOH in tip cleaning plate after plasma sample extraction and desorption.

7.3.1.4 Fiber-to-fiber Reproducibility

It is desirable to achieve excellent fiber-to-fiber reproducibility for high throughput sample analysis with multiple SPME fibers in parallel extraction format; however, fiber-to-fiber reproducibility will not impair the precision and accuracy of SPME method if internal standard could compensate any differences among different

fibers, especially when stable isotopic labeled internal standard is applied. This was demonstrated from the uniformity of aspiration and dispenses experiment where in house tailor-made Oasis HLB-coated polymer monoliths, PDMS-DVB and Varian C18 μ -SPE tips were compared. 12 tips of each type were used for extraction in plasma matrix simultaneously; the %CV value from absolute peak areas was 14.9%, 18.6%, and 5.3% for Oasis HLB-coated polymer monoliths, PDMS-DVB and Varian C18 μ -SPE tips, respectively. When labeled compound was used as an internal standard, the %CV value was down to 2.5%, 2.8%, and 2.4% for three different tips, which were fabricated from completely different approaches. Many factors could affect the performance of the fiber in biological fluids extraction and, therefore, the results of fiber-to-fiber reproducibility, such as uniform fiber fabrication during preparation, effect of extraction speed, and carry-over of analyte in the extraction phase, etc. It is expected that in high throughput multiple fibers SPME analysis, the automation of the coating procedure and sample extraction and desorption will improve inter-fiber reproducibility and, consequently, system performance.

7.3.1.5 Multiple-extraction Reproducibility

As a non-exhaustive extraction method, SPME provides very low recovery in comparison with other sample preparation methods such as LLE and SPE. Sensitivity is often a critical issue in SPME method, which may prevent its application in determination of target analyte at very low concentrations. This disadvantage could be overcome by multiple solid phase extraction (MSPME), a stepped consecutive extraction procedure that could result in either an increase in extraction yield or a decrease in extraction time because of the exponential relationship between the time and the

extracted amount. Based on previous studies^{201,202} with multiple extractions under non-equilibrium conditions, considerably less time is required to obtain an extraction amount that is equal to that of one extraction at equilibrium. On the other hand, the extraction yield can be increased if multiple extractions are performed with the same total time as is needed for one extraction at equilibrium time. This interesting feature is extremely useful in compensating the disadvantage of relatively low recovery of SPME especially when extraction time is very long in order to achieve equilibrium. However, MSPME has not been widely used, as expected, mainly because of the intensive labor and lack of reproducibility of using one single fiber manually. A comparison experiment was performed using automated in-tip SPME approach to evaluate the reproducibility and sensitivity between single SPME and multiple SPME. Ten individual PDMS-DVB fibers were used to extract benzodiazepines solutions from plasma samples at a concentration from 5 to 5000 ng/mL in triplicate, the aspiration and dispense cycles were 1×80 times and 4×20 times, respectively. For desorption, the total aspiration and dispense cycles was kept the same between single SPME (1×40 times) and multiple SPME (4×10 times). It was found that the extraction amount increased consistently across the concentration range for each compound in multiple SPME. The mean absolute peak area ratios between multiple and single SPME with %CV values from 10 concentration points for diazepam, lorazepam, nordiazepam, oxazepam, and internal standard, d₅-diazepam were 2.7 (6.8), 2.8 (4.8), 2.7 (5.6), 2.8 (3.6), and 2.6 (10.0), respectively.

7.3.1.6 Selection of Optimal Calibration Methods

Selection of an appropriate calibration method is essential in high throughput bioanalytical analysis. This is even more important in multiple fibers parallel extraction as the fiber-to-fiber reproducibility could be significantly improved with the utilization of stable isotopic labeled internal standard as discussed previously. Several calibration methods have been proposed in the literature⁶⁷ for quantitative determination of analyte concentrations in SPME, such as external calibration, standard addition, internal standard, standard-in-fiber calibration, and equilibrium extraction calibration. In high throughput biological sample analysis using SPME, the best approach is to use internal standard calibration because it is simple and easy without requiring any additional experiments. However, stable isotopic labeled internal standard is not always available and analog compound has to be used which may not be able to compensate the different variations during sample preparation and analysis. Figure 7-1 compares the results obtained from 12 random selected fibers of Oasis HLB-coated polymer monoliths and PDMS-DVB, as well as Varian C18 μ -SPE tips for the extraction of diazepam using different calibration methods tested (1) calibration without internal standard, (2) standard-in-fiber calibration, and (3) d_5 -diazepam as the internal standard.

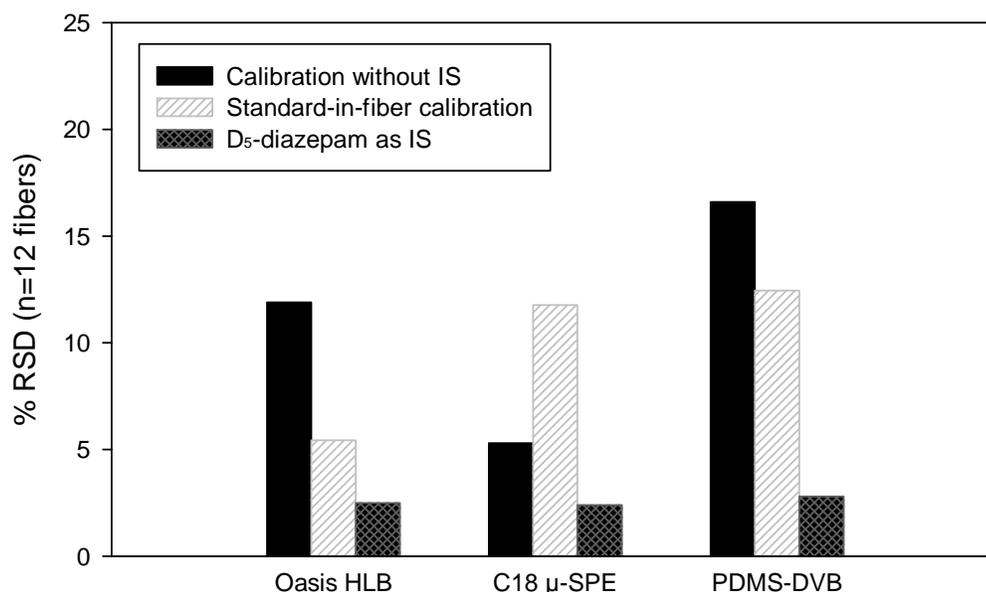


Figure 7-1 Comparison of calibration without internal standard, standard-in-fiber calibration and D₅-diazepam as internal standard calibration methods for the determination of diazepam using different SPME fibers and C18 μ-SPE tips

7.3.1.7 Overall Performance of Different SPME Fibers and Varian C18 μ-SPE Tips

Representative extraction time profiles for diazepam using Oasis HLB-coated polymer monoliths, PDMS-DVB fibers, and Varian C18 μ-SPE tips are depicted in Figure 7-2. Equilibrium was reached quite rapidly for Varian C18 μ-SPE tips in 40 aspirations/dispenses cycles, which were about 5 minutes; whereas for SPME fibers, equilibrium was not reached even after 320 aspirations/dispenses cycles in both cases. This clearly indicated the different extraction mechanism between exhaustive extraction of SPE and equilibrium extraction of SPME. The extraction recoveries from C18 μ-SPE tips for benzodiazepines were generally higher than those from SPME fibers (Table 7-1), however, the relatively lower values compared with other SPE methods were probably

because the elution conditions were not optimized.²⁰³ It was very promising to notice that Oasis HLB-coated polymer monoliths provided from 13.7 to 46.5% recoveries for SPME where absolute recoveries were often less than 1% for many SPME fibers. The larger surface areas of polymer and the weak intermolecular interactions and hydrophobic interactions because HLB particles all played important roles in increasing analyte extraction efficiency. No significant matrix effects were observed for SPME fibers in benzodiazepines as shown in Table 7-1, the absolute matrix effects were in the range between 71.4 and 111.2%. It was unexpected that C18 μ -SPE tips shown relative larger matrix effects than SPME fibers with ion enhancement for diazepam and ion suppression for lorazepam, nordiazepam, and oxazepam.

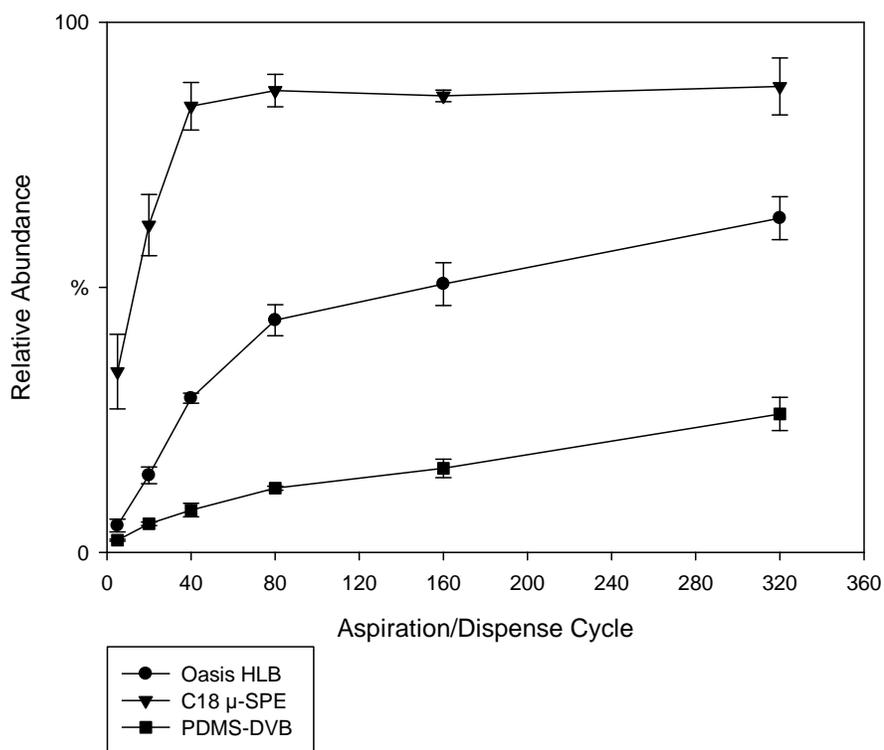


Figure 7-2 Extraction time profile of 200 ng/mL diazepam in human plasma using Oasis HLB-coated polymer monoliths, PDMS-DVB fibers, and Varian C18 μ -SPE tips under conditions as described in the Experimental Section

Table 7-1 Calculated Recovery and Matrix Effects for Various Compounds using SPME Fibers and Varian C18 μ -SPE Tips ^a

Analyte	Recovery (%)			Matrix effects (%)		
	Oasis HLB	PDMS-DVB	Varian C18 μ -SPE	Oasis HLB	PDMS-DVB	Varian C18 μ -SPE
Diazepam	46.5 [9.7]	12.3 [9.2]	73.9 [10.4]	96.2 [7.1]	91.6 [6.8]	110.5 [9.0]
Lorazepam	18.6 [3.2]	3.7 [24.0]	34.6 [8.1]	88.1 [13.9]	111.2 [18.2]	69.2 [8.2]
Nordiazepam	22.2 [7.0]	5.8 [15.5]	46.5 [14.2]	91.7 [13.6]	108.9 [13.6]	72.8 [11.2]
Oxazepam	13.7 [8.7]	3.3 [27.0]	26.4 [7.0]	71.4 [11.7]	98.1 [21.7]	62.8 [17.1]

^a Recovery and “absolute” matrix effects were evaluated at 0.1 μ g/mL for benzodiazepines in five different lots of human control plasma under conditions as described in the experimental section

The selected conditions for the determination of benzodiazepines in human plasma were partially validated to compare the suitability of using Oasis HLB-coated polymer monoliths, PDMS-DVB fibers, and Varian C18 μ -SPE tips for quantitative bioanalytical analysis. Five standard curves were prepared in five different lots of human control plasma based on what was proposed by Matuszewski et al. for bioanalytical assay validation.^{89,90} Under the same LC-MSMS conditions, for Oasis HLB-coated polymer monoliths and Varian C18 μ -SPE tips, the calibration curves were linear in the range of 5-1000 ng/mL using 100 μ L of plasma for all benzodiazepines; and in the range of 20-1000 ng/mL for PDMS-DVB fibers. As shown in Tables 7-2 and 7-3, for all compounds and all levels, accuracy of the results does not deviate more than 15% from nominal concentration and precision is within 20% for both Oasis HLB-coated polymer monoliths and Varian C18 μ -SPE tips, which demonstrates that both are suitable for high throughput quantitative bioanalysis. On the other hand, the accuracy results from PDMS-DVB are not very satisfactory according to SOPs for method validation of bioanalytical

assays even the calibration range is less than 4 times comparing with that of using Oasis HLB-coated polymer monoliths (Table 7-4).

Table 7-2 Calibration Curves for the Determination of Diazepam, Lorazepam, Nordiazepam, and Oxazepam in Human Plasma using Varian C18 μ -SPE Tips

Nominal Conc. (ng/mL)	Accuracy (%) ^a [%CV] (n= 5)			
	Diazepam	Lorazepam	Nordiazepam	Oxazepam
5	100.7 [5.9]	93.2 [11.0]	101.0 [11.3]	95.8 [2.2]
10	101.9 [6.6]	108.1 [9.9]	101.0 [4.0]	105.1 [6.9]
20	94.0 [3.6]	110.1 [8.5]	96.2 [5.9]	107.8 [9.1]
50	100.1 [1.6]	105.9 [2.6]	97.3 [9.7]	100.4 [6.5]
100	97.1 [1.2]	101.6 [14.9]	96.8 [5.6]	98.9 [11.9]
200	95.0 [0.4]	85.0 [11.3]	89.4 [11.3]	85.3 [10.0]
500	103.6 [2.0]	96.5 [12.6]	103.7 [7.7]	99.3 [9.5]
1000	107.5 [2.8]	99.6 [17.8]	114.5 [3.4]	107.4 [13.7]
R ² ^b	0.9991	0.9988	0.9958	0.9969

^a Expressed as [(mean calculated concentration)/(nominal concentration)] \times 100%

^b Linear regression of peak area ratio of analyte/internal standard vs. concentration (x), y= intercept + slope * x, using 1/x² weighing factor, with correlation of coefficient (r²)

Table 7-3 Calibration Curves for the Determination of Diazepam, Lorazepam, Nordiazepam, and Oxazepam in Human Plasma using Oasis HLB SPME

Nominal Conc. (ng/mL)	Accuracy (%) ^a [%CV] (n= 5)			
	Diazepam	Lorazepam	Nordiazepam	Oxazepam
5	101.1 [11.7]	99.1 [12.5]	103.2 [2.4]	101.5 [14.5]
10	99.2 [3.8]	98.1 [5.2]	92.5 [1.9]	95.6 [10.8]
20	95.9 [1.5]	102.7 [5.2]	98.6 [2.8]	99.4 [2.1]
50	105.2 [3.0]	112.9 [11.5]	109.6 [5.5]	111.5 [10.1]
100	99.9 [3.7]	98.4 [4.5]	100.4 [6.5]	96.3 [7.6]
200	96.1 [0.6]	96.7 [7.7]	95.4 [6.3]	99.2 [10.1]
500	102.4 [1.9]	99.8 [6.5]	101.9 [5.4]	100.8 [11.4]
1000	100.3 [2.1]	92.4 [2.4]	98.4 [3.8]	95.6 [5.4]
R ² ^b	0.9998	0.9986	0.9996	0.9993

^a Expressed as [(mean calculated concentration)/(nominal concentration)] × 100%

^b Linear regression of peak area ratio of analyte/internal standard vs. concentration (x), y= intercept + slope * x, using 1/x² weighing factor, with correlation of coefficient (r²)

Table 7-4 Calibration Curves for the Determination of Diazepam, Lorazepam, Nordiazepam, and Oxazepam in Human Plasma using PDMS-DVB SPME

Nominal Conc. (ng/mL)	Accuracy (%) ^a [%CV] (n= 5)			
	Diazepam	Lorazepam	Nordiazepam	Oxazepam
20	112.2 [1.7]	113.5 [4.4]	108.6 [8.0]	114.8 [8.9]
50	83.8 [0.8]	83.7 [1.2]	92.3 [3.7]	88.2 [4.3]
100	98.6 [1.0]	91.2 [13.2]	95.2 [0.5]	90.8 [13.9]
200	103.7 [1.4]	114.6 [0.5]	104.2 [3.0]	112.8 [0.3]
500	94.6 [2.4]	106.8 [1.4]	99.4 [0.9]	103.9 [0.6]
1000	107.4 [1.6]	90.7 [9.8]	100.7 [0.5]	90.3 [5.1]
R ² ^b	0.9958	0.9888	0.9998	0.9913

^a Expressed as [(mean calculated concentration)/(nominal concentration)] × 100%

^b Linear regression of peak area ratio of analyte/internal standard vs. concentration (x), y= intercept + slope * x, using 1/x² weighing factor, with correlation of coefficient (r²)

In summary, the performance of Oasis HLB-coated polymer monoliths is comparable to that of Varian C18 μ -SPE tips in quantitative drug analysis using benzodiazepines as model compounds. Varian C18 μ -SPE tips provided relatively better extraction recovery and less extraction time to achieve required assay sensitivity, but with relatively larger matrix effects. In general, extraction efficiency is fairly low from fiber based extraction such as PDMS-DVB, which further demonstrates that SPME is more suitable for drug analysis in PK studies when dose levels are relatively high, unless more specific and selective coatings or other types of SPME fiber configuration such as Oasis HLB-coated polymer monoliths using photo-polymerization are developed. Carryover using 1000 ng/mL of diazepam extracted from 250 μ L of plasma was found to be 4.1, 10.6, and 5.8 after the initial desorption with ACN (0.1 % formic acid) as desorption solvent for Oasis HLB-coated polymer monoliths, PDMS-DVB and Varian C18 μ -SPE tips, respectively. Although Varian C18 μ -SPE tips are not designed to be used repeatedly, it was found that the extraction amount did not decrease up to 5 times in plasma samples, however, tip blocking was observed for several tips where no sample extraction occurred. The phenomenon was not observed in both SPME coatings and the tips were used more than 10 times. It can be concluded that in-tip SPME has the ability to handle very complex samples such as whole blood¹⁴ with no sample pretreatment, which results in significant timesavings, versus μ -SPE tips, which require sample pretreatment in general.

