High-Throughput Method Development Approaches for Bioanalytical Mass Spectrometry

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Abstract

A rational approach to the development and optimization of solid-phase extraction (SPE) methods is described. The semiautomated scheme allows for the simultaneous testing of multiple chemistries using a custom multiple-sorbent 96-well method development plate. Optimized extraction conditions for up to five analytes are determined in a single 2.5-h experiment. The experiment can be tailored to determine SPE conditions (including wash protocols) for related analytes. Data obtained by liquid chromatography–atmospheric pressure ionization-mass spectrometry allows the quantitation of absolute recovery and selection of the best extraction conditions for approximately 100 analytes of diverse structure. Optimized extraction protocols yielding at least 80% recovery are determined for 81% of the analytes. For 96% of the analytes screened, extraction conditions resulting in recoveries of ≥ 60% are determined. The most generic set of SPE conditions consist of either C8 or C18 sorbent with an eluent composition of acetonitrile with 5mM nitric acid added.

Introduction

The development and utilization of high-speed synthesis and combinatorial chemistry techniques for drug synthesis have accelerated the drug discovery process. Large sets of compounds (combinatorial libraries) are created in short time frames and are rapidly screened for biological activity using automated high-throughput systems. Drug metabolism plays an increasingly critical role in support of drug discovery when evaluating the pharmacodynamic and pharmacokinetic properties of candidate compounds. Liquid chromatography interfaced with atmospheric pressure ionization mass spectrometry (LC–API-MS) is the current method of choice for the analysis of drugs in biological fluids and the generation of drug metabolism parameters. This is due to the speed, high specificity, and sensitivity of LC–API-MS and its applicability to compounds of diverse structure. In addition, compounds entering drug development require a quick and efficient bioanalytical method development to provide timely drug metabolism information in support of ongoing drug safety and clinical studies.

A significant advance in high-throughput solid-phase extraction (SPE) has been the development of the 96-well microtiter plate format in a flow-through system (1). This technique utilizes single blocks or plates having 96 wells that contain disks or packed beds of sorbent particles arranged in an 8-row by 12-column rectangular matrix. Many high-throughput bioanalytical techniques have been successfully demonstrated using SPE plates packed with bonded silica or copolymer sorbent beds (2–6). Although 4- and 8-tip liquid handling workstations can be used for automation of the 96-well SPE methods, our laboratory has previously demonstrated that higher throughput can be obtained by adopting a 96-tip liquid delivery system (3).

The development of SPE methods for new drugs requires a thorough optimization of the SPE method for each compound, including sorbent chemistry, sample load conditions, and wash and elution solvents. An automated approach for SPE method development has been described using individual SPE cartridges to test sorbent chemistries (7). The use of microtiter SPE plates configured with multiple sorbents per plate can simplify and expedite SPE method development. Only recently have multiple sorbents formatted within the same extraction microtiter plate been commercially available from several manufacturers.

The present work describes an approach for the rapid development of optimized SPE methods for analytes in biological fluids. It utilizes microtiter extraction plates containing multiple sorbents and automated 96-tip liquid dispensing. The technique can be used to routinely develop methods for multiple analytes by examining a set of eluents and sorbent chemistries to rapidly identify the optimal sorbent chemistry and eluent composition for each analyte. In addition, insight into the SPE chemistry controlling extraction is obtained in a single experiment, resulting in a wealth of analytical information. The current approach has been applied to more than 100 drug prototypes from various therapeutic programs. These results allowed the identification of a generic SPE scheme for most analytes.

Experimental

Chemicals and reagents

All solvents were of high-performance liquid chromatography (HPLC)-grade or better. Water was purified with a Milli-Q system

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(Millipore, Marlborough, MA) and had resistivity of 18.2 W/cm. Nitric acid and calcium acetate were obtained from Fisher Scientific (Fair Lawn, NJ).

Control plasma was obtained from Cocalico Biologicals (Reamstown, PA). All samples and controls were stored at –20°C. Approximately 100 drugs were examined in these studies, and all were obtained from Pfizer Inc. (Groton, CT).

