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Evaluation of automated micro solid phase extraction tips (μ-SPE) for the validation of a LC–MS/MS bioanalytical method

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Abstract

Automated μ-SPE tips were successfully utilized for the determination of posaconazole in rat plasma. The bioanalytical method using μ-SPE tips was successfully qualified for routine quantitation of posaconazole over the concentration range of 10.0–10,000 ng/mL in rat EDTA plasma. Inter-assay precision, based on percent relative deviation for $n = 18$ replicate quality controls, was ≤5.7%. Inter-assay accuracy based on $n = 18$ replicate quality controls was ±7.7%. Complete solid phase extraction using μ-SPE tips was demonstrated on a Tomtec liquid handler where >95% recovery for posaconazole was obtained. The μ-SPE tips had sufficient capacity to extract at least 100 μL plasma fortified with 10 μg/mL of posaconazole and the analyte could be efficiently eluted with as little as 60 μL of methanol. Of particular note is the unique ability of these μ-SPE tips to perform exhaustive solid phase extraction more commonly performed when using liquid/liquid extraction.

Keywords: Solid-phase extraction; SPE; Extraction methods; LC–MS/MS; Preclinical studies; Posaconazole; OMIX

1. Introduction

State-of-the-art LC–MS/MS instrumentation is the workhorse of the modern bioanalytical laboratory because of its sensitivity, ruggedness and specificity [1–3]. Since, its introduction, LC–MS/MS as a technique has increased the productivity of the modern bioanalytical laboratory many folds as advancements in the areas of sample preparation, mass spectrometry, HPLC instrumentation and column technologies have matured. For a period after its introduction, it was believed that LC–MS/MS was sufficiently specific that little or no sample cleanup was required. During this period, the most common method of sample extraction consisted of acetonitrile precipitation of plasma proteins followed by rapid gradient or isocratic chromatographic separation on a short column (generally 3 cm or less) [4,5]. Subsequently, it became apparent that inadequate sample cleanup and inefficient chromatography often led to ion suppression which resulted in imprecision and lack of reproducibility in bioanalysis [6–9]. As a result, the most recent trends in both clinical and non-clinical bioanalysis are toward traditional sample preparation techniques such as liquid/liquid extraction and solid phase extraction in combination with higher resolving power chromatography [10,11].

Solid phase extraction has established itself as an important sample preparation technique in the field of bioanalysis and is preferred over other traditional extraction procedures such as liquid/liquid extraction [12] because it is more efficient, more easily automated and less time-consuming. Numerous publications review the topic of solid phase extraction technique for the analysis of biological samples [13,14]. Special attention has been given to the development of SPE devices that can be automated for higher throughput. For example, modern LC–MS/MS instrumentation is capable of injecting an extracted analytical sample every 2–3 min. In order to keep such LC–MS/MS systems fully utilized, analytical runs with batch sizes of 300 samples are often generated. Compared to the traditional cartridge type SPE set-up where only 8 or 16 analytical samples could be prepared at the same time, utilization of the 96-well plate format allows rapid, concurrent sample cleanup for 96 samples using automated robotic liquid handlers. In addition, the small-volume 96-well SPE devices have the advantage of lower solvent requirements, minimal desorption volumes, and decreased void volumes [15,16].

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However, the 96-well plate format still suffers from a few drawbacks. Because 96-well SPE plate flow is unidirectional, exhaustive extraction of the same plasma sample is time consuming and requires a second sample transfer after elution. Secondly, the plate format is not flexible which makes screening multiple sorbants difficult without wasting unused SPE wells. Finally, the 96-well SPE plate is expensive, costing as much as $3 per well.

Compared to their large syringe-barreled cartridge predecessors, three types of small volume SPE devices in the 96-well footprint are in common use today. The most popular type are 96-well SPE formats made from 40 μm to 60 μm d_p chemically bonded reverse phase silica. This modern day 96-well version of the classic cartridge contains bonded silica typically having a bed mass between 25 mg and 100 mg and is packed in a polyethylene cartridge body with frits that sandwich the extraction media. Available in a myriad of chemical compositions, some examples of these plates include trade names such as Bond Elut®, DISCOVERY® and Sep-Pak®. The second most popular type of 96-well SPE plate is based on the styrene-divinylbenzene and *N*-vinylpyrrolidone copolymer sorbents. The high specific area and the ability to work under deweted conditions make these types of sorbents the best choice when a "universal" extraction is required. These plates are sold under the trademarks of Oasis® and STRATA® by Waters and Phenomenex, respectively. The third type of 96-well SPE plate utilizes monolithic silica disc technology. The advantage of this format is that it allows higher flow-rates without channeling effects because of its large cross-sectional area. The thin disc design of these plates makes them the least likely to clog which translates to more uniform flows across the plate. Available products under this category are sold under the trademarks of SPEC® and Empore® by Varian and 3 M, respectively.