7.3.2 Comparison of Different Automated SPME Approaches

The automation of SPME in a 96-well plate format was successfully achieved

with the first commercially available SPME robotic station (Concept 96, PAS Technology), which is capable of preparing up to 96 samples in a fully automated way. Both fiber geometry and thin-film configuration was applied in the automation processes for high throughput parallel SPME sample preparation. A thorough evaluation of the Concept 96 autosampler was given to describe the advantages and limitations of the system in bioanalytical analysis. Head-to-head comparisons between automated in-tip SPME and other SPME configurations, such as blade and thin-film geometries using Concept 96 autosampler were performed in terms of SPME conditions, automation performance, and assay validation results.

7.3.2.1 Evaluation of Concept 96 Autosampler

The first prototype of Concept 96 consisted of a three-arm robotic auto-sampler that was fully controlled with Concept software and two orbital agitators.⁷⁸ One XYZ arm of the auto-sampler was to hold, transport, and position the 96-fiber SPME device for the extraction and desorption steps of SPME. The second arm was equipped with a N₂ below-down device to perform solvent evaporation and analyte pre-concentration steps if necessary. Finally, one XYZ arm was equipped with a 250 µL syringe with dual function of liquid/solvent dispensing and direct injection of samples into LC-MSMS instruments. The initial multi-fiber SPME device was based on a commercially available pin-tool replicator to support different types of SPME coatings¹⁰⁹ and the replicator was attached to the one of auto-sampler arms with the appropriate screws. A new custom-built multi-fiber SPME device was later developed to accommodate 0.061" thickness of fibers in order to increase surface area of coatings, as well as to improve the robustness of the automated system since fiber binding was encountered using the pin-tool replicator.

Recently, the configuration of the SPME device has been changed from commonly used fiber or blade geometry to thin-film geometry and 96 thin-film devices are commercially available from PAS technology.^{65,66}

It was very clear that the original idea of the Concept 96 was aimed at achieving a higher degree of automation and high sample throughput of SPME. However, the system was not designed in a way that sample preparation and injection could be performed simultaneously and, therefore, sample high throughput could not be achieved as the system claimed. In addition, since the system had to be connected to a LC-MSMS system for sample injection after sample preparation, it was not good laboratory practice from a safety point of view as the whole system was in an open environment, especially when biological samples and organic solvents were used in sample preparation process. In Concept 96, the sample plate was vibrated by the orbital agitator at a certain speed; fiber bending could not be avoided using the similar size of any conventional SPME fibers; thus, in order to immobilize multi-fibers, in either blade or thin-film geometry, the size of the individual fibers was much larger. The increasing of extraction phase volume and surface area resulted in higher extraction efficiency (higher absolute recovery) and enhanced extraction rate; on the other hand, large volumes of biological samples and desorption solvents were necessary to completely immerse the full length of the coated blade/thin-film in order to achieve reasonable method accuracy and precision. For example, 800 μL of blood sample and 800 to 1000 μL of desorption solvent were used in one of the reported studies,⁷⁸ while in current bioanalytical analysis, sample volumes from 25 to 100 μL were routinely used in drug discovery and development, and the

consumption of organic solvent less than 500 μL was quite often in PPT and SPE methods.

7.3.2.2 Comparison of In-tip SPME, Blade SPME and Thin-film SPME

The performance of automated in-tip SPME approach was compared against blade and thin-film geometries using Concept 96 automation system. A detailed comparison is summarized in Table 7-5 including (1) SPME conditions, such as fiber coating type, sample volumes, sample pretreatment, extraction and desorption time, etc; (2) LC-MSMS conditions; (3) automation performance, such as fiber position, agitation speed, sample throughput, and inter-fiber reproducibility, etc, and (4) validation results.

In terms of sample throughput, all approaches are compatible with 96 samples prepared in about 90-100 minutes, and the main steps including fiber precondition, extraction, and desorption are fully automated. Inter-fiber reproducibility significantly improved with the utilization of an internal standard in both in-tip and blade geometries, which indicated that different automation systems did not play critical roles in the performance of various multi-fiber SPME configurations. Assay validation was performed independently for in-tip SPME using Oasis HLB polymer monoliths in plasma and for blade-geometry SPME using RP-amide C16 in blood. For in-tip SPME, the results of five-curve validation prepared in five different lots of plasma are very encouraging with linear range of 5-1000 ng/mL for all benzodiazepines, and the overall intraday accuracy and precision is 92-113% and 1-14%, respectively. Absolute recoveries are about 14-46% and the absolute matrix effects are 71-96%. For blade-geometry SPME using Concept 96 auto-sampler, five-curve validation was prepared in two different lots of blood, the linear range for diazepam and nordiazepam is from 4-1000 ng/mL, and 4-

500 ng/mL for lorazepam and oxazepam. The overall intraday accuracy and precision is 87-111% and 2-17%, respectively, with absolute recoveries about 10-30%, and the absolute matrix effects about 95-102%. The relative deviations from nominal concentrations of benzodiazepines in five-curve validation using different approaches are shown in Figure 7-3. Assay validation was not conducted with thin-film geometry using Concept 96 system; however, because of the increase of absolute recovery, a low limit of quantification (LLOQ) of 0.2 ng/mL could be achieved, and the %CV of inter-fiber reproducibility is about 7% without utilizing any internal standard when 96 fibers were extracted in PBS buffer.

The biggest advantages of blade or thin-film geometry using Concept 96 system include the dramatic increase of absolute extraction recovery compared with conventional SPME fibers and robustness and reusability of the fiber coatings. For example, absolute recovery of diazepam is about 50% using C18 thin-film which is excellent for a microextraction; consistence extraction efficiency and reusability of C18-PAN coated thin-film fibers for more than 100 extractions of diazepam from PBS and human plasma have been observed. In addition, blade or thin-film geometry is particularly suitable for complex biological matrix, such as blood, tissues, etc. without any additional sample pretreatment. The main limitations of the above automated approach are that a large volume of biological samples and desorption solvent has to be used in order to achieve acceptable accuracy and precision and the the automation processes are totally dependent on Concept 96 system which is not very well designed and is not compatible to the widely used commercially available liquid handling systems in the pharmaceutical industry.

Table 7-5 Comparison of Different Automated SPME Approaches for Benzodiazepines Determination in Biological Fluids

Parameter	In-tip SPME	Blade-geometry SPME	Thin-film SPME
SPME conditions			
Analyte	Benzodiazepines	Benzodiazepines	Benzodiazepines
Matrix	Plasma	PBS/Blood	PBS/Urine
Fiber coating	Oasis HLB polymer monoliths	RP-amide C16	C18
Sample volume	250 μ L	800 μ L	1000 μ L
Fiber pre-conditioning	<5 min, 50% ACN	30 min, 50% MeOH	15 min, 50%MeOH
Sample pre-treatment	No	No	No
Internal standard	D ₅ -diazepam	D ₅ -diazepam	--
Extraction time	40 min	30 min	25 min
Desorption time	5 min	30 min	30 min
Desorption solvent	100 μ L ACN	800 μ L 50% ACN	1000 μ L 80% MeOH
Evaporation/reconstitution	Yes/150 μ L 50%ACN	No	No
LC-MSMS conditions			
Column	Restek BDS Hypersil C18 (50 x 2.1 mm, 3 μ m)	Waters Shield RP18 (50 x 2.1 mm, 5 μ m)	Waters Shield RP18 (50 x 2.1 mm, 5 μ m)
Mobile phase	A: H ₂ O (0.1% formic acid) B: ACN (0.1% formic acid)	A: 90% H ₂ O/10% ACN (0.1% acetic acid) B: 90% ACN/10% H ₂ O (0.1% acetic acid)	A: 90% H ₂ O/10% ACN (0.1% acetic acid) B: 90% ACN/10% H ₂ O (0.1% acetic acid)
Flow rate	0.4 mL/min, gradient	0.5 mL/min, gradient	0.5 mL/min, gradient
Injection volume	15 μ L	20 μ L	20 μ L
MS conditions	API 3000, TIS, positive	API 3000, TIS, positive	API 3000, TIS, positive
Automation performance			
Fiber position	Non critical	Critical	Critical
Agitation speed	Aspiration/dispense cycle	Orbital agitation, 850rpm	Orbital agitation, 850rpm
Sample throughput	96 samples/90 min	96 samples/100 min	96 samples/95 min
Instrumentation	No extra device, coupled directly with commercially available liquid handling systems	Utilizing customer designed "The Concept 96 robotic station"	Utilizing customer designed "The Concept 96 robotic station"
Inter-Fiber reproducibility	15% RSD (n=96, no IS, in plasma); 6% RSD (n=96, with IS, in plasma)	12% RSD (n=96, no IS, in PBS); 7% RSD (n=96, with IS, in PBS)	7.1% RSD (n=96, no IS, in PBS)
Validation results			
Calibration range	5-1000 ng/mL in plasma	4-1000 ng/mL (diazepam and nordiazepam); 4-500 ng/mL (lorzepam and oxazepam) in blood	0.2-500 ng/mL in PBS
Intraday accuracy	92-113%	87-111%	--
Intraday precision	1-14%	2-17%	--
Absolute recovery	14-46%	10-30%	17-51%
Matrix effects	71-96%	95-102%	--
Carry-over	Non, disposable	<0.3% in PBS	<0.3% in PBS

In-tip SPME demonstrated its unique automated approach of directly coupling any SPME fibers with commercially available automation systems, such as Tomtec, without introducing any additional devices. More importantly, in-tip SPME maintains the simplicity and advantages of conventional fiber SPME technique, and the approach is amendable to all fibers types possessing a wide range of different coating materials, which will overcome the drawback of limited selections of commercial available fibers and broaden its use with HPLC. This feature is extremely useful in the processes of developing automated SPME methods because different multiple fibers could be evaluated simultaneously for the determination of optimal extraction efficiency which will dramatically reduce the total method development time. Currently, two formats are commonly used for in-tip SPME, one is fiber-packed in-tip, where SPME fibers are put inside the pipette tips; the other is sorbent-packed in-tip, such as Oasis HLB polymer monoliths where extraction sorbent is prepared inside the tips through photopolymerization. For fiber-packed in-tip SPME, low recovery is still the biggest concern in the determination of target analyte in a very low concentration unless very selective fiber coatings are developed, otherwise, it may only be applicable as an alternative approach in pharmaceutical biofluid sample analyses to support PK studies at higher doses of analytes. Sorbent-packed in-tip SPME has shown great potential in high throughput drug analysis because of its simplicity and easy to fabricate, with a low-cost, enhanced extraction recovery, and no carry-over effects if used as a disposable. In addition, the SPME method selectivity could be greatly improved with a wide range of chemistries for reactions and enhanced mass transfer in polymer monoliths preparation.

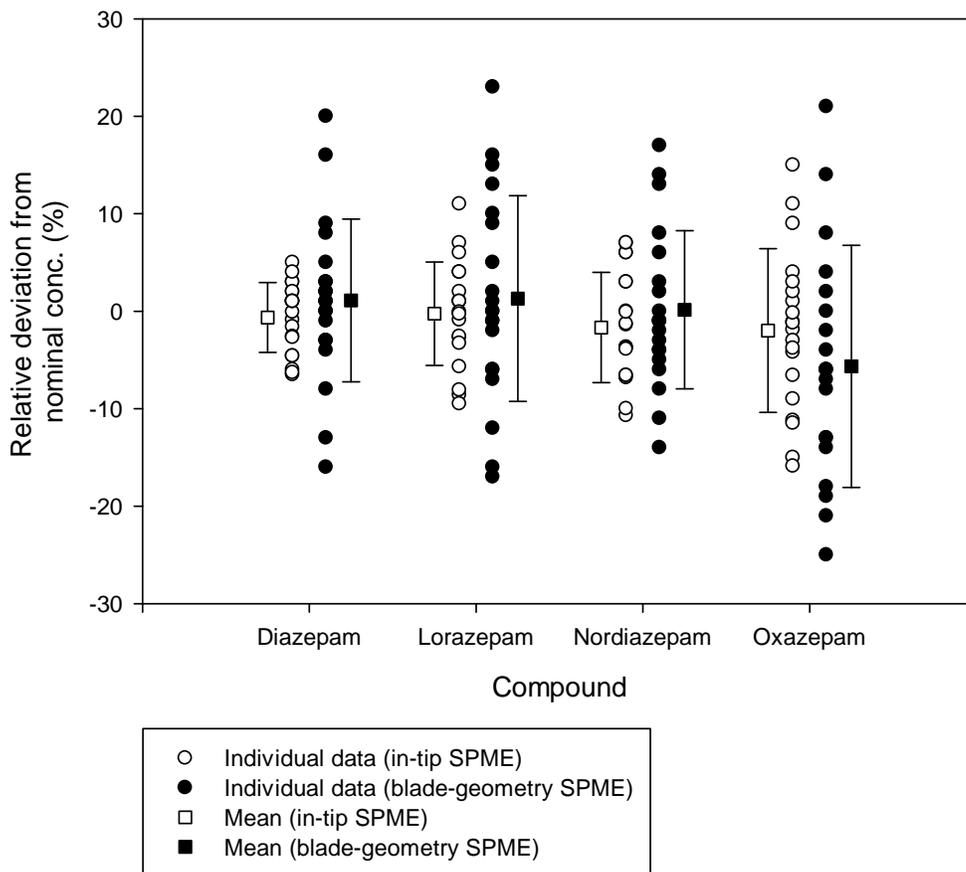


Figure 7-3 Comparison of relative deviations from nominal concentrations in 5-curve validation of benzodiazepines using in-tip SPME (plasma) and blade-geometry SPME (blood)

Vuckovic et al. listed detailed troubleshooting procedures for automated thin-film SPME for high throughput analysis of biological fluids using Concept 96 system.¹⁹⁶ Some of the potential problems during SPME experiment such as sample or solvent spills, particle loss from the coatings, and improper thin-films lineup in the center of the wells are all due to the specific design of the Concept 96 system. However, these issues will

never be encountered with in-tip SPME approach, which is a biggest advantage because it is essential to have simple, reproducible, and robust methods in routine drug analysis.

7.3.3 Strategies for In-tip SPME Method Development and Validation

SPME is a simple, solvent-free extraction technique, which combines sampling, sample clean up, and pre-concentration into a single step. However, SPME method development is tedious and time consuming because many parameters and steps are usually required to be investigated when a new SPME method is developed.¹⁹⁰ Using the traditional approach with one single SPME fiber, it is practically impossible to investigate so many parameters in a short period of time, which prevents its applications in routine bioanalytical analysis. Since automated in-tip SPME has demonstrated great potential to be used for high throughput quantitative determination of drugs in bioanalysis, it is necessary to develop some new strategies to minimize labor intensive and to shorten the time in method development and validation. These strategies will mainly focus on method development scheme and key experiments involved to generate different SPME conditions simultaneously and systematic procedures in SPME assay validation for quantitative drug analysis.

Table 7-6 listed some typical parameters and steps for optimization in SPME method development and validation. For high throughput drug analysis using automated in-tip SPME-LC/MSMS, some of the parameters are usually not necessary to be evaluated such as extraction mode, separation and detection method, agitation method and analyte derivatization, etc. This will dramatically simplify the whole process for

SPME method development and, therefore, the total method development time will be compatible to that of other sample preparation approaches such as LLE and SPE.

For automated in-tip SPME methods development, the following experiments should be considered and performed in sequence:

A. Evaluate different fiber coatings and optimize fiber extraction conditions. In the experiments, various SPME fibers are screened in biological matrix under three conditions: no sample treatment, pH adjustment, and salt effects. Sample volumes should start with 50 or 100 μL . The preliminary information on recovery and matrix effect could also be obtained if sample plate layout is arranged as shown in Figure 7-4. In addition, analyte extraction in matrix free samples such as PBS buffer could also be evaluated. A combination of following parameters could be selected as the default conditions: extraction time (30 minutes), desorption time (10 minutes), extraction speed (high), desorption solvent (100 μL of ACN, 0.1% formic acid), washing step between extraction and desorption (50 μL of water, 0.1% formic acid). In SPME method development, extraction at equilibrium is not necessary as long as enough sensitivity has been achieved. In general, the extraction time should not exceed 60-min otherwise SPME should not be considered if all other options such as fiber coatings, pH adjustment, salt effects, sample volume, etc. have been explored. Fiber conditioning procedure should follow the requirements from different fibers.

	Fiber #1				Fiber #2				Fiber #3			
Pre-Spike	X	A	B	C	X	A	B	C	X	A	B	C
	X	A	B	C	X	A	B	C	X	A	B	C
	X	A	B	C	X	A	B	C	X	A	B	C
Post-spike	X	A	B	C	X	A	B	C	X	A	B	C
	X	A	B	C	X	A	B	C	X	A	B	C
	X	A	B	C	X	A	B	C	X	A	B	C
Neat	Neat	Neat	Neat		Neat	Neat	Neat		Neat	Neat	Neat	

Note: Treatment X: PBS buffer pH 7.4
Treatment A: No sample treatment
Treatment B: pH adjustment
Treatment C: Salt effect
Neat: neat standard solution

Figure 7-4 96-well plate layout for evaluation of recovery and matrix effects under different experimental conditions with various SPME fibers using automated in-tip SPME

B. Select one (or two) fibers with the best extraction efficiency from section A and perform experiments of optimization on desorption conditions and sample volume, as well as evaluation of carry-over effects. Different compositions of desorption solvent mixtures such as MeOH/water, ACN/water, ACN/water/0.1% acetic acid or any other combinations should be evaluated. Desorption solvent that is compatible to the mobile phase will be helpful to achieve acceptable peak shape and eliminate the need for an evaporation/reconstitution step if no pre-concentration is required. The total desorption time should be less than extraction time with typical 10 to 30-min. Effect of sample volume of extraction efficiency may not be dramatic when 25-250 μ L of biological fluids are evaluated, which also depends on the magnitude of analyte distribution constant

between fiber and matrix. Carry-over should be assessed by injecting an extracted double blank immediately after an extracted highest standard and the analyte response in the double blank should not exceed 20% of the LLOQ. Disposable tips should be considered if carry-over is difficult to minimize with too much effort.