Deep-well (1.2 mL) polypropylene microtiter plates, used as sample blocks and for eluate collection, were obtained from Marsh Biomedical (Rochester, NY). Empore™ microtiter SPE plates for method development, containing C_2, C_8, C_{18}, and mixed-phase cation (MPC) sorbents within the same plate, were experimental products fabricated by 3M Company (St. Paul, MN) at our request. Microlute™ extraction plates containing LMS sorbent (10 mg) were obtained from Varian Sample Prep Products (Harbor City, CA).

SPE and assay setup

Two solutions were prepared in 5-mL volumes, and up to five analytes were spiked into a plasma (extraction stock) and into methanol (100% recovery stock) at a final concentration in the lower quartile of the expected standard curve range (50–250 ng/mL). A 100-µL aliquot of the plasma stock solution was then transferred to each well of a 32-well rectangular layout in a deep-well polypropylene plate (e.g., rows A–H columns 1–4 were filled), and a diluent volume of 200 µL of 1% aqueous acetic acid was then added to each well.

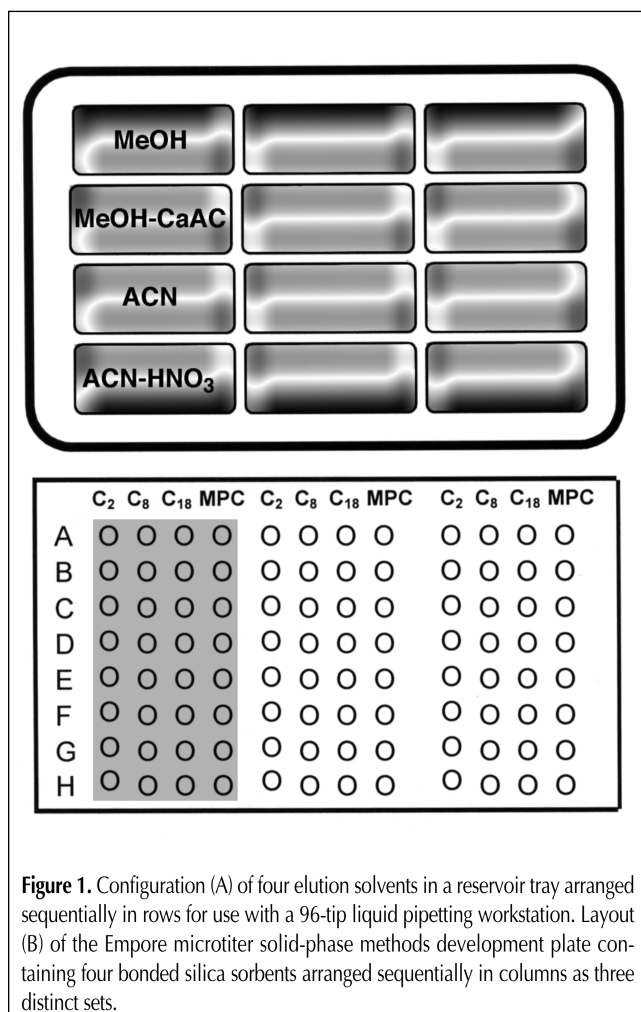

Figure 1. Configuration (A) of four elution solvents in a reservoir tray arranged sequentially in rows for use with a 96-tip liquid pipetting workstation. Layout (B) of the Empore microtiter solid-phase methods development plate containing four bonded silica sorbents arranged sequentially in columns as three distinct sets.

The transfer was accomplished manually using an 8-channel articulating pippetor (MATRIX, Lowell, MA) or via automation using a MultiPROBE 104DT liquid handling workstation (Packard Instruments, Meriden, CT). The prepared deep-well sample plate was then processed using a programmable 96-tip pipetting workstation (Quadra 96, Tomtec, Hamden, CT) outfitted in-house with a QIAvac vacuum manifold (Qiagen, Chatsworth, CA) and a specially designed valve/vent system (3). A divided eluent reservoir was used to partition selected eluents to be tested (Figure 1).