In addition to 96-well SPE plates, recent interest in proteomics and metabolomics have prompted the development of newer formats such as micropipette tip-based SPE. These micropipette tips typically contain less than 1 mg of solid phase material and fit onto single or multichannel pipettes. These formats are capable of purifying miniscule quantities of analyte. Available products include tips sold under the trademark ZipTip® from Millipore. Shukla and Majors have published a comprehensive discussion on the use of micropipette tip-based protein sample preparation [17]. Of particular note is a recent study for simultaneous determination of 10 antihistamine drugs in human plasma using MonoTip C18 solid phase extraction pipette tips conducted by Hasegawa et al. [18]. Although readily compatible, the use of automated tip-based SPE for use in bioanalytical quantitation of small molecule drugs has not been previously reported.

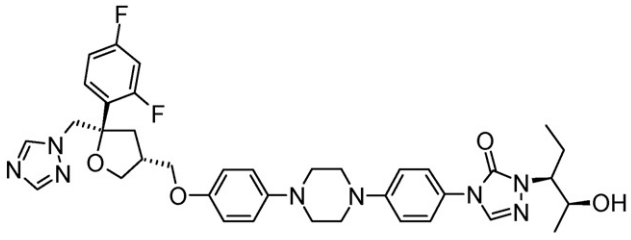
In the pharmaceutical area, before a new chemical entity (NCE) can be tested in human subjects, extensive animal testing is conducted to evaluate systemic exposure of the drug and its metabolites to help correlate exposure to toxicity. These toxicological tests are carried out in animal species such as rat, mouse, monkey or dog. The bioanalysis used to determine the concentration of the drug and metabolite in vivo is conducted in accordance with the principles of good laboratory practices (GLP). Regulatory agencies have required that bioanalytical methods be validated to ensure that the method is reliable and reproducible for its intended use [19]. In this report, we evaluate the use of automated micro solid phase extraction tips (μ-SPE) in a bioanalytical method to support toxicology studies. The bioanalytical method was subsequently validated to determine the accuracy, precision, selectivity, sensitivity, and reproducibility.

The model compound used in this study was posaconazole, a novel broad-spectrum triazole antifungal compound discovered by Schering-Plough Research Institute. Posaconazole is a potent, selective inhibitor of the enzyme 14α-demethylase (CYP 51) which is important in cell wall biosynthesis for yeast and molds [20]. Because of its novel structure, development of resistance to posaconazole is expected to be slower than for other triazoles. To support non-clinical toxicokinetic studies, an LC–MS/MS method was required that had a quantitative range of 10–10,000 ng/mL and used a 50 μL aliquot of EDTA rat plasma.

2. Experimental

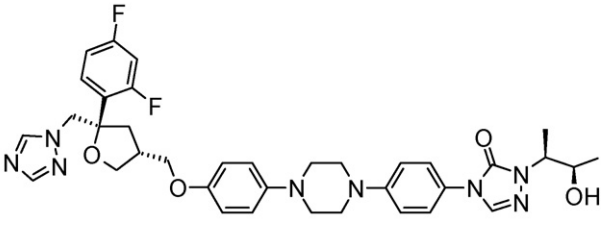
2.1. Materials and reagents

Certified posaconazole and its internal standard (IS) SCH 56984 (Fig. 1) were synthesized at Schering-Plough Research Institute (Kenilworth, NJ) with a purity of 99.7% and 98.6%, respectively. Rat plasma with ethylenediaminetetraacetic acid (EDTA) as the anticoagulant was obtained from Bioreclamation Inc. (Hicksville, NY). The blank matrix was used as received for the preparation of calibration standards (STD) and qual-



Posaconazole

Formula Weight: 700.8



SCH 56984

Formula Weight: 686.8

Fig. 1. Structures of posaconazole and SCH 56984.

ity control (QC) samples. HPLC grade methanol, acetonitrile, water and ACS grade formic acid and ammonium hydroxide were obtained from Fisher Scientific (Fair Lawn, NJ). All other chemical reagents used were reagent grade or higher and used without further purification.