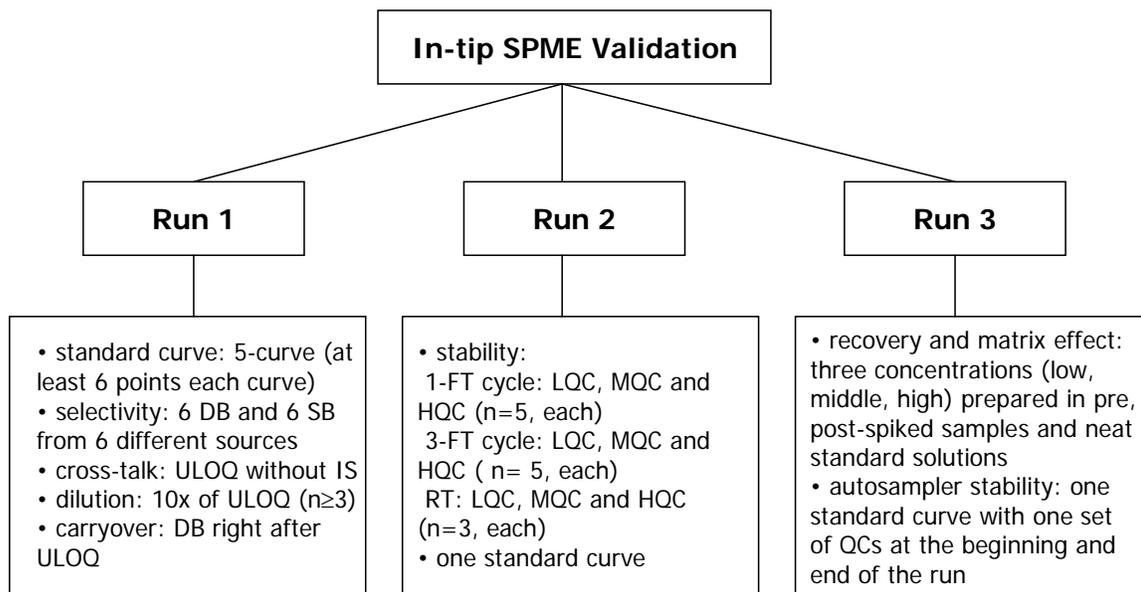
C. Select the fiber with the most optimized extraction and desorption conditions to evaluate linear curve range with an appropriate calibration method. The stability of quality control (QC) samples could also be evaluated in this step with one selected concentration point. Assay sensitivity could be increased by adding an evaporation/reconstitution step if all other parameters are optimized, or by increasing injection volume with no significant matrix effects. QC samples should be prepared as close as possible to real samples with the amount of organic solvent in the spiked biological samples kept below 1% (vol/vol). However, for calibration standard samples preparation, incubation time is not necessary to establish binding equilibrium between analyte and biological protein as pointed in many other studies^{105, 196} as make-up solvent will be added to real biological samples during sample preparation.

D. Perform in-tip SPME method validation according to different requirements for discovery and development assays. Currently, there are no standard validation procedures for bioanalytical assays before drug development stage in industry, while in clinical sample analysis; assay validation should follow FDA guidelines. In general, selectivity, sensitivity, standard curve, intraday variation, recovery, stability, matrix effect, sample dilution, and carryover should be determined during assay validation procedures. To assess intraday precision and accuracy, at least five replicates of standard curves should be prepared and analyzed. The precision value for these standard curve slopes should be less than 3-4% for the method to be considered free from

“relative” matrix effect. Stable isotopic labeled internal standard is highly recommended if it is available. Recovery experiments should be performed at three concentrations (low, middle, and high), and results reported as a mean concentration at each level obtained from five different lots of matrix. Storage stability of the analyte in the biological matrix and the influence of freeze-thaw cycles should be examined by using a set of QC prepared by spiking biological fluids with analyte to yield low QC (within 3x the concentration of the LLOQ), middle QC (near the center), and high QC (near the upper boundary) of the standard curve. All validation procedures can be accomplished with three experimental runs as shown in Figure 7-5.

Table 7-6 Typical Parameters and Steps that Require Optimization during Traditional SPME and In-tip SPME Method Development Procedures

Typical parameters and steps for optimization in SPME method development and validation	Parameters optimization for in-tip SPME method development and validation	Comments
<ul style="list-style-type: none"> ○ selection of fiber coating ○ selection of extraction mode ○ selection of separation and detection method ○ selection of agitation method ○ selection of derivatization reagent, if required ○ optimization of sample volume ○ optimization of extraction conditions (pH, ionic strength, temperature) ○ optimization of water content and organic solvent content ○ determination of extraction time ○ optimization of desorption conditions ○ selection of calibration method ○ method validation 	<ul style="list-style-type: none"> ○ selection of fiber coating ○ optimization of sample volume ○ optimization of extraction conditions (pH, ionic strength) ○ determination of extraction time ○ optimization of desorption conditions ○ selection of calibration method ○ method validation 	<ul style="list-style-type: none"> ○ in-tip SPME is normally performed with direct extraction due to polar compounds for majority of drug candidates ○ HPLC/UPLC-MSMS is the best option in terms of column separation and sensitivity ○ sample aspiration and dispense is the only process for in-tip SPME sample extraction ○ temperature and derivatization are normally not considered in method development



Note: DB: double blank, control blank biological sample without drug and internal standard (IS);
 SB: single blank, same as DB except with IS;
 ULOQ: upper limit of quantification;
 FT: freeze thaw, quality control (QC) samples should be stored at -20°C or other temperatures adequate for long-term storage of clinical specimens for at least 24 hr and thawed unassisted at room temperature (RT) for the first FT cycle. When completed thawed, the samples should be refrozen for at least 12 hrs under same conditions and thawed a second time. The FT cycle should be repeated and then analyzed on the third cycle;
 LQC, MQC and HQC: low QC (within 3x the concentration of LLOQ, low limit of quantification); middle QC (near the center); HQC (near the upper boundary)
 RT stability: QC samples should be thawed at RT and kept for at least 4 hr or longer before analyzed.

Figure7-5 Experimental runs for fully in-tip SPME assay validation

7.3.4 Potential Applications and Future Directions of In-tip SPME in Bioanalysis

To understand the advantages and limitations of SPME in bioanalysis would be extremely helpful for the development and application of SPME automation, especially compared with other commonly used sample preparation techniques, such as PPT, LLE, and SPE. Many reviews have been published on similar topic; however, there is no comprehensive summary available to compare SPME with other approaches in terms of method development and assay performance. In addition, many claimed advantages of SPME may not be applicable in routine quantitative drug analysis, and a big gap still exists between applications of SPME in the laboratory and in the pharmaceutical industry. Automated in-tip SPME demonstrated a great potential to bridge this gap and to overcome the challenges of applying SPME as an alternative approach for high throughput bioanalysis, while still maintaining the simplicity of SPME technique.

Table 7-7 summarized method development and assay performance based on PPT, LLE, SPE, and SPME, respectively. A decision tree for sample preparation method selection is also illustrated in Figure 7-6. Despite the facts that PPT provides very poor sample clean up which may results in significant matrix effects; LLE and SPE requires more solvent, significant method development time, and considerable sample pre-treatment, in reality, PPT is the main sample preparation method in use, about 90% in drug discovery bioanalysis; and SPE is always the solution when PPT and LLE cannot meet the assay requirement in clinical sample analysis. Although there are many claimed advantages of SPME over traditional techniques for bioanalytical applications, such as very clean sample extracts, similar sample throughput, capable of handling complex, heterogeneous samples including whole blood without any sample pre-treatment, ability

to obtain free and total concentration from a single biofluid sample, and very low cost of analysis per sample, up to now, SPME technique has not been accepted by the pharmaceutical industry. Considering the requirements and trends in high throughput bioanalytical analysis and the principle disadvantages of SPME technique, it can be concluded that SPME could only be considered as an alternative sample preparation approach, and its applications may be limited to certain areas.

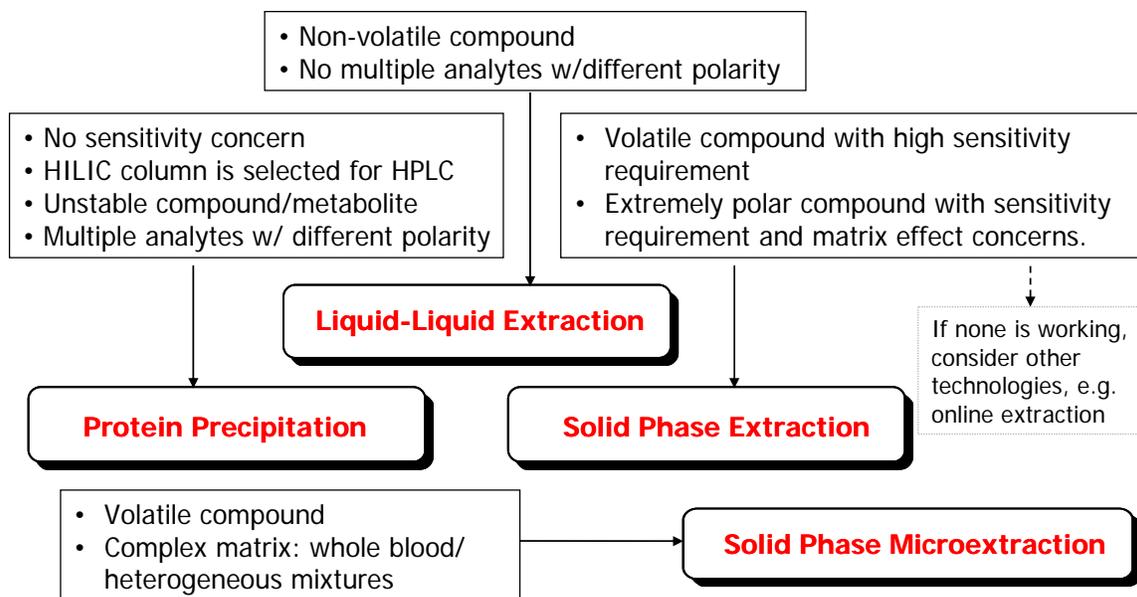


Figure 7-6 A decision tree for sample preparation method selection

Table 7-7 Comparison of Different Sample Preparation Approaches in Bioanalysis

	PPT	LLE	SPE	SPME
Method Development				
Analyte	Useful to multiple analytes/metabolites with different polarities; unstable compounds	Suitable for non-volatile compounds	Applicable to volatile and extremely polar compounds	Good for volatile compounds
Matrix	Plasma, blood, serum, tissue homogenates, in vitro incubation mixtures	Plasma, blood, serum, urine	Plasma, serum, urine, in vitro incubation media	Whole blood, urine, serum, plasma, complex heterogeneous mixtures
Sample pretreatment	Not necessary in general	Requires pH modification to increase analyte recovery	Requires dilution, centrifugation, pH adjustment, and filtration to ensure analyte retention	Simple to no sample pretreatment
Simplicity	Simple, generic, and universal procedure	Relative simple to do as a routine	Versatile, complex, and labor intensive method development, despite generic sorbents and methods have been developed	Relative simple but labor intensive method development
Method development time	No method development time: easy to perform, simply mix solvents, centrifuge/filtration	Moderate method development time: multiple transfer steps and limited amount of non-water soluble extraction solvents, requires to examine the influence of pH, ionic strength, etc.	Long method development time: difficult to grasp the chemistry of the technique due to great selectivity of SPE sorbents and the many choices for manipulating pH and solvent conditions	Long method development time: requires to examine too many parameters including fiber coating, extraction mode, agitation method, pH, ionic strength, extraction time and desorption time and solvent, calibration method, etc.
Automation	Semi-automated procedure as plate sealing, vortex mix and centrifugation steps could not be automated	Semi-automated procedure as plate sealing, vortex mix, centrifugation, and evaporation steps could not be automated	Fully automated and integrated process for on-line SPE; not a fully automated process for off-line SPE	Fully automated and integrated process for in-tube SPME; not a fully automated process for fiber based SPME such as in-tip and thin-film SPME

Assay Performance				
Selectivity	Non selective	Moderate selective (limited in solvent selections)	Very selective	Moderate selective (limited in coating selections)
Sensitivity	Low sensitivity method, typically, 1-10 ng/mL from 50 μ L of sample with LC/MSMS	Low LOD's are possible	Most sensitive sample preparation method	Relatively low sensitive, suitable for low volatile drugs with concentration range from 1 to 100 μ g/mL
Recovery	High recovery (>95%) even with the extent of protein binding exceeds 99%	Analyte recovery depends on sample pH and the characteristics of the organic solvent	High recovery with evaporation steps needed	Considerably low recovery range from 0.1% to 30%
Reproducibility	Less reproducibility, relatively high chances on validation and analytical run failures due to matrix effects and potential recovery losses	Good reproducibility and good chances on successful validation and analytical run	Very good reproducibility and high chances on successful validation and analytical run	Less reproducibility, relatively high chances on validation and analytical run failures due to low recovery and potential matrix effects
Matrix effects	Poor and limited sample cleanup with high potential of significant matrix effects in LC-MSMS analysis	Very clean extracts and relatively low matrix effects with proper selection of organic solvent and adjustment of sample pH	Very selective sample cleanup with minimized matrix effects; most effective method to remove phospholipids in plasma	Matrix effects vary with fiber coatings and more prone to errors due to changes of matrix

Overall				
Advantages	Simple, low cost, fast and universal sample preparation method dominantly used in drug discovery bioanalysis; applicable to very small sample volume (20-50 µL); easy for method transfer; first choice and good starting point for method development	Wide applicability for many drug compounds; clean extracts with good selectivity and sample enrichment; relatively short method development time besides PPT; easy for method transfer; one of the most widely used methods in drug development bioanalysis	Very selective sample extracts with minimized matrix effects; wide variety of sample matrices accepted and applicable for almost all compounds; high recovery with good reproducibility; low sample and solvent volumes; useful for volatile compounds where high assay sensitivity is required; suitable for full automation; easy for method transfer	Rapid and solvent-less sample preparation method with sampling, extraction and concentration in one step; applicable to extreme sample volume and complex, heterogeneous samples such as whole blood without pretreatment; suitable for obtaining free and total concentration information from a single biofluid sample by appropriate calibration strategies, suitable for automation with relatively low cost
Disadvantages	Dirty extracts with potential significant matrix effects; LC column and mass interface fooling due to ineffective protein removal; sensitivity limitations because of no sample pre-concentration; possible low recovery for heat labile compounds when extra dry-down is necessary to increase sensitivity	Very labor intensive and difficult for fully automation; environmental unfriendly because of large amount of organic solvent required, not suitable for volatile and oxygen labile reactive analytes due to a must dry-down procedure; potential emulsion formulation problem	Relative time-consuming in method development because of difficulty to master the theory and usage; potential clogging problems and sample pre-treatment always necessary; by far, the most expensive sample preparation method	Very limited selections of SPME coatings; sensitivity limitation because of low extraction recovery; very time-consuming in method development; lack of enough data to demonstrate the robust and reproducibility of SPME in routine high throughput drug analysis by LC-MS/MS; difficult for method transfer

In the drug discovery process, many compounds have to pass through a series of stages, which represent various *in vitro* and *in vivo* tests in order to qualify to be a development compound. The unique environment of drug discovery requires bioanalytical methods to be able to analyze many samples from many discrete compounds in various biological matrices in a high throughput fashion and a timely manner, hence, it is highly desirable to have a generic sample preparation method regardless of compound properties. Compared with PPT which is the dominant method before any compound reaches development stage, in-tip SPME is not suitable for the drug discovery environment, but could be used in some special cases especially when biological sample volume is extremely small (less than 25 μL); and when complex heterogeneous samples are encountered such as blood and tissue samples. One successful study of using in-tip SPME in drug discovery has been conducted in our laboratory recently when mouse blood volume was only available at 20 μL , SPME demonstrated its advantage over PPT that samples could be repeatedly extracted which was not feasible for PPT method when sample re-assay was needed. However, in-tip SPME technique is more suitable for the drug development environment, especially in later stage clinical studies, where robust clinical assays have been developed and applied to analyze thousands of samples and higher doses are normally selected. The advantages of in-tip SPME such as simplicity, solvent-less, and low cost will be notable over other sample preparation methods.

The capability of determining total and free drug concentrations in the same time is one of the most remarkable advantages that are often claimed for SPME in bioanalytical analysis. However, in the pharmaceutical industry, equilibrium dialysis,

ultrafiltration, and ultracentrifugation are the widely used methods for quantification of the binding of small molecule drug candidates to plasma proteins in drug discovery and development.²⁰⁴ These well established methods with commercially available 96-well devices allow automation and rapid determination for multiple compounds in a batch, and the significant bottleneck of plasma protein-binding (PPB) methods due to the lack of automation does not exist anymore. It is impractical that SPME will be used for high throughput screening purposes given the fact that each compound has to be calibrated in both biological fluid and buffer solution in order to obtain the free drug concentration, let alone that SPME conditions have to be optimized for each individual compound. This explains the situation that up to now the free drug concentration determination using SPME is only limited to laboratory researches.^{187, 205,206} Nevertheless, SPME is very promising for very hydrophobic molecules that have limited water solubility because the free drug is never partitioned into a solution that is void of protein. In addition, for highly protein bound compounds, SPME may be applicable to determine free drug concentration more accurately with very selective fiber coatings. The success of the automation approaches such as in-tip SPME or any other configurations will definitely make microextraction technique more compatible with other widely used approaches.

One of the most potential applications of in-tip SPME is in DBS sampling. Recently, there has been increasing interest in the use of DBS to support PK/TK studies in small molecule drug discovery and development due to low blood volume requirement and the potential for simplified sample collection, storage, and shipment conditions.^{57,58} The standard sample preparation approach for DBS analysis consists of punching out a disk from the card that contains the DBS followed by extraction of the analyte and then

analyzing by LC-MSMS. Although this standard procedure is most likely suitable for most of the compounds, performing this process manually is very time consuming and labor intensive. Automation of the sample preparation process is very necessary and it could be achieved by using different format of in-tip SPME other than fiber-packed in-tip (Figure 7-7A) and sorbent-packed in-tip (Figure 7-7B). Extraction sorbent (disk/film) could be packed within pipette tips as shown in Figure 7-7C, after blood sampling, known aliquot of blood samples will be collected in each individual tip and desorbed simultaneously afterwards for analysis. The whole sample preparation procedure could be easily automated to increase efficiency. In addition, sample throughout could be also achieved by using 96 or even 384 well collection plate. A workflow of using in-tip SPME during DBS sample preparation and analysis is illustrated in Figure 7-7D.