The SPE plates were processed using a delivery sequence in which the 96 tips aspirated, in succession, 300 µL diluted plasma followed by 25 µL air and then 50 µL methanol. The Quadra 96 dispensed these volumes into the SPE in succession with the vacuum adjusted as previously described (3). Elution volumes of 100 µL were used, and eluate was later diluted with water (50 or 100 µL) to achieve a solvent strength similar to an HPLC mobile phase. A 100-µL aliquot of the methanol stock was added to clean the wells of the collection plate after the elution step. These served as recovery standards and were treated in a similar fashion to the eluate. A polypropylene lid (Whatman-Polyfitronics, Lowell, MA) was then heat-sealed onto the surface of the collection plates. A Gilson 233 XL autosampler injected 50-µL volumes for LC–MS analysis.

The mean peak area for the recovery standards was compared to the peak areas obtained for the sorbent–eluent combinations after LC–MS analysis. A Microsoft (Redmond, WA) Excel spreadsheet with embedded formulas was used for data analysis.

LC–MS conditions

Analysis utilized two model PU 980 HPLC pumps along with a DG-980-50 membrane degasser (Jasco, Easton, MD). The autosampler was a model 233 XL (Gilson, Middleton, WI). An API-150 single quadrupole MS (PE-Sciex , Ontario, Canada) equipped with an atmospheric pressure chemical ionization (APCI) probe was used for mass analysis. Stock solutions of the test analytes were prepared at concentrations between 10 and 50 µg/mL. The stock solutions were used to determine the molecular ion for MS analysis and for preparing solutions in the test matrix (e.g., dog plasma). Generic LC conditions were utilized; they consisted of a linear high-pressure gradient from 10 to 90% organic mobile phase component over 2 min; equilibration time was 30 s. Short (20–30 mm) HPLC columns, such as a HIACART C_{18} column (20 × 3.2 mm, 5 µm, Higgins Analytical, Mountain View, CA) or equivalent, were used for all analyses. The organic component of the mobile phase consisted of acetonitrile, and the aqueous component was 5mM ammonium acetate. Injection volumes ranged between 10 and 40 µL of diluted eluate.

Results and Discussion

The analytical method requirements for drug discovery support are different from those needed for drug development and GLP bioanalysis. In drug discovery applications, the required

limit of quantitation is often higher than that for clinical or GLP toxicokinetic studies. Also, the number of compounds requiring in vitro/in vivo analysis is high, but the number of samples per compound is low. The present goal was to develop a fast SPE method development protocol and investigate the potential for a generic set of SPE conditions that would be applicable to most analytes. This approach required the evaluation of various elution solvents and modifiers. The elution solvents used were methanol, methanol–1mM calcium acetate, acetonitrile, and acetonitrile–5mM nitric acid. Calcium acetate was included because the doubly charged cation is thought to disrupt cation-exchange interactions on the silica surface. Nitric acid was chosen because the strong acid is expected to neutralize secondary ion-exchange interactions with the silica surface (8). The evalution of different sorbents utilized a method development plate designed in collaboration with 3M. The plate was configured with four bonded-silica sorbents (C_2, C_8, C_{18}, and MPC), the latter consisting of C_8 and benzene sulfonic acid functional groups bonded to the same silica particles. These four sorbents in membrane format are arranged sequentially in columns as three distinct sets. This arrangement (Figure 1) allows for the conducting of experiments using partial plates in blocks of 32 wells at a time. At the time the plates were fabricated, the choice of empore sorbents was limited. A styrene divinylbenzene polymer sorbent (LMS, Varian) was also tested to investigate a wider range of sorbent chemistries. It was decided not to include the Empore MPC sorbent in this evaluation because its requirements, relative to precise pH control, were not compatible with our automated approach.

Table I. Percent Recovery for Diverse Compound Set (*n* ~ 100)

Percent recovery	Percentage of compounds
> 90%	65%
80–90%	16%
70–79%	9%
60–69%	6%
30–60%	3%
< 30%	1%

Table II. Percentage of Compounds Yielding Recovery of Greater Than 50% and 70% on Sorbents and Eluents Tested

Elution solvent	Recovery	C_2	C_8	C_{18}	LMS*
Methanol	> 50%	65	63	71	48
	> 70%	51	42	59	19
Methanol +†	> 50%	69	72	79	44
	> 70%	45	45	54	23
Acetonitrile	> 50%	55	56	65	60
	> 70%	34	29	50	38
Acetonitrile +‡	> 50%	65	81	80	63
	> 70%	38	63	70	38

* LMS, polystyrene divinylbenzene (Varian Sample Prep Products, Harbor City, CA).
† Methanol plus 1 mm calcium acetate.
‡ Acetonitrile plus 5 mm nitric acid.