2.2. Liquid chromatography–tandem mass spectrometry

The LC–MS/MS system consisted of an integrated Shimadzu high performance liquid chromatography system and an Applied Biosystems/MDS Sciex (Concord, Ontario) API 3000 triple quadrupole mass spectrometer. The LC system had two Shimadzu 10ADvp LC pumps (Shimadzu Corporation, Columbia, MD, USA), a Shimadzu SCL-10A system controller, and a Shimadzu DGU-14A degasser. The autosampler used was a LEAP CTC PAL (Leap Technologies, Carrboro, NC) with a cooled sample storage compartment. The HPLC column was a Varian (Varian Corp, Walnut Creek, CA) Polaris C-18A 2.0 mm × 50 mm column with 5 μm particle size. A gradient elution program (Table 1) was used to separate posaconazole and its internal standard from the bulk of the endogenous matrix components. Mobile phase A consisted of water–methanol–formic acid (90:10:0.1, v:v:v) solution and mobile phase B consisted of acetonitrile–methanol–formic acid (90:10:0.1, v:v:v). The retention times for posaconazole and its IS were 2.0 and 2.1 min, respectively.

Unless otherwise noted, the mass spectrometer was operated in the multiple reaction monitoring (MRM) mode. Sample introduction and ionization was by atmospheric pressure chemical ionization (APCI) in the positive ion mode. The corona discharge needle was set to 4.5 μA. The declustering potential (DP) was set to 80 V and the collision energy (CE) was set to 47 eV. The probe temperature (TEM) was set to 425 °C. The collision gas was set to maintain a pressure of approximately 3.1×10^{-5} Torr. To assay all analytes, the mass spectrometer was operated at unit mass resolution and set to select the MRM transitions for the $[M+H]^+$ ions of *m/z* 701 → *m/z* 683 for posaconazole and *m/z* 687 → *m/z* 669 for SCH 56984. The dwell time for both posaconazole and SCH 56984 was 250 ms.

MRM data was acquired and integrated by using the Sciex Analyst software package (version 1.3.1). Data regression was accomplished using the Watson (Thermo LabSystems, Philadelphia, PA) Laboratory Information Management System (version 6.4.0.03).

Table 1
Shimazu HPLC gradient program

Time (min)	% Mobile Phase B	% Mobile Phase A	Flow rate (μL/min)
0.3	10	90	250
1.3	75	25	250
2.5	75	25	250
2.6	100	0	500
3.5	100	0	500
3.6	10	90	400
4	10	90	250

2.3. Preparation of calibration standards and quality controls

Individual fresh stock solutions for posaconazole and SCH 56984 were prepared in methanol at a concentration of 1.0 mg/mL. Ten posaconazole calibration standards were prepared in blank rat EDTA plasma at the nominal concentrations of 10.0, 20.0, 40.0, 100, 200, 500, 1000, 4000, 8500 and 10,000 ng/mL by serial dilution from the stock solution. Three levels of posaconazole quality controls were prepared in blank rat EDTA plasma at the nominal concentrations of 30.0 (QCL), 1500 (QCM) and 7500 ng/mL (QCH) by serial dilution from the stock solution. All samples were stored frozen at −20 °C until use.

2.4. Prototype OMIX™ C18 μ-SPE tips

All experiments described herein were performed on prototype OMIX™ Tomtec C18 μ-SPE tips from Varian Corp. (Fig. 2) (Walnut Creek, CA). The μ-SPE tip was constructed by inserting a plug of OMIX™ C18 SPE material into the tip section of a Tomtec 450 μL pipette. The OMIX™ C18 media used was based on a monolithic glass fiber sorbent bed functionalized with nonpolar octadecylsilane (C18), which provided superior flow characteristics compared to a packed bed. No additional filters or frits were present in this prototype of the μ-SPE tip. During extraction, solvents/samples were aspirated/dispensed across the solid phase media. The use of a monolithic glass fiber results in a design that has less sorbent density than that used in a traditional plate format thereby enabling solvents/samples to flow freely across the media without assistance of vacuum.