The comparison results from in-tip SPME and rod or thin-film SPME clearly demonstrated that the performance of various automated SPME approaches is not completely dependent on an automation system; on the contrary, limited selection of SPME fiber coatings is still the bottleneck of preventing the technique to be widely accepted in routine bioanalysis. Thus, for future development of in-tip SPME or any other types of automated SPME, commercialization of simple, flexible, low-cost, and reproducible procedures for the preparation of SPME coatings for use with automated multi-fiber SPME is the key to promote the widely applications of the technique. In addition, the applicability of automated SPME for high throughput bioanalytical analysis should not be limited to certain selected compounds and matrices and the assay validation procedures for quantitatively determination of drug development candidates should be thoroughly performed according to FDA guidelines. Communications and collaborations

between researchers and pharmaceutical companies are important and mutually beneficial to fill up the gaps between applications of SPME in the laboratory and in the pharmaceutical industry. Finally, development of user friendly commercial SPME kits for certain applications such as dry blood spots will be an interesting direction for facilitating SPME technique.

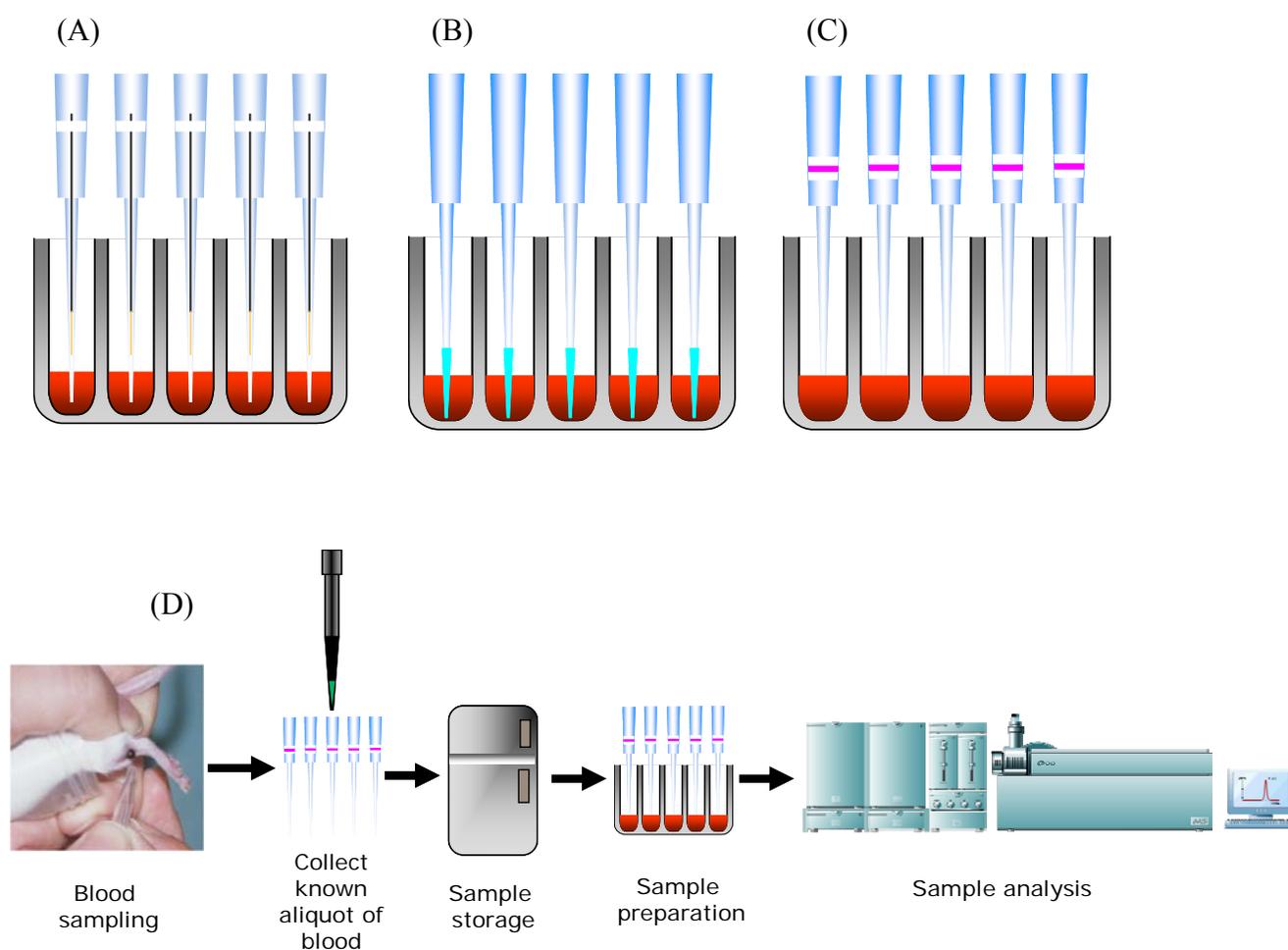


Figure 7-7 Configurations of (A) fiber-packed in-tip SPME, (B) sorbent-packed in-tip SPME, (C) disk/film-packed in-tip SPME, and (D) a workflow of using in-tip SPME in DBS sample preparation and analysis

Chapter 8

SPME Modeling and Simulation

8.1 Introduction

Unlike other exhaustive extraction methods such as SPE and LLE, where the goal is to extract as near as possible to 100% of the analyte from a sample, SPME is an equilibrium extraction. The extraction efficiency is determined by the partitioning of analyte between the sample matrix and the extraction phase. The higher the affinity the analyte has for the extraction phase relative to the sample matrix, the greater the amount of analyte extracted. Partitioning is controlled by the physicochemical properties of the analyte, the sample matrix, and the extraction phase. Typically, SPME extraction is completed when the analyte concentration has reached the equilibrium distribution between sample matrix and the extraction phase, where the amount of analyte extracted is constant and independent of a further increase in extraction time. However, in many cases, the time to reach equilibrium could be more than 10 h, which is impractically long. Therefore, some level of sample agitation is required in order to facilitate rapid extraction and transport analytes from the bulk of the sample to the extraction phase, and sometimes SPME is performed under pre-equilibrium conditions. For fiber SPME, the mass transfer from the sample matrix to the fiber coating is modeled using a zone referred to as the *Prandtl* boundary layer, which is considered as a region where analyte flux is progressively more dependent on analyte diffusion and less on agitation, as the fiber coatings approached. The analyte flux in the bulk of the sample is assumed to be controlled by agitation, and the analyte flux within the boundary layer is assumed to be

controlled by diffusion. The presence of another binding matrix or hydrophobic phase, such as serum protein or humic acids, besides the SPME fiber, may strongly influence the extraction efficiency and complicates the calibration procedure. Binding matrices may interact by adsorbing to the fiber surface, thus, possibly blocking the exchange of analyte across the fiber boundary. This may also lead to an overestimation of the concentration in the fiber coating as the matrix-bound analyte adsorbed to the fiber coating is measured along with the analyte in the fiber coating. Therefore, it would be valuable to have a model that can be used to analyse measured concentrations in the fiber coating in a very complex sample matrix as a function of time.

In SPME, depending on the types of fiber coatings, the extraction mechanism can be distinguished as absorption for liquid coatings and adsorption for solid coatings. When liquid coatings are used, the analytes partition onto the extraction phase and the analyte molecules are dissolved by the coating molecules.^{61,180,207,208} The magnitude of the analyte diffusion coefficient in the liquid coating allows the molecules to penetrate the entire volume of the coating with a reasonable extraction time. On the other hand, solid coatings possess complex crystalline structures which lead to reduced analyte diffusion coefficients within the structure, therefore, the extraction normally occurs on the surface of the coating. Several approaches have been proposed to mathematically model the kinetics of the absorption process to SPME liquid fibers such as PDMS. Vaes et al.⁸⁰ and Heringa et al.²⁰⁹ model the fiber as a classical one compartment, first-order kinetic model with absorption and desorption rate constants as parameters. Such a model uses abstractly defined mass transfer coefficients between medium and fiber which are dependent on both the physicochemical processes and the experimental setup. Although the model is

simple to use, it is not explicitly based on processes like diffusion and partitioning of the analyte and on the experimental conditions such as medium volume and fiber geometry. A mechanistically based modeling approach¹⁸⁰ has also been developed in which the mass transfer of the analyte from the bulk to the fiber coating is considered to be controlled by the molecular diffusion in the stagnant boundary layer around the fiber coating. The influence of the agitation condition on the uptake kinetics can be explained by this model. It can also be applied to predict kinetics based on parameters such as distribution coefficient, diffusion coefficient, and diffusion boundary layer thickness. However, additional parameters, such as fluid linear speed at the fiber surface, the fluid's kinematic viscosity and the diffusion coefficient of the analyte in the medium, are required to determine the boundary layer thickness. Recently, others have introduced mass transfer forced by the concentration difference between bulk medium and outer fiber surface,^{81,82} instead of mass transfer from bulk medium to fiber through an explicitly modeled stagnant layer around fiber. In this approach, the mass transfer coefficient is interpreted as the ratio of the unknown intra-layer diffusion (D) of the analyte and the unknown effective layer thickness (δ), where it is assumed that the diffusion process is instantaneously at steady state. Furthermore, the model includes binding of the analyte to a matrix of bovine serum albumin (BSA) to investigate the possible influence of a binding matrix on the uptake kinetics of analytes into the fiber. In this model, since the fiber coating thickness and the initial concentration of the analyte are known, the model requires the estimation of many parameters including diffusion coefficients, boundary layer thickness, fiber-medium partition coefficient, and association and dissociation constants. These parameters are obtained by fitting the model to the experimental data,

however, from a numerical point of view, the model calculation times or numerical stability can become prohibitive for some combinations of these parameter values.

An understanding of the fundamental of thermodynamics and mass transfer of analytes in multiphase systems will provide insight and direction when developing SPME methods and identifying parameters for rigorous control and optimization. For example, based on the dynamic model proposed by Ai,^{68,69} Chen et al.^{70,210} demonstrated the isotropy of absorption and desorption in the SPME liquid coating fiber and proposed a new concept called "in-fiber standardization technique". Later this was termed as the "kinetic calibration method". The method uses the desorption of standards, which are preloaded in the extraction phase, to calibrate the extraction of the analytes. More recently, the one-calibrant kinetic calibration technique was developed. This desorption of a single calibrant to calibrate all extracted analytes. The technique eliminates the requirement of several isotopical compounds or high-concentration standards, and it simplifies the standard loading and quantitation procedures. Despite all the efforts and successful applications of SPME modeling and simulation, the need still exists to develop simple and accurate models both for liquid coating fibers and for solid coating fibers as well. The increasing computation capabilities and the advances in the application of numerical techniques make it possible to include all transport steps in kinetic modeling and simulation. The effective use of the modeling approach will minimize the number of experiments needed in SPME method development and, therefore, shorten the total cycle time. In the current work, several model approaches were evaluated to investigate the kinetics of SPME extraction of the target analyte in a sample matrix containing dissolved organic matter using both liquid and solid fiber

coatings. Model validation was performed by comparing the predicted results to previous experimental data in the literature. Different applications of SPME modeling approaches, including estimation of boundary layer thickness, time to reach extraction equilibrium, and the total extraction amount as a function of time are discussed.

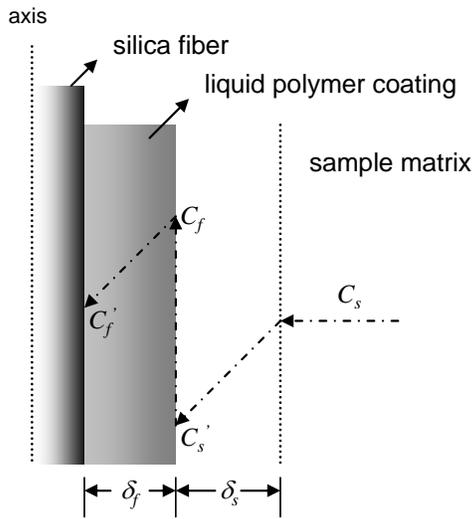
8.2 Theory

When a SPME fiber is exposed to an agitated sample matrix, mass transfer from the sample matrix to the surface of the SPME fiber coating occurs. The analytes can further diffuse from the surface of the fiber coating to its inner layer if the fiber is a liquid coating. The mass transfer of the analytes based on diffusion is considered the rate-determining step, and, thus, the process follows Fick's first law of diffusion:

$$J \equiv \frac{\partial n}{\partial t} = -D_s A \frac{\partial C_s}{\partial x} = -D_f A \frac{\partial C_f}{\partial x} \quad \text{Equation 8-1}$$

where J is the mass flux of the analyte from the sample matrix to the SPME fiber, A is the surface area of the fiber, and ∂n is the amount of the extracted analyte during sampling time ∂t . D_s and D_f are diffusion coefficients of the analyte in the sample matrix and the fiber coating, respectively; C_s and C_f are the concentrations of the analyte in the sample matrix and the surface of the fiber coating, respectively. Figure 8-1 illustrates a schematic of the absorption and desorption processes between liquid fiber and the sample matrix; and Figure 8-2 depicts the adsorption and desorption process between solid fiber and the sample matrix.

(A)



(B)

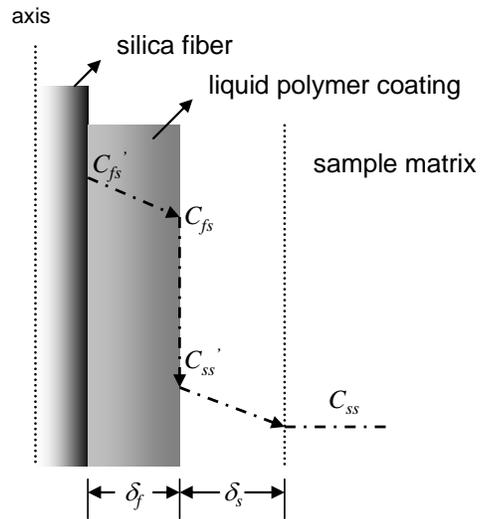
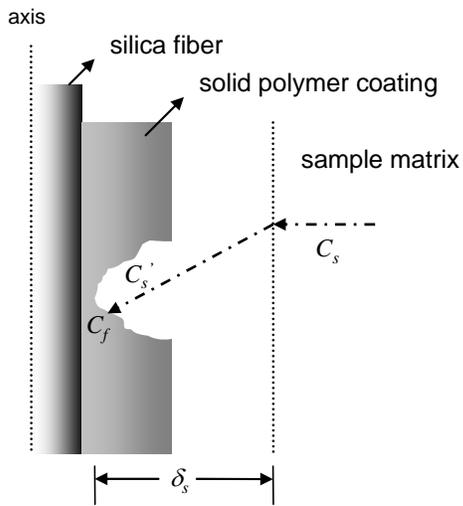


Figure 8-1 Schematic of the absorption and desorption processes between the liquid fiber and the sample matrix.

(A)



(B)

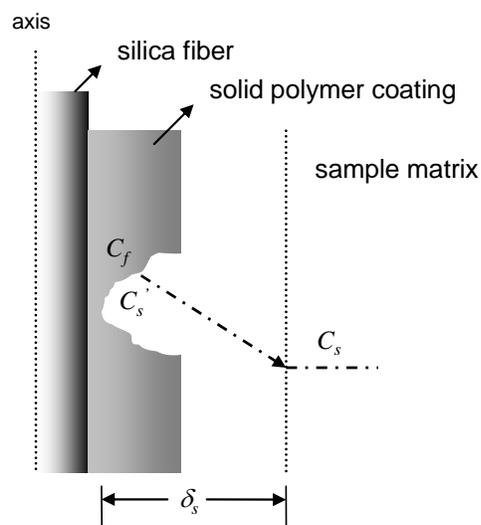


Figure 8-2 Schematic of the adsorption and desorption processes between the solid fiber and the sample matrix.

In the sample matrix containing dissolved organic matter, equilibrium can be reached very quickly for the association and dissociation between the analyte and the binding matrix:



where K_a is the equilibrium binding constant; C_s , C_b , and C_{bs} are the analyte concentration, the binding matrix concentration and the bounded analyte concentration in bulk solution, respectively.

8.2.1 Boundary Layer Model (Model A)

Adsorption kinetics for solid-coated SPME fiber:

According to Equation 8-1, a steady-state mass transfer for a solid fiber can be established when agitation is applied effectively in the sample matrix, therefore, a linear concentration gradient in the boundary layer is assumed:

$$\frac{1}{A} \frac{\partial n}{\partial t} = -D_s \frac{\partial C_s}{\partial x} = \frac{D_s}{\delta_s} (C_s - C'_s) = h_s (C_s - C'_s) \quad \text{Equation 8-3}$$

where δ_s is the thickness of the boundary layer and C'_s is the concentration of the analyte in the boundary layer at the interface of the fiber coating and the boundary layer. The mass transfer coefficient of the analyte in the fiber coating, h_s , which is defined as D_s/δ_s , is a constant for a steady-state diffusion process in an effectively agitated sample matrix.

At the interface of the fiber coating and the boundary layer, there is a quick equilibrium for the analyte between sample matrix and the coating surface, therefore:

$$K = \frac{C_f}{SC'_s} \Rightarrow C'_s = \frac{C_f}{KS} = \frac{n}{AKS} \quad \text{Equation 8-4}$$

where K is the equilibrium constant of the analyte between the coating surface and the sample matrix, C_f is the concentration of the analyte on the surface of the fiber coating, and S is the concentration of unoccupied sites on the surface of the sorbent, which is usually a constant when the limited sites are occupied. It is assumed that the SPME coating has a uniform pore distribution and surface area throughout its bulk, and C_f is determined by a ratio of n/A where n is the extracted amount.