The method development approach described above was applied to more than 100 compounds from various therapeutic programs. The extraction of each compound was evaluated by at least 16 different conditions (4 sorbents × 4 eluents). The compounds were then grouped by highest recovery obtained (Table I). Sorbent and eluent conditions yielding better than 90% recovery were found for 65% of the compounds tested. Conditions yielding better than 70% recovery were found for 90% of the test analytes. These can be considered tailored methods that are optimal for a given compound across all the SPE combinations. In many cases, the method development approach led to optimized conditions that differed for structurally related compounds; that is, although the core structure of the analytes was similar, the required SPE chemistry (as indicated from extraction recovery) was influenced by a unique structural modification (e.g., caboxylic acid group).

The results are presented in Table II as the percentage of compounds having a recovery greater than 50% or 70% under a given set of sorbent and eluent conditions. Although recovery of greater than 70% might be considered optimal for most applications, SPE conditions yielding recovery in excess of 50% represents an acceptable generic method for multiple compounds in drug discovery applications. It was found that acetonitrile with an acidic additive (in this case, 5mM nitric acid) would yield recoveries of better than 50% for 8 out of every 10 compounds tested (i.e., 80% of the time). In this study, therefore, the SPE conditions having the broadest applicability were the C_8 or C_{18} sorbent along with acetonitrile–5mM nitric acid as the eluent.

These results indicated that on the silica-based phases, methanol is generally a stronger eluent than acetonitrile. The difference in eluent strength between methanol and acetonitrile is more pronounced on the C_2 phase, where polar interaction dominates, and is less pronounced on the C_{18} phase, where non-polar interaction dominates. Methanol can penetrate deeper into the phase, thereby disrupting polar interactions on the silica surface, and these retention mechanisms play a larger role on the C_8 and C_2 phases (9). There was no significant difference in recovery for the methanol–calcium acetate additive in comparison with methanol alone. Acidified actetonitrile was the strongest eluent, yielding better than 70% recovery for 70% of the compounds tested on the C_{18} sorbent. The LMS polymer sorbent performed best using acetonitrile as the elution solvent (> 50% recovery for 60% of compounds, > 70% recovery for 38% of compounds). In the case of the LMS phase, modifiers did not noticeably influence analyte desorption from the sorbent bed. This was most likely due to the absense of silanol interactions.

Once the relationship between sorbent, eluent, and analyte is understood, the choice of optimal wash solvent can be deduced. For example, if secondary interactions control extraction chemistry, C_2 with acidified methanol may provide optimal analyte recovery. Acetonitrile, in this case, may yield no or very low recovery. When the results of the recovery experiment are considered, it becomes clear that acetonitrile (possibly diluted) can be used as a wash solvent. Therefore, all of the information

necessary to design optimized SPE protocols can be obtained from a single method development experiment.

This scheme allows SPE conditions to be optimized for a single compound or a structural series of multiple compounds. Using multiple analytes whose core structure is similar but contain unique functional groups can provide insight into the most important structural features and how they influence SPE recovery. The results can then be used to tailor an SPE protocol for a series of compounds. In this case, the SPE conditions might be biased to the most difficult analyte at the expense of optimal recovery across the entire set.

The time required to evaluate four sorbents and four elution solvents in duplicate (approximately 40 samples, including recovery standards) is about 2.5 h. This includes setup, extraction, analysis, and data interpretation. The present array method could be expanded to include more sorbents or eluents in the 96-well format, but expanding the experiment beyond four sorbents or eluents may be a case of diminishing returns. In other words, for a relatively small time investment, a great deal of information is obtained. It may be more effective to keep the initial experiment small. In those rare cases where satisfactory SPE conditions are not found, a second experiment based on the initial results can be designed rather than expanding the first experiment to include many alternatives.

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