In the bulk of the sample matrix, the mass balance for the analyte in the system can be expressed as:

$$C_s^0 = C_s + C_{bs} + \frac{n}{V_s} \quad \text{Equation 8-5}$$

where C_s^0 is the total concentration of the analyte in the system, and V_s is the volume of sample matrix. Based on Equation 8-2, C_s is obtained by:

$$C_{bs} = K_a C_s C_b^0 \Rightarrow C_s = \frac{C_s^0 - \frac{n}{V_s}}{1 + K_a C_b^0} \quad \text{Equation 8-6}$$

Substitution of Equations 8-4 and 8-6 into Equation 8-3 gives:

$$\frac{\partial n}{\partial t} = Ah_s \left(\frac{C_s^0 - \frac{n}{V_s}}{1 + K_a C_b^0} - \frac{n}{AKS} \right) = \frac{Ah_s C_s^0}{1 + K_a C_b^0} - \left(\frac{ASKh_s + h_s V_s (1 + K_a C_b^0)}{SKV_s (1 + K_a C_b^0)} \right) n$$

$$\text{Equation 8-8}$$

Let

$$a = \frac{ASKh_s + h_s V_s (1 + K_a C_b^0)}{SKV_s (1 + K_a C_b^0)}, \quad b = \frac{Ah_s C_s^0}{1 + K_a C_b^0}$$

Equation 8-8 can be simplified as:

$$\frac{\partial n}{\partial t} = b - an \quad \text{Equation 8-8}$$

Equation can be solved with the initial condition: $t = 0, n = 0$:

$$n = \frac{b}{a} [1 - \exp(-at)] \quad \text{Equation 8-9}$$

Substitution of a and b into Equation 8-9 results in:

$$n = \frac{ASKh_s V_s C_s^0}{ASKh_s + h_s V_s (1 + K_a C_b^0)} \left[1 - \exp\left(-\frac{ASKh_s + h_s V_s (1 + K_a C_b^0)}{SKV_s (1 + K_a C_b^0)} t\right) \right] \quad \text{Equation 8-10}$$

The extraction profile with the presence of binding matrix in the sample can be predicted using Equation 8-10. The parameter a in the equation is a time constant that can be used to describe how quickly the equilibrium can be reached.

Desorption kinetics for solid-coated SPME fiber:

When a solid fiber preloaded with an analyte is exposed to an agitated sample matrix, desorption of the analyte from the coating surface to the sample occurs. The desorption process can be treated as the reverse process of adsorption. Similarly, the desorption process can be described as:

$$\frac{1}{A} \frac{\partial q}{\partial t} = -h_s (C_s - C'_s) \quad \text{Equation 8-11}$$

where ∂q is the amount of the analyte desorbed from the fiber surface during time period ∂t . If the initial amount of analyte preloaded on the coating surface is q_0 , the remaining concentration of the analyte on the fiber is:

$$C_f = \frac{q_0 - q}{A} \quad \text{Equation 8-12}$$

At the interface of the fiber coating and the boundary layer, there is a quick equilibrium for the analyte between sample matrix and the coating surface, therefore:

$$K = \frac{C_f}{SC'_s} \Rightarrow C'_s = \frac{C_f}{KS} = \frac{q_0 - q}{AKS} \quad \text{Equation 8-13}$$

In the bulk of the sample matrix, similarly, C_s is obtained by:

$$C_s = \frac{q_0 - q}{V_s(1 + K_a C_b^0)} \quad \text{Equation 8-14}$$

Substitution of Equations 8-13 and 8-14 into Equation 8-11 gives:

$$\frac{\partial q}{\partial t} = -Ah_s \left(\frac{q_0 - q}{V_s(1 + K_a C_b^0)} - \frac{q_0 - q}{AKS} \right) = \left(\frac{ASKh_s - h_s V_s(1 + K_a C_b^0)}{SKV_s(1 + K_a C_b^0)} \right) q + \frac{h_s V_s(1 + K_a C_b^0) - ASKh_s}{SKV_s(1 + K_a C_b^0)} q_0$$

$$\text{Equation 8-15}$$

Let

$$a' = \frac{ASKh_s - h_s V_s(1 + K_a C_b^0)}{SKV_s(1 + K_a C_b^0)}, b' = \frac{h_s V_s(1 + K_a C_b^0) - ASKh_s}{SKV_s(1 + K_a C_b^0)} q_0$$

Equation 8-15 can be simplified as:

$$\frac{\partial q}{\partial t} = a'q + b' \quad \text{Equation 8-16}$$

Therefore, Equation 8-16 can be solved and arranged as Equation 8-17:

$$q = \frac{b'}{a'}(1 - \exp(-a't)) + q_0 \exp(-a't) \quad \text{Equation 8-17}$$

Absorption and Desorption kinetics for liquid-coated SPME fiber:

The absorption and desorption processes of a liquid SPME fiber in an agitated sample matrix have been described previously⁷¹ and are expressed in Equation 8-18 and Equation 8-19, respectively:

$$n = \frac{K_{fs} V_f V_s C_s^0}{K_{fs} V_f + V_s (1 + K_a C_b^0)} \left[1 - \exp \left(-A \frac{2K_{fs} h_s V_f + 2h_s V_s (1 + K_a C_b^0)}{V_s V_f (2K_{fs} h_f + h_s)(1 + K_a C_b^0)} t \right) \right]$$

Equation 8-18

and

$$\frac{\partial q}{\partial t} = -A \left(\frac{2K_{fs} h_{fs} V_f + 2h_{fs} V_s (1 + K_a C_b^0)}{V_s V_f (2K_{fs} h_{fs} + h_{ss})(1 + K_a C_b^0)} \right) q + \frac{2AK_{fs} h_{fs}}{V_s (2K_{fs} h_{fs} + h_{ss})(1 + K_a C_b^0)} q_0$$

Equation 8-19

where K_{fs} is the distribution coefficient of the analyte between the fiber coating and the sample matrix; h_s or h_{ss} and h_f or h_{fs} are mass transfer coefficients in the boundary layer and the fiber coating, respectively.

8.2.2 Compartment Model (Model B)

When considering measurements in the dynamic stages of SPME, the classical one-compartment, first-order kinetic model with absorption and desorption rate constants

as parameter for SPME liquid fibers has been applied in the past.^{81,209} Although the model is not based on processes such as diffusion and partitioning of the analyte and it does not take into consideration of sample matrix volume and fiber geometry, it is very simple to use. This feature is particular useful when limited data is available and if the time to reach extraction equilibrium and the total concentration of analyte in the system needs to be determined during SPME method development. A modified compartment model has been developed in a sample medium containing a binding matrix. The model not only considers the concentration time courses of an analyte in the sample matrix, the analyte absorbed or adsorbed to the fiber coating, but also the binding matrix and the bounded analyte in the bulk solution. In addition, the volumes of the sample matrix and the fiber coating are also incorporated in the calculations. A simple schematic illustration of the model is shown in Figure 8-3.

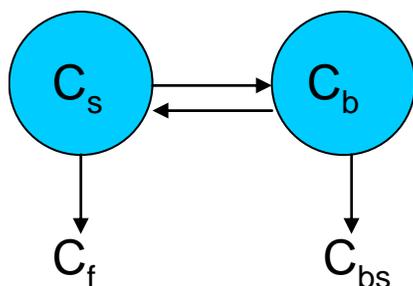


Figure 8-3 Simplified schematic of compartment model in a sample matrix.

In this model, it is assumed that chemical equilibrium reaches rapidly between unbound analyte and the binding matrix, and the binding matrix and bounded analyte will not be absorbed or adsorbed to the fiber coating, but the free analyte. In addition, it is

assumed that the bulk medium is well mixed and diffusion occurs only in a small film close to the fiber. Similarly, the fiber volume is well mixed and the diffusion in the fiber will not be modeled. The concentration of the analyte in the sample matrix can be described as:

$$\frac{\partial C_s}{\partial t} = k_1 \left(\frac{C_f}{K} - C_s \right) - k_a C_s C_b + k_b C_{bs} \quad \text{Equation 8-20}$$

where k_1 is a composite parameter that is estimated by fitting the model to the experimental data. K is the partitioning coefficient of the analyte between the fiber coating and matrix for a liquid fiber, or the equilibrium coefficient for a solid fiber. k_a and k_b refer to the rate constants of association and of dissociation the analyte to the binding matrix, respectively. Similarly, based on mass balance, the following equations can be derived:

$$\frac{\partial C_f}{\partial t} = -k_1 \left(\frac{C_f}{K} - C_s \right) \quad \text{Equation 8-21}$$

$$\text{and } \frac{\partial C_b}{\partial t} = -k_a C_s C_b + k_b C_{bs} \quad \text{Equation 8-22}$$

$$\text{and } \frac{\partial C_{bs}}{\partial t} = k_a C_s C_b - k_b C_{bs} \quad \text{Equation 8-23}$$

The initial concentrations of the analyte and the binding matrix are known, as well as the volumes of the sample matrix and the fiber coating. The amount of the analyte extracted or remaining on the fiber coating depending on absorption/adsorption or depletion processes is the model outcome and will be corrected according to the ratio of $k_2 (V_s / V_f)$, and k_2 is another composite parameter that is obtained from fitting the model to the experimental data.

8.2.3 COMSOL Multiphysics Model (Model C)

Recently, the influence of the important variables on the kinetics of SPME including physicochemical properties of the analyte, physical dimensions of the fiber, the presence of binding matrix in the sample, and the flow velocity across the fiber has been modeled and simulated using commercial software called COMSOL Multiphysics. The software provides a powerful interactive environment for modeling and solving different type of scientific and engineering problems such as fluid flow, heat transfer, and chemical reactions based on partial differential equations in one or more physical domains simultaneously. In general, the steps followed to solve a problem using COMOSL Multiphysics are: 1) defining geometrical domain; 2) domain meshing; 3) establishing the equations in the domain and determining the boundary conditions; 4) simulation to solve the equations; 5) post-processing; and 6) parametric studies.

Based on previous studies, a two-dimensional axis-symmetric geometry was built to simulate the extraction process of a liquid SPME fiber in an agitated sample matrix. As shown in a snapshot of COMSOL model setup in Figure 8-4, rectangle sub-domain R1 represents the dimension of a liquid fiber with a coating thickness of 8 μm and a fiber length of 0.5 cm, while sub-domain R2 is the diffusion layer with an arbitrarily selected boundary layer thickness (20 μm).

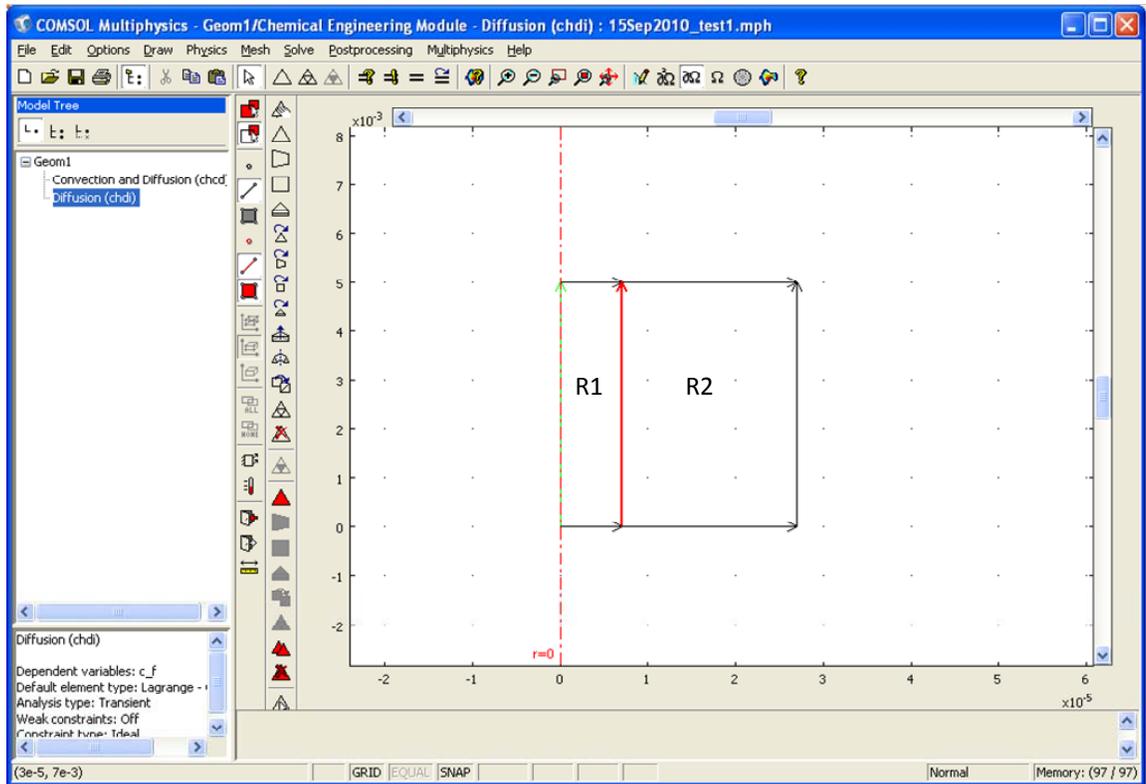


Figure 8-4 A snapshot of a model setup in COMSOL

A structured mesh is applied in order to yield more accurate solution as solution is always dependent on the grid size – resolution. The schematic diagram of meshes generated in COMSOL is displayed in Figure 8-5.

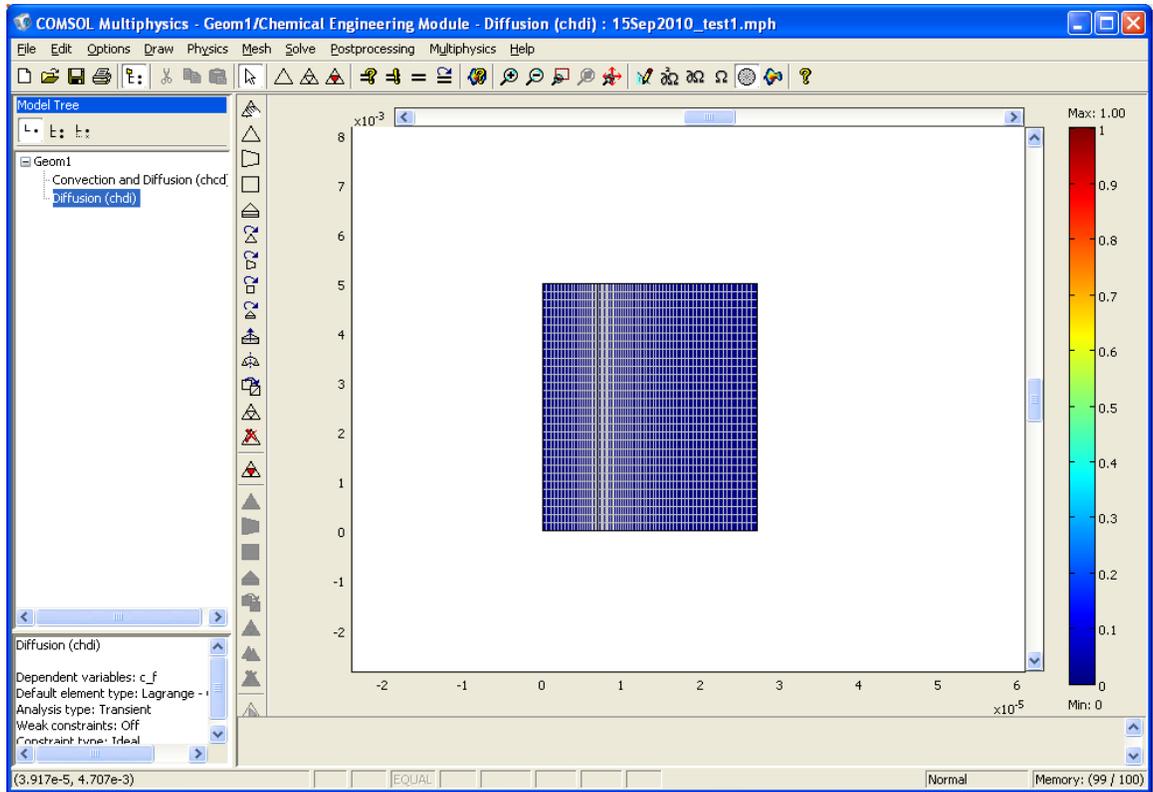


Figure 8-5 A schematic diagram of meshes generated in COMSOL

The analyte is transported by diffusion and convection in the sample matrix, but only diffusion in the fiber coating. The sub-domain settings used in COMSOL are:

$$\frac{\partial C_s}{\partial t} + \nabla(-D_s \nabla C_s + C_s \mathbf{u}) = R$$

$$R = -k_a C_s C_b + k_b C_{bs}$$

$$\frac{\partial C_b}{\partial t} + \nabla(-D_b \nabla C_b + C_b \mathbf{u}) = R$$

$$R = -k_a C_s C_b + k_b C_{bs}$$

Equation 8-24 (in sample)

$$\frac{\partial C_{bs}}{\partial t} + \nabla(-D_{bs} \nabla C_{bs} + C_{bs} \mathbf{u}) = -R$$

$$R = -k_a C_s C_b + k_b C_{bs}$$

$$\frac{\partial C_f}{\partial t} + \nabla(-D_f \nabla C_f) = 0 \quad \text{Equation 8-25 (in fiber)}$$

where D_s , D_b and D_{bs} is the diffusion coefficient of analyte, binding matrix and bounded analyte in aqueous phase, respectively; D_f is the diffusion coefficient of the analyte in the fiber coating; u is the velocity vector in aqueous phase.

As shown in Figure 8-1 (A), there will be discontinuities in the concentration profile at the boundaries between aqueous and fiber coating phases, in order to obtain continuous flux over the phase boundaries, a stiff-spring method is used to apply a special type of boundary condition. Instead of defining Dirichlet concentration conditions according to the distribution coefficient K_{fs} ($K_{fs} = C_f / C_s'$), which would destroy the continuity of the flux, continuous flux conditions are defined in order to, at the same time, force the concentrations to the desired values. These boundary settings for sub-domains R1 and R2 are, therefore, described in Equations 8-26 and 8-28, respectively:

$$\begin{aligned} (-D_s \nabla C_s + C_s u) n &= M' (C_f - K_{fs} C_s) \\ C_b &= C_b^0, C_{bs} = K_a C_s C_b^0 \end{aligned} \quad \text{Equation 8-26}$$

$$(D_f \nabla C_f) n = M' (K_{fs} C_s - C_f) \quad \text{Equation 8-28}$$

where M' is set as 10000, which is sufficiently large to give continuity in flux. In the sub-domain of sample matrix, it is assumed that there is no transport at the horizontal insulation/symmetry boundaries. It is also assumed that there is symmetry at the horizontal boundaries of the fiber coating in the sub-domain of the liquid SPME fiber.

8.3 Experimental Section

8.3.1 Chemical and Supplies

[2,4,6,8-³H]18 β -estradiol (2628 GBq/mmol, 38 MBq/mL) was purchased from New England Nuclear (Boston, MA) and used within two months to ensure radiochemical purity (>98%). Pyrene (98%) was obtained from Aldrich Chemie BV (Zwijndrecht, The Netherlands). Bovine serum albumin (BSA) was bought from Sigma Chemical Co. (St. Louis, MO). Polyacrylonitrile (PAN, monomer as impurity less than 4.85 ppm) and verapamil (>98%) were bought from Sigma (ON, Canada). The C18-silica (5 μ m), RP-amide-silica (5 μ m) and HS-F5-silica (5 μ m) were obtained from Supelco (Bellefonte, PA). A length of 50 m of SPME fiber with a coating of 7 μ m of polyacrylate (PA) was purchased from Supelco (Bellefonte, PA) and was cut into pieces \sim 1.5 cm before use. Glass fibers with a core diameter of 110- and 28.5- μ m poly(dimethylsiloxane) (PDMS) coating (volume 12.4 μ L/m) were obtained from Poly Micro Industries (Phoenix, AZ). Tailor-made PAN/C18, PAN/RP-amide and PAN/HS-F5 SPME fibers with coating thickness of 60 μ m and length of 1.5 cm were prepared according to a procedure described previously.¹⁸⁹

8.3.2 Instrumentation

The radioactivity of [³H]estradiol was counted on a Minaxi Tricarb 4000 scintillation counter of Packard Bioscience Co. (Meriden, CT). All experiments were carried out at room temperature. Disintegration per minute (dpm) was calibrated with a series of the same solution that was used to prepare the samples. Analyses determining pyrene concentrations were performed using HPLC fluorescence. The HPLC system was

equipped with a Shimadzu DGU 14 A degasser, a Varian 9012 pump, a Basic Marathon autosampler (Middelburg, The Netherlands), a Merck Hitachi F-1050 fluorescence spectrophotometer (Maarsse, The Netherlands), and a 100 mm × 3 mm, 5 μm PAH ChromSpher 5 C18 column. All analyses were performed with a flow rate of 400 μL/min and an injection volume of 20 μL. The excitation and emission wavelengths of pyrene were set at 284/400 nm. Concentrations of verapamil were determined by LC-MS using an Agilent 1100 series liquid chromatography (Palo Alto, CA) equipped with a vacuum solvent degassing unit, a binary high-pressure gradient pump, an autosampler, a column thermostat, and a variable wavelength UV-vis detector coupled on-line with an Agilent 1100 series MSD single quadrupole instrument with atmospheric pressure electrospray ionization. Chromatographic separations were carried out on a Discovery C18 column (50 mm × 2.1 mm, 5 μm). Detailed LC-MS conditions were described previously.¹⁸⁹ For optimization experiments, a positive ion of verapamil was monitored at m/z 455.3.

8.3.3 Absorption Experiments of [³H]estradiol

To determine whether protein affects the uptake of [³H]estradiol into the fiber, absorption profiles of [³H]estradiol were measured at different BSA concentrations. Concentrations were 7.1 × 10⁻⁹ M [³H]estradiol, and 0, 6.5 × 10⁻⁶, 1.6 × 10⁻⁵, 6.4 × 10⁻⁵, and 1.0 × 10⁻³ M BSA all in 50 mM Tris buffer (pH 7.4). Mixtures were left to incubate for 10 min at 20°C. Absorption times were 2, 5, 15, 30, 60, 90, 120, 180, 300, and 420 min in duplicate. The 1.5 cm PA fiber was pierced through the septa of the lids of 2-mL glass vials with the length of fiber sticking into to the vial of 0.5 cm. Sample volumes were 1.6 mL throughout the study. After a certain absorption time, the vial was taken off and the

fiber was pulled out of the septum and transferred wholly into a scintillation vial. It was left for desorption with 3.8 mL Ultima GOLD for at least 3 hr, then vortexed before counting radioactivity.

8.3.4 Depletion Experiments of Pyrene

All PDMS fibers were cut to a length of 2.5 cm and loaded with pyrene by exposing them to a 30 mL sterilized 1:1 methanol-water (v/v) mixture spiked with 2 mg/L pyrene for 24 hr on a shaker (rocking 5 cycles/min, turning 5 rpm). Five fibers were immediately analyzed for their pyrene concentration after dosing to determine the initial pyrene concentrations in the fiber.

The loaded fibers were fully submerged in 5 mL of culture medium supplemented with heat-inactivated newborn calf serum (NCS) at 0, 1.4, and 23.34 μ M BSA in 5-mL glass vials on the shaker. The experiment was carried out in triplicate. Three non-loaded fibers were exposed for 48 hr in bare cell culture medium to serve as negative control. The samples were then sampled after 0.1, 0.25, 0.5, 1, 2, 4, 24, and 48 hr. After exposure, each fiber was gently blotted dry with a tissue. They were put into 1.8-mL autosampler vials containing 250- μ L glass insert with 200 μ L of acetonitrile for 24 hr to extract the pyrene out of the fiber coating. The samples were stored at -20°C prior to analysis.

8.3.5 Adsorption Experiments of Verapamil

Plasma samples were thawed at room temperature and aliquots of 1.5 mL were transferred to clean vials. Appropriate amounts of spiked standards were added followed

by vortex mixing for 1 minute. The time required to reach equilibrium for plasma samples for verapamil (5×10^{-7} M) was determined by measuring the amounts extracted at different time points. For extraction, the samples were placed on a digital vortex platform and the SPME fibers were immersed in the sample for a precise period of time. The fiber was briefly rinsed with water and desorbed with a desorption solution prepared from acetonitrile/water/acetic acid (50:49:1) for analysis.

8.4 Results and Discussion

8.4.1 Model Performance

The experimental results of absorption of [^3H]estradiol and depletion of pyrene from the literature were used to evaluate the performance of different model approaches for both absorption and desorption kinetic processes using liquid SPME fibers. The parameters used for model validation are listed in Tables 8-1 and 8-2.

Table 8-1 The Parameters Used in the Model Validation for Absorption Process of [^3H]estradiol in a Sample Matrix

Parameter	Value
Concentration of analyte (M), C_s^0	7.1×10^{-9}
Concentration of binding matrix (M), C_b^0	0, 6.5×10^{-6} , 1.6×10^{-5} , 6.4×10^{-5} , 1.0×10^{-3}
Thickness of fiber coating (μm)	7
Distribution coefficient, K_{fs}	5.04×10^3
Binding constant (M^{-1}), K_a	8.9×10^4
Sample volume (mL), V_s	1.6
Diffusion coefficient in sample matrix (m^2/s), D_s ($D_s = h_s \times \delta_s$, δ_s , boundary layer thickness)	8.96×10^{-10}
Diffusion coefficient in fiber coating (m^2/s), D_f ($D_f = h_f \times \delta_f$, δ_f , fiber thickness)	3.33×10^{-15}

Table 8-2 The Parameters Used in the Model Validation for Desorption Process of Pyrene in a Sample Matrix

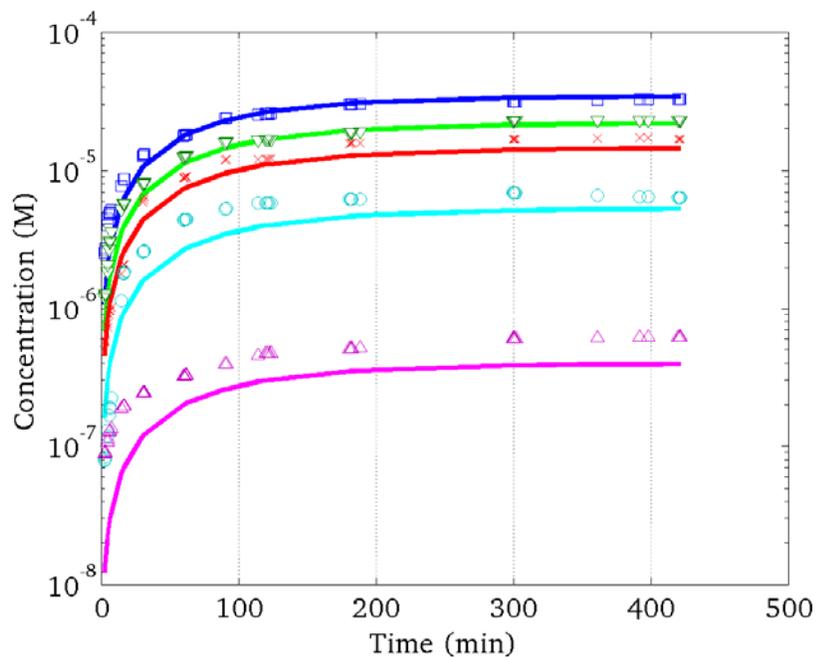
Parameter	Value
Concentration of analyte on fiber (M)	2.8×10^{-9}
Concentration of binding matrix (M), C_b^0	0, 1.4×10^{-6} , 2.3×10^{-5}
Thickness of fiber coating (μm)	30
Distribution coefficient, K_{fs}	1.95×10^4
Binding constant (M^{-1}), K_a	8.58×10^6
Sample volume (mL), V_s	5
Diffusion coefficient in sample matrix (m^2/s), D_s ($D_s = h_s \times \delta_s$, δ_s , boundary layer thickness)	4.38×10^{-10}
Diffusion coefficient in fiber coating (m^2/s), D_f ($D_f = h_f \times \delta_f$, δ_f , fiber thickness)	1.05×10^{-12}

In Models A and B, equations are implemented in MATLAB 7.8.0 (R2009a) software with initial concentrations of the analyte and the binding matrix as well as other necessary parameters listed in Tables 8-1 and 8-2 to obtain the amount of [^3H]estradiol extracted or the fraction of pyrene in the fiber over time. Equations in Model C are solved using COMOSL Multiphysics when all the sub-domains and boundary conditions are established.

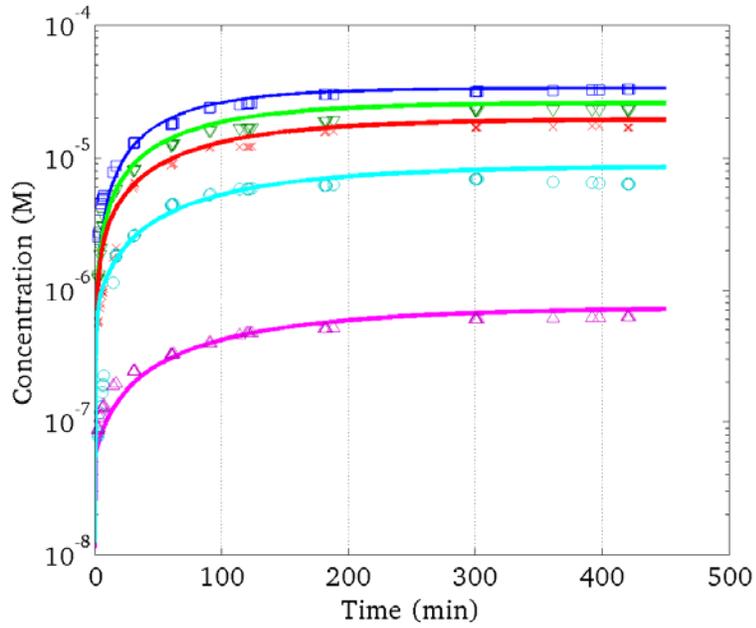
Figure 8-6 illustrates the comparison between experimental data and prediction results of the extraction profiles of [^3H]estradiol under different BSA concentrations using three different model approaches. It can be seen that higher concentrations of binding matrix do not change the shape of the absorption profile, but only the maximum concentration of the [^3H]estradiol in the fiber. This maximum is related to the free concentration in the solution, which is lower at the high binding matrix concentrations. Therefore, the presence of the binding matrix does not seem to have an effect on the uptake kinetics of this particular compound into this type of fiber. In general, the

prediction results from all three models are in good agreement with the experimental data; however, there are some differences among these three models. Model A predicts the extraction profiles very well when the concentrations of the binding matrix are low, but deviations increase with increasing of BSA concentrations. Model C accurately predicts the concentration data after the extraction reaches equilibrium; however, it appears that the equilibrium is reached much faster in the model than in the real situation. Overall, Model B gives the best results in terms of the shape of the extraction profile. The diagnose plots (Figure 8-7) between experimental concentrations and predicted data at all conditions further demonstrate the precision of each model with standard deviation of 1.49×10^{-6} , 1.42×10^{-6} , and 4.32×10^{-6} , respectively.

(Model A)



(Model B)



(Model C)

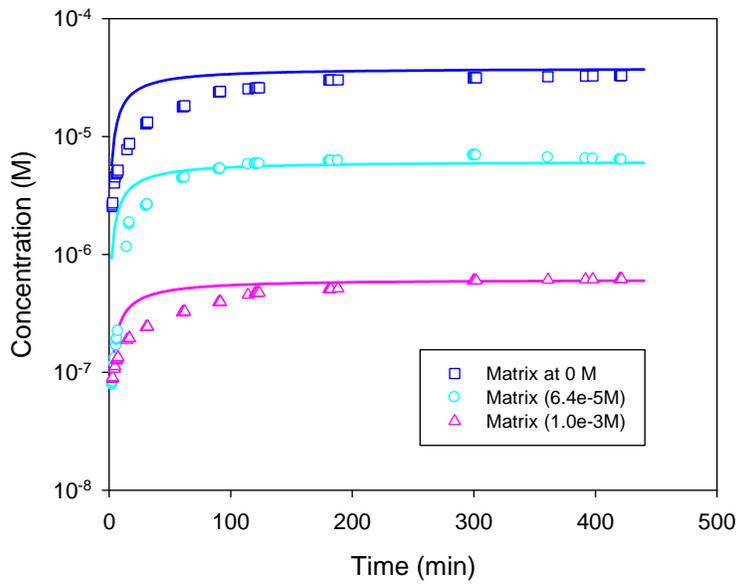
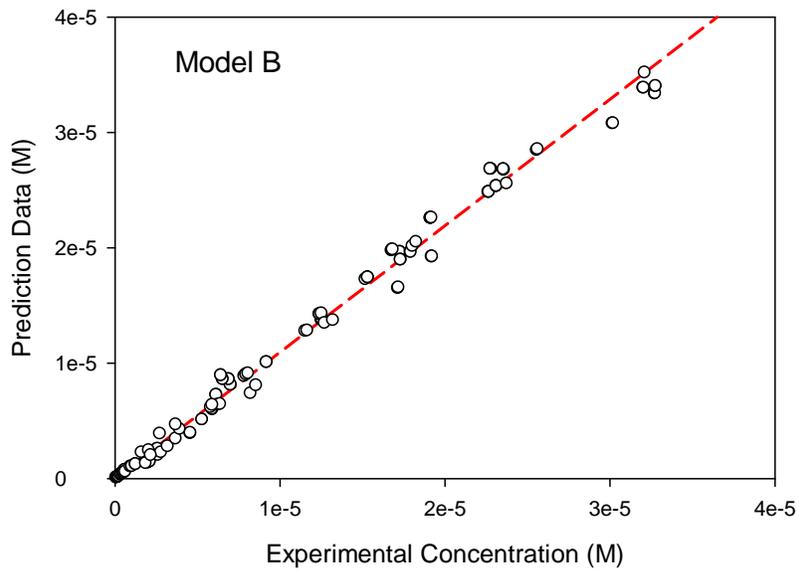
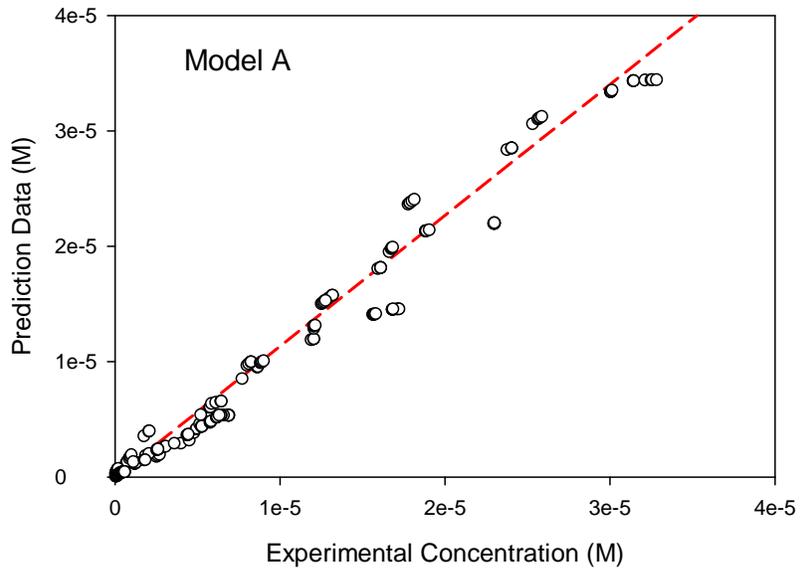


Figure 8-6 Extraction profiles of [³H]estradiol at different BSA concentrations: The points are experimental results and the curves are obtained from models A, B, and C, respectively. BSA concentrations from top to bottom are: 0 (□), 6.5×10^{-6} (∇), 1.6×10^{-5} (×) 6.4×10^{-5} (O) and 1.0×10^{-3} M (Δ).



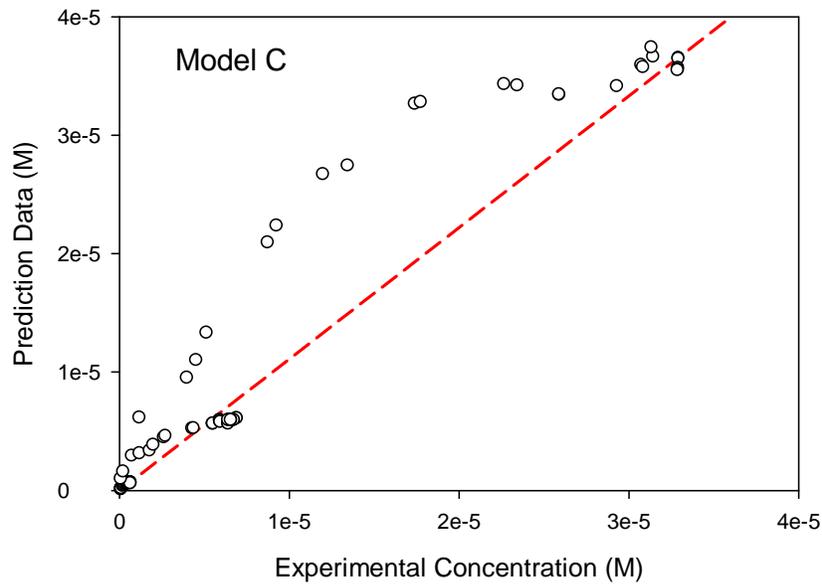
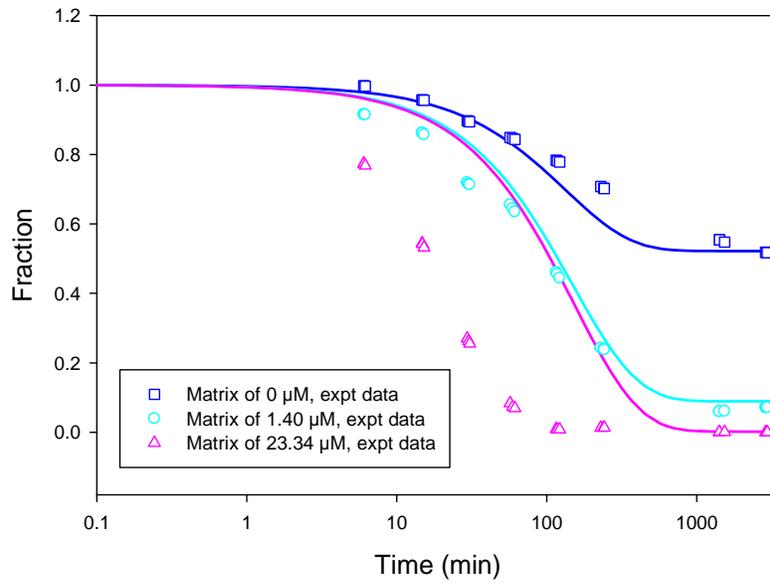


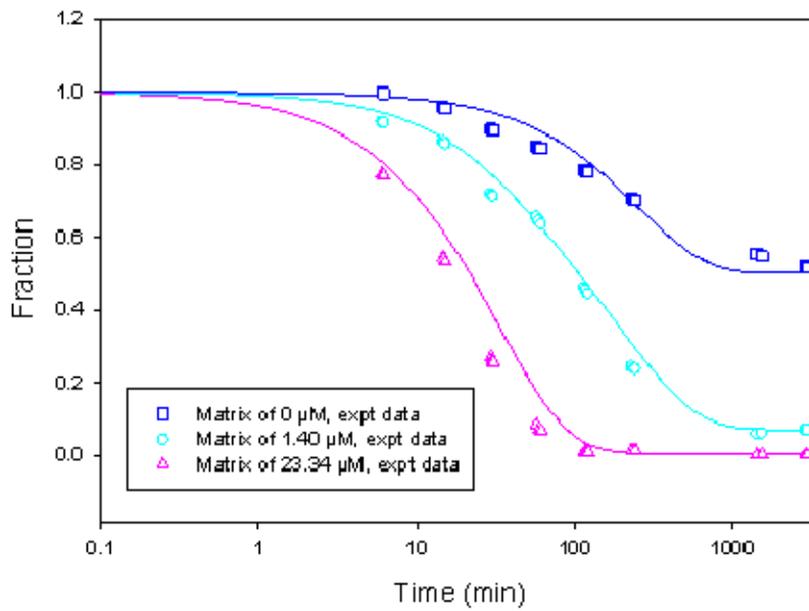
Figure 8-7 Diagnose plots between experimental concentrations from extraction of [³H]estradiol at different BSA concentrations and predicted data obtained from models A, B and C, respectively.

Figure 8-8 shows the comparison between experimental data and prediction results of the desorption profiles of pyrene under different BSA concentrations using three different model approaches. Compared with previous modeling approaches for the extraction processes of [³H]estradiol, it is interesting to see that only Model B provides a reasonable simulation of the experimental data; in contrast, Model A cannot provide accurate prediction at binding matrix concentration of 23.24 μM , and Model C has difficulties in predicting the desorption profile without matrix effects. The reasons for these large deviations are unclear. The accuracies of the parameters used in the models and the model setup could all contribute to these deviations.

(Model A)



(Model B)



(Model C)

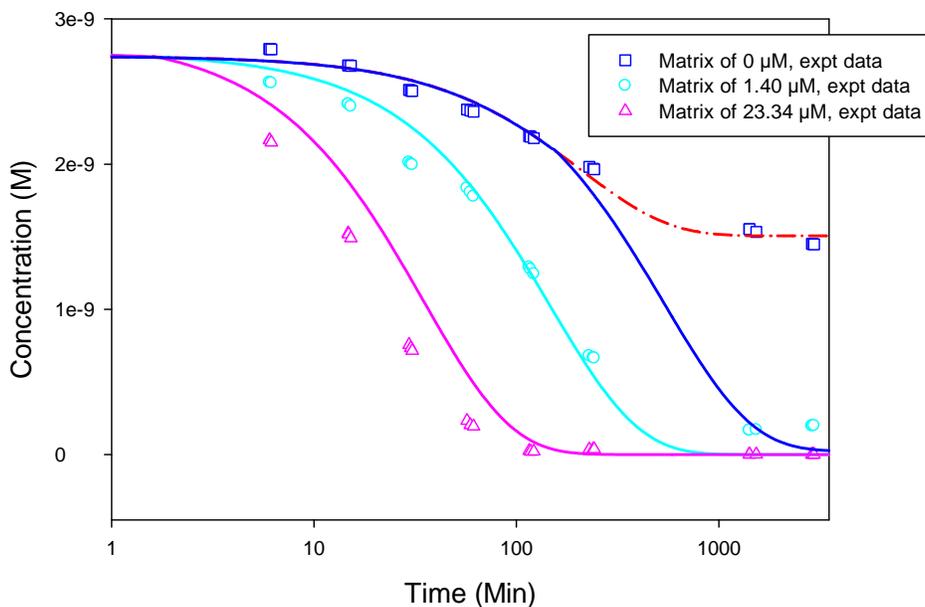


Figure 8-8 Desorption profiles of pyrene under different BSA concentrations: The points are experimental results and the curves are obtained from models A, B and C, respectively.

In summary, in Model A, many parameters are needed. These include mass transfer coefficients, distribution coefficients, binding constant and physical dimensions of the sample matrix, and the SPME fiber coating in order to calculate the extraction or desorption profiles with the presence of a binding matrix. As the thickness of the fiber coating is normally known, to obtain the thickness of the boundary layer is important as the mass transfer coefficient can be determined. The model predicts fairly well for the absorption profile of [³H]estradiol, but not the desorption profiles of pyrene in the presence of BSA, and the deviation increases with increasing concentrations of BSA. Model B provides a better prediction overall than the other two model approaches with fewer requirements of necessary parameters. The disadvantage is that the model does not

provide detailed information about all the parameters involved in sorption processes. The composite parameter k_1 is used to determine the shape of the extraction or desorption curves, and k_2 has a big impact to the analyte concentrations in the fiber. The advantage of using COMSOL Multiphysics is that mass transfer and chemical reaction processes can be described by one or more physical domains simultaneously based on various partial differential equations and boundary conditions. However, the geometry of the model has to be set up properly in order to provide reasonable predictions, in addition, all required parameters are necessary in model set up before solving the equations, although unknown parameters can be obtained through curve fitting of experimental data, in this regard, the sub-domain setup will be much more complicated.

8.4.2 Model Applications

Several different model approaches have been proposed to investigate the kinetics of SPME extraction and desorption of the target analyte in a sample matrix containing dissolved organic matter. The purpose of SPME modeling and simulation is to provide a more fundamental understanding of the experimental data which can be used to optimize experimental conditions. Although all models are wrong, some are useful.²¹¹ Applications of various SPME kinetic models are applied to estimate boundary layer thickness, time to reach extraction equilibrium and total amount of analyte extracted at a given time using SPME solid fibers.

8.4.2.1 Boundary Layer Thickness Estimation

Boundary layer thickness is an important piece of information as it determines the equilibrium time and the extraction rate because extraction rate is controlled by diffusion

from the sample matrix through the boundary layer to the extraction phase. In the mechanistically based model based on Fick's law, a boundary layer thickness can be estimated under the assumption that the flow around the SPME fiber is steady and laminar. For such conditions to be met and controlled, the sample has to be contained in a vertically standing vial with stirring taking place by a magnetic stirring bar during SPME setups. The thickness of the boundary layer can be calculated by the following semi-empirical equation:

$$\delta_s = 2.64(b/R_e^{0.50}S_c^{0.43}) \quad \text{Equation 8-28}$$

where b is the radius of the fiber, R_e is the Reynolds number $\left(R_e = \frac{2ub}{\nu}\right)$, and S_c is the Schmidt number $\left(S_c = \frac{\nu}{D_s}\right)$, u is the linear velocity of the sample, and ν is the kinematic viscosity of the matrix medium.

The flow velocity u around the fiber can be calculated according to the following equation:

$$u = 1.05\pi Nr \left[2 - (r/0.74R_s)^2\right] \quad \text{Equation 8-29}$$

where N is the magnetic stirrer speed in revolutions per second, r is the distance between the fiber and the center of the vial, and R_s is the radius of the stirring bar.

Although the model with a defined film thickness layer is useful for steady, laminar conditions, in many cases there are other types of agitation regimes that will not fulfill the conditions for the estimation of a stagnant thickness. For example, ultrasound agitation⁹ and agitation by the fiber itself²¹² have been applied and reported. In the

experiments of Heringa et al.,²⁰⁹ disposable fibers are exposed and mechanically agitated by placing the vials horizontally on a rotating plate which results in a non-steady, periodically accelerated movement of the fiber with the medium. As a result, flow velocities vary from about plus to minus 17 cm/s every 0.085 seconds. The method of least-squares is applied in Model A to estimate the boundary layer thickness during pyrene desorption process. The method of least-squares assumes that the best-fit curve of a given type is the curve that has the minimal sum of the deviations squared (least square error) from a given set of data. Suppose that the data points are (x_1, y_1) , (x_2, y_2) , ..., (x_n, y_n) where x is the independent variable and y is the dependent variable. The fitting curve $f(x)$ has the deviation (error) d from each data point, i.e., $d_1 = y_1 - f(x_1)$, $d_2 = y_2 - f(x_2)$, ..., $d_n = y_n - f(x_n)$. According to the method of least squares, the best fitting curve has the property that:

$$\Pi = d_1^2 + d_2^2 + \dots + d_n^2 = \sum_{i=1}^n d_i^2 = \sum_{i=1}^n [y_i - f(x_i)]^2 = \text{a minimum}$$

Equation 8-30

In Equation 8-28, all parameters are known except h_s , as $h_s = D_s / \delta_s$; the boundary layer thickness δ_s can be obtained from curve fitting of the experimental data to estimate h_s . In the simulation, the range of the boundary thickness layer was assumed from 1 to 100 μm with an interval of 2, therefore, 50 iterations were performed and the least square error generated an estimated value of δ_s to be 45.9 μm . Selected plots from this exercise are illustrated in Figure 8-9. In the mass transfer model where mass transfer is forced by the concentration difference between bulk medium and outer fiber surface,⁸² the boundary

layer thickness was estimated from 18.5 to 41.7 μm for pyrene desorption process using different diffusion coefficients of bound pyrene. The current model prediction result is in agreement with the literature data (41.7 μm) when assuming the diffusion coefficient of bound pyrene is the same as the diffusion coefficient of albumin in water.

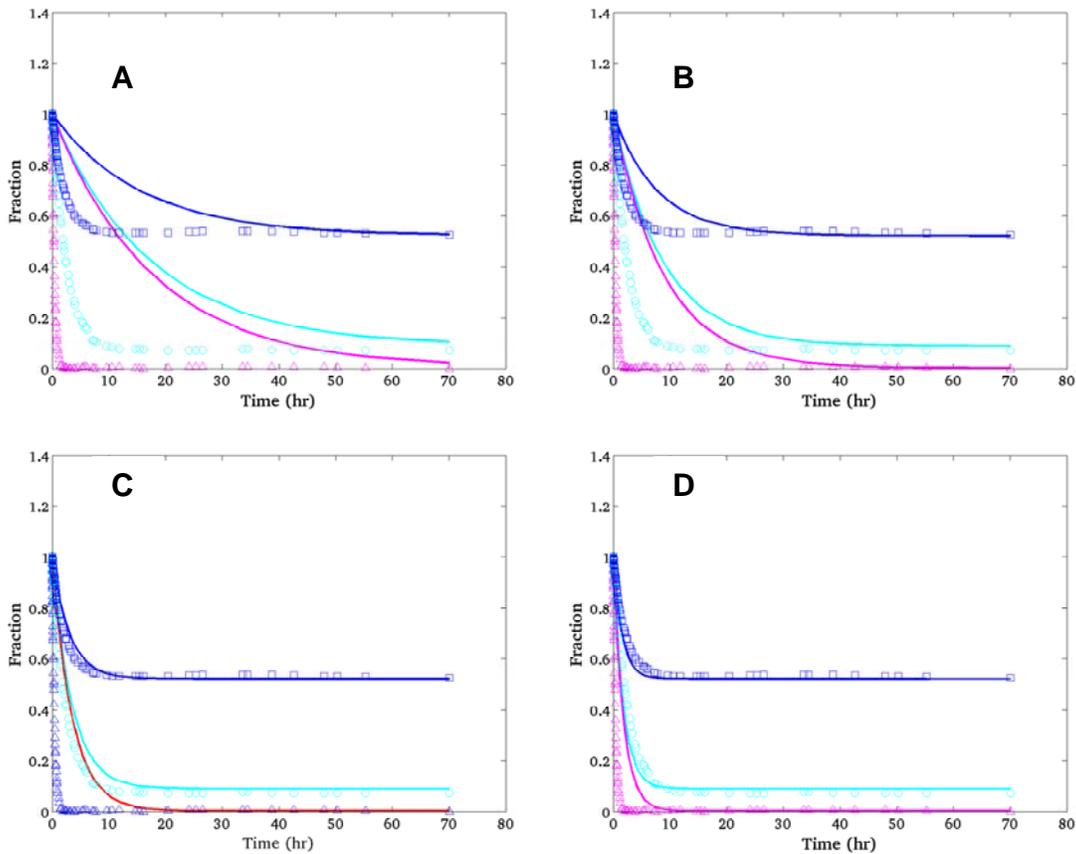


Figure 8-9 Selected plots for model simulation using least-squares method.

Similarly, an attempt has been made to estimate the boundary layer thickness in the absorption experiments of $[^3\text{H}]$ estradiol, and the estimated value of δ_s from Model A is about 11.0 μm . Although there is no published literature data for comparison, it is

invalid to calculate the theoretical δ_s according to Equations 8-28 and 8-29 since an accelerated flow regime is applied in the experiments. However, if a steady flow rate is assumed from 10 to 100 cm/s, a range that is often used in SPME experiments, the corresponding calculated boundary layer thickness using the two equations is from 1.1 to 4.7 μm . Based on the predicated results from both processes including desorption of pyrene and absorption of [^3H]estradiol in a sample matrix, it can be concluded that it is feasible to estimate the boundary layer thickness using Model A.

8.4.2.2 Extraction Equilibrium Time Prediction

In SPME method development, the process limiting step is to determine the time to reach extraction equilibrium. As SPME is a non-exhaustive extraction technique, it is desirable to perform extraction at equilibrium to reach maximum extraction efficiency. However, SPME experiments are often performed under non-equilibrium conditions as for more hydrophobic compounds for which equilibrium times can be very long. The total method development time will be greatly shortened if mechanistic models can accurately predict the extraction equilibrium time, which, in the meantime, will help to optimize the experimental conditions.

Both Models A and B provide very close estimations on the times to reach equilibrium in all five uptake curves in the experiments of [^3H]estradiol absorption. The extraction amount appears to reach steady state after about two hours. It can be seen from Figure 8-6 that the presence of a binding matrix does not impact the uptake kinetics of an analyte to the fiber coating and, therefore, the increasing concentrations of the binding matrix will not affect the time to reach extraction equilibrium. As shown in Equations 8-

10 and 8-18, the extract amount of analyte is proportional to the total concentration of the analyte, providing the sample, fiber, and the concentration of the binding matrix are held constant, which indicates that the equilibrium time is independent of the concentration of the analyte in the sample matrix. The equilibrium binding constant should also not affect the equilibrium time, but the amount of analyte extracted in the fiber coating, as the higher affinity of the analyte to the binding matrix, the more the analyte bounded by the binding matrix and the less free analyte present in the sample matrix. Factors that will change the time to reach equilibrium include agitation speed and the distribution coefficient of the analyte between the fiber coating and the sample matrix. By increasing the speed of agitation, the boundary layer thickness will decrease, as a result, the mass transfer rate will be increased and the time to reach equilibrium becomes much shorter. For an analyte with a larger distribution coefficient, the equilibrium time can last several hours. For example, the time to reach equilibrium is about 30 minutes for an analyte with a distribution coefficient of 1000; however, under the same extraction conditions, it will take seven hours when the distribution coefficient is above 100,000.

It appears that Model C overestimates the equilibrium time which generates very quick diffusion in the fiber with respect to the reaction rates. The concentration gradient evens out more or less instantaneously in the fiber, but the level builds up gradually in time.

8.4.2.3 Total Concentration Estimation

To predict the total amount of target analyte extracted at a given time in the adsorption experiments of verapamil using different solid fiber coatings, it is important to

know the equilibrium or distribution constants of the analyte between the fiber coating surface and the sample matrix. The equilibrium constant can be calculated from the total number of moles of analyte extracted by the coating at equilibrium through the following equation:

$$K = \frac{nV_s}{V_f(C_0V_s - n)} \quad \text{Equation 8-31}$$

where C_0 is the initial concentration of a given analyte in the sample, V_s and V_f are the sample volume and fiber coating volume, respectively. For any new coatings, the new volume of coating can be estimated from the coating length (b), coating thickness (d) and radius of the supporting wire (r):

$$V_f = \pi b[(r + d)^2 - r^2] \quad \text{Equation 8-32}$$

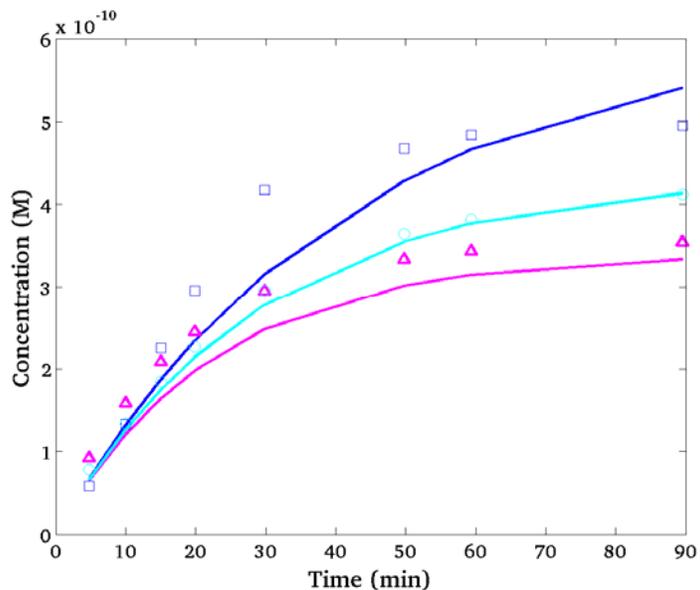
As mentioned previously, since the concentration of the sample analyzed by SPME has no impact on the extraction time profile and equilibrium time, and the agitation conditions, coating thickness, equilibrium constant, and diffusion coefficient of the analyte play very important roles in determining the equilibrium time, it is essential to keep the experimental conditions constant for different fiber coatings and the extraction time should be equal to or longer than the equilibrium to minimize potential errors caused by different sampling times. The time required for verapamil to reach equilibrium was found to be 60 minutes in three cases, the total concentration of verapamil extracted at equilibrium and the calculated equilibrium constants for fiber coatings PAN/C18, PAN/RP-amide and PAN/HS-F5 are reported in Table 8-3.

Because no binding matrix was involved in the experiments, Equation 8-10 from model A could be simplified to estimate the total amount of verapamil extracted at any given time. The adsorption profiles of verapamil to three different fiber coatings obtained from Equation 8-10 are depicted in Figure 8-10, and the total amount of verapamil extracted at equilibrium are calculated in Table 8-3. It can be seen that the data from model prediction are in reasonable agreement with the experimental results. Model B was also applied to simulate the experimental results of verapamil adsorption in three different fiber coatings. It is interesting to see in Figure 8-10 that the model predicts the experimental results extremely well with differences less than 3% in total amount extracted at equilibrium (Table 8-3).

Table 8-3 Equilibrium Constants and Total Amount Extracted at Equilibrium for Adsorption of Verapamil to Three Different Types of Solid Fiber Coatings

Fiber	K	Total_Expt. (M)	Total_Model A (M)	Difference (%)	Total_Model B (M)	Difference (%)
PAN/C18	3.27×10^3	4.96×10^{-10}	6.08×10^{-10}	22.6	5.04×10^{-10}	1.6
PAN/ HS-F5	2.33×10^3	4.12×10^{-10}	4.33×10^{-10}	5.1	4.21×10^{-10}	2.2
PAN/ RP-amide	1.83×10^3	3.54×10^{-10}	3.40×10^{-10}	-4.0	3.64×10^{-10}	2.8

(Model A)



(Model B)

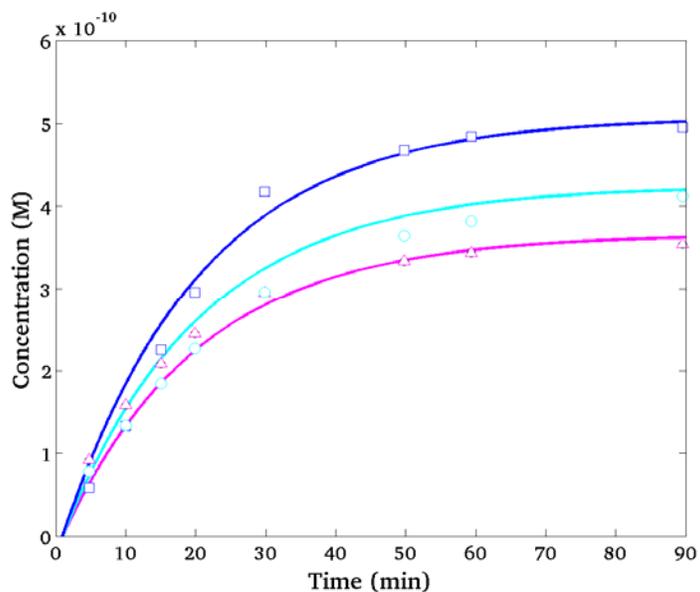


Figure 8-10 Adsorption profiles of verapamil to PAN/C18 (\square), PAN/HS-F5 (O) and PAN/RP-amide (Δ). The points are experimental results and the curves are obtained from models A and B, respectively.

8.5 Conclusion

Several mathematical models have been proposed to describe the kinetics of the extraction and desorption processes of different types of SPME fibers in complex sample matrixes. The simulation results clearly indicated that SPME modeling and simulation is a useful tool to provide more fundamental understanding of the experimental data which can be used to optimize experimental conditions. The performance of the models is dependent on the physicochemical properties of the target analytes and fiber coatings, agitation conditions, as well as sample parameters. With further improvement of the model setup and investigation of the causes of deviations from experimental results, and more implementation of the modeling approach in various SPME applications and rapid development of SPME database, it is believed that SPME modeling and simulation will have a profound impact on increasing the utilization of the technique in bioanalysis.

Chapter 9

Summary and Future Directions

9.1 Summary

Since its invention in the early 1990s, SPME has gained wide interest and has become one of the most active research areas in sample preparation in the past twenty years. The original paper published in *Analytical Chemistry* is among the Top 20 Most Cited articles published by the journal on the All Time lists. This is largely due to the unique characteristics of SPME technique that integrates sampling, extraction, concentration, and introduction of a sample to an analytical instrument into a single solvent-free step. SPME was originally developed in an attempt to address the need for rapid sampling and sample preparation in both laboratory and on-site situations and now has been widely applied to environmental, food, metallic, forensic, and pharmaceutical analysis. However, in contrast to the rapid growth and development of SPME technique including the development of various types of fiber coatings and new SPME formats, advances in the automation of SPME processes and contribution to high throughput sample analyses, and in spite of the many claimed advantages of SPME over traditional sample preparation methods such as PPT, LLE, and SPE, in reality, the goal of using SPME technique as an alternative approach for quantitative determination of analytes especially in pharmaceutical bioanalysis has not been realized. Up until now, SPME has not been used in any pharmaceutical companies for routine sample preparation.

One of the major reasons that prevent widespread applications of SPME in biological sample analysis is the lack of investment from manufacturers in this unique

technique because of the big gap between the academic research and the actual needs of customers from the pharmaceutical industry. The literature data has not built up enough confidence by the manufacturers that SPME will be generally accepted by users for bioanalysis because most of the research is either limited to a few compounds and confined to certain specialized laboratories, or because inadequate and insufficient validation results are provided. For example, matrix effects are rarely investigated during the method development; assay validation is normally performed in a single lot/source instead of different lots/sources of biological fluids; and QC samples freeze/thaw stabilities are seldom tested. All these factors are important indicators for the reproducibility and robustness of bioanalytical assays. One of the objectives of this thesis is to bridge this gap and to explore the feasibilities of bringing SPME technique from laboratory to industry for routine drug analysis. Based on the results from a proof-of-concept study, in the current work, an interesting approach using in-tip SPME has been proposed and developed for high throughput bioanalysis. In-tip SPME fibers are prepared through two different formats: sorbent-packed and fiber-picked. A polymerization mixture consisting of ethylene glycol dimethacrylate (EDMA), dimethoxy- α -phenylacetophenone (DMPA), and 1-decanol was used to prepare the sorbent-packed in-tip SPME fibers, and 96 fibers were fabricated at one time using a commercially available automation device. Fiber reproducibility of the sorbent-packed in-tip SPME with immobilized Oasis HLB particles was found to be 15.4% (R.S.D.) from absolute peak areas of 96 fibers and the value dropped to 5.6% when an isotopic labeled internal standard was applied. The current study clearly showed that in-tip SPME provided comparable results with those from SPE using an Oasis HLB μ Elution plate when a head-

to-head comparison experiment was conducted with a clinical development drug candidate. In fact, SPME was much easier to use than SPE with fewer sample preparation steps and less consumption of organic solvent. The remarkable advantages of in-tip SPME technique include that automation of sample extraction and desorption can be easily achieved using commercially available liquid handling systems. More importantly, in-tip SPME maintains the simplicity and advantages of conventional fiber SPME technique, and the approach is amendable to all fibers types possessing a wide range of different coating materials. This will overcome the drawback of limited selections of commercial available fibers and broaden its use with HPLC-MS/MS.

The successful applications of in-tip SPME were further demonstrated through two challenging studies on quantitative determination of vitamin D₃ in human serum with derivatization and three polar analytes, imipenem (IMP), cilastatin (CIL), and an investigational β -lactamase inhibitor (BLI) simultaneously in rat plasma and mouse blood, for 3 analytes in 2 systems. Vitamin D₃ and its metabolites have no easily ionizable polar functional groups in their molecules and the ionization efficiency of these compounds using either ESI or (HN) APCI is very limited. The required sensitivity for trace determination of these compounds in biological fluids is difficult to achieve. In addition, the determination of vitamin D₃ in the presence of a number of metabolites circulating in human biofluids exhibiting similar chemical and HPLC behavior as the parent compound may be highly non-selective, and an efficient separation of a large number of equimolar interferences, metabolites or their MS fragments, and large numbers of endogenous impurities from plasma, may be quite challenging. Although the desired sensitivity was not achieved in the study with 70 μ g dose of vitamin D₃, in-tip SPME clearly showed its

merit in that it can be used for a complex sample preparation process in which a derivatization reaction was applied in order to increase sensitivity and selectivity of vitamin D₃ determination. Furthermore, in-tip SPME required only a small volume (0.1 mL) of biological fluid for analysis (as opposed to 1.0 and 0.4 mL required in conventional tube-LLE and plate-LLE methods, respectively); and the extraction recovery, although not high in the absolute terms, was proven to be consistent between different plasma lots and was unusually high (~24-29%) in comparison with other methods based on SPME (<1%). Historically, methods to analyze Primaxin[®] were reported as separate assays for the two analytes, IMP and CIL, respectively. It would be very challenging to quantitatively determine the three very polar, non-structurally related compounds simultaneously, without considering the stability issues of all the analytes in biological fluids. In addition, in drug discovery preclinical environment, sample volume from different animal species is often very limited. For instance, in the current study; sample volume from mouse blood is only 20 µL. With the help of HILIC-MS/MS, it is very encouraging to see that PK parameters such as AUC, C_{max}, clearance (CL), and volume of distribution (V_{ss}) generated from in-tip SPME approach are in excellent agreement with those from PPT method.

Matrix effects have been thoroughly evaluated in this thesis. Some interesting findings may be useful for future SPME assay development and validation in bioanalysis. The hypothesis that SPME should provide sample clean-up as effective as or better than solid phase extraction (SPE) with no or minimal matrix effects is not supported by the experimental data. It was found that SPME was not as effective as LLE and SPE in terms of extract cleanliness for sample preparation when SPME was performed at equilibrium.

The ultimate test to demonstrate the absence of a “relative” matrix effect is to determine slopes of standard lines constructed in at least five different lots of a biological fluid (plasma, for example) and confirm the coefficient of variation (%CV) of these slopes is less than 3-4%. The results of this thesis indicated that isotopic stable labeled internal standard played a critical role to minimize "relative" matrix effects in SPME bioanalysis assays and should be utilized as much as possible. In terms of high throughput drug analysis, the current study has clearly shown that in-tip SPME performed as well or better than other automated approaches including blade-geometry SPME and thin-film SPME, which both heavily relied on a Concept 96 autosampler which is specifically designed for SPME automation. A comprehensive comparison between SPME and other traditional sample preparation methods in terms of method development and assay performance summarized in this thesis would certainly enhance the understanding of the advantages and limitations of SPME in bioanalysis, and the strategies with systematic experimental design for in-tip SPME method development and validation would provide general guidelines for any potential users of this technique.

SPME modeling and simulation provides more fundamental understanding of the experimental data through mechanistically-based models that describe the kinetics of the partition processes to SPME fibers. Several different modeling approaches introduced in this study gave reasonable fits of the experimental data by predicting the sorption profiles as a function of time in a complex sample matrix for both liquid and solid SPME fibers. Further improvement of these kinetic models would be highly useful in developing SPME assays in bioanalysis and to identify parameters for rigorous control and optimization.

9.2 Future Directions

The successful applications of the automated in-tip SPME technique to various drug compounds from preclinical and clinical studies clearly show that SPME can be a very useful tool in bioanalysis. However, it is important for future SPME development to understand that, while SPME cannot replace traditional sample preparation methods such as PPT, LLE, and SPE, it is an alternative approach for drug analysis. Therefore, future research in bioanalysis using SPME should focus on its specialized application. For example, because of its time consuming and compound dependent method development and validation process, SPME is not suitable for the drug discovery environment where simple, generic methods are often required for high throughput and fast sample turn around. Since SPME is simple and easy to use with low cost, its advantages will be more substantial in clinical drug development when bioanalytical assays are fully established and a large amount of samples need to be analyzed. To promote the SPME technique in bioanalysis, the areas and directions outlined below should be considered.

9.2.1 SPME Fiber Coatings and Devices

Fiber commercialization is the key for widespread acceptance of SPME technique by bioanalytical chemists in pharmaceutical industry. This should be accomplished through close collaboration between researchers, customers, and manufacturers. It is useful to develop a broad selection of new types of SPME fiber coatings, but it is even more important to have a product in the market that can be used by any customer for routine drug analysis. Sorbent-packed in-tip SPME has a great potential to become the first line of high throughput automated SPME fiber products for bioanalysis if the fiber

fabricating procedure can be further improved to increase inter-fiber reproducibility and fiber extraction capacity and efficiency. Research on in vivo SPME sampling has been extensively conducted recently due to the very promising applications of the technique because it eliminates the need for blood withdrawal during pharmacokinetic studies and allows the study of various biochemical processes directly in vivo. However, for most SPME in vivo studies, the sample preparation process, especially the analytes desorption step, is done manually, this will greatly hamper the wide applications of the technique. The fiber-packed in-tip SPME would, potentially, be a solution to increase sample throughput for in vivo SPME if the fiber preparation procedure can be further modified to assemble a fiber easily into the individual pipette tip after in vivo sampling.

Dried Blood Spot (DBS) and Microdosing techniques are two interesting new trends in drug discovery and development. Although the two techniques provide great benefits to pharmacokinetic studies, it has to be mentioned that some challenges in terms of sample preparation are enormous. For example, in DBS, the standard sample preparation procedure is very time consuming and labor intensive as it consists of punching out a disk from the card that contains the DBS followed by extraction of the analyte. Automation of sample preparation is an emerging need to increase efficiency and throughput. Currently, there are no effective sample preparation methods to obtain trace amounts of analytes from biological fluids when drug dose level does not exceed 100 μg in Microdosing studies. SPME can potentially be used in these two areas if appropriate SPME devices are developed with extremely selective fiber coatings. An initial study using C18 disk/film-packed in-tip SPME format indicated that the absolute extraction recovery could be increased 4-5 fold compared to C18 sorbent-packed in-tip under the

same extraction conditions. As shown in Figure 7-7, blood samples can potentially be collected directly in the film-packed in-tip SPME fibers so that sample preparation procedure will be fully automated. With the development of highly selective fiber coatings, such as molecular imprinting on SPME fibers, and the help from modern instrument including UPLC and API 5000 mass spectrometer, it is quite possible to detect analytes and metabolites at very low concentrations for microdosing studies.

9.2.2 SPME Application Kit

One of the limitations of SPME is that each application of interest involving different target analytes and sample matrices requires its own separate SPME method development procedure and extraction conditions must be consistent for all of the samples analyzed in one batch. Although many guidelines have been given for general SPME method development, in the practical performance of SPME, there are a number of SPME parameters must be carefully considered and specified for the problem under investigation. These include fiber coating, extraction mode, agitation method, sample volume, pH, ionic strength, water and organic solvent, extraction time, desorption conditions, calibration method, etc. For any applications that will not need high sample throughput, such as in vivo SPME sampling in animal tissues and cerebrospinal fluids (CSF), to make the technique more user friendly, SPME application kits should be developed with detailed information of the properties of the fiber coating, in vivo sampling and calibration procedures, potential target analytes/biologics (proteins and peptides) for best performance, desorption conditions, and sampling devices as well as any necessary accessories. The robustness and reproducibility of the SPME application

kits will make SPME a unique tool and a specialized approach in certain areas that traditional methods cannot achieve.

9.2.3 SPME Modeling and Simulation

SPME modeling and simulation is an interesting area that needs to be further explored because it will be a useful tool to provide insight and direction when developing SPME methods and identifying parameters for rigorous control and optimization. Currently, mechanistically based models to describe the kinetics of the partition processes to SPME fibers mainly focus on liquid fibers such as PDMS. In addition, some important parameters that are necessary to the models including distribution coefficient, binding constant, and diffusion coefficients in sample matrix and fiber coating, etc. may not always be available for the interested analytes which will affect the performance of the models. For any unknown compound, it would be ideal for an appropriate model to predict the concentrations of the target analyte in the fiber as a function of time based on the known parameters such as the types and thickness of the fiber coatings, the chemical properties of the compound, the geometries of the fiber and sample matrix, and the agitation conditions. To achieve this, a large SPME database needs to be built to determine the correlation between distribution coefficients, binding constants and diffusion coefficients, and the physicochemical properties including, but not limited to, structure, molecular weight, and polarity, of various compounds with the literature data and additional experimental results. It is expected that effective use of the SPME modeling and simulation will minimize the number of experiments that need to be performed and, ultimately, decrease the total cost and cycle time of the SPME approach.

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