2.5. Extraction procedure

Unless otherwise noted, all μ-SPE tip experiments were performed on a Tomtec (Tomtec Corp, Hamden, CT) Quadra96 model 320 liquid handler. The C18 μ-SPE tips were supplied in a format compatible with the standard Quadra96 tip racks. The Tomtec Quadra 96 model 320 was programmed using Command

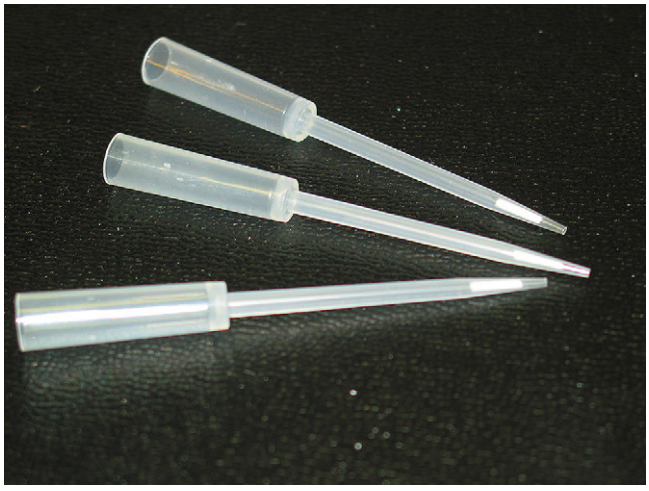


Fig. 2. Varian OMIX® Tomtec μ-SPE Tips.

and Control version 3.2. The procedures comprised of what is typical for SPE; i.e., conditioning the μ-SPE tips, application of samples into the μ-SPE tips, wash steps, and finally elution. To minimize cross well contamination, wash and elution solutions were pre-aliquoted into individual wells of 96-well blocks before being placed on the Tomtec liquid handler to be used during extraction.

Prior to aspiration of sample/reagent, a 50–150 μL air gap was drawn into the μ-SPE tips followed by an aliquot of sample/reagent and finally another 5–10 μL air gap. The entire tip contents were dispensed in a single step with the top air-gap acting as an air pump to expel all liquid from the μ-SPE tips. After each dispense cycle, 25 μL of air from the system air compressor was blown into the tips to dislodge any remaining liquid. The entire extraction procedure took place without operator intervention and without the need to apply vacuum.

2.6. Optimization and validation of extraction procedure

Two-dimensional (2D) experiments were performed to determine the composition of organic solvent needed to optimize the wash and elution steps. For this experiment, 50 μL of 10 μg/mL posaconazole in phosphate-buffered saline (PBS) solution were diluted with 100 μL of 3% ammonium hydroxide in water. Following conditioning with 50 μL of methanol and 50 μL of water, the diluted posaconazole sample in PBS was applied to a μ-SPE tip, then eluted with two 50 μL aliquots of a methanol–water mixture. The methanol composition of the mixture was sequentially increased by 10%. A total of eleven samples were eluted using methanol compositions from 0% to 100%. The experiment was repeated under acidic conditions where 2% formic acid was added to each elution mixture. All eluates were collected separately and analyzed using the LC–MS/MS conditions described above.

The capacity experiments, exhaustive extraction experiments, and elution volume experiments were performed using 10 μg/mL posaconazole in rat EDTA plasma. Following each experiment, the collected eluates were evaporated to near dryness under a stream of nitrogen and reconstituted in 250 μL of 1:1 mobile phase A–mobile phase B. External standards were added into each well to minimize detector drift.

For the method validation, experiments were performed using 50 μL aliquots of fortified rat EDTA plasma pipetted into 96-well format dilution tubes. A 25 μL aliquot of IS working solution was added to all samples except matrix blanks. Samples were capped and vortexed for 60 s on a multi-tube vortexer. A 200 μL aliquot of 3% ammonium hydroxide in water was then added to each tube and the tubes were vortexed again. The C18 μ-SPE tips were conditioned using 150 μL of methanol and 300 μL of 3% ammonium hydroxide in water. The samples were exhaustively extracted by aspirating and dispensing the plasma samples seven times from the dilution tube. The C18 μ-SPE tips were then washed with 90 μL of 3% ammonium hydroxide in water, followed by 100 μL of methanol–water (20:80, v:v). Finally, the C18 μ-SPE tips were eluted with two 50 μL aliquots of methanol–water (90:10, v:v) into a clean 96-well block. The extracts were evaporated to near dryness under a heated (50 °C) stream of nitrogen before being reconstituted with 200 μL of mobile phase A–mobile phase B (50:50, v:v). A 10 μL aliquot was injected into the LC–MS/MS system for analysis.

3. Results and discussion

3.1. 2D experiments

Two-dimensional experiments have been routinely used to optimize reverse phase solid phase extraction conditions during method development. In one example, Cheng et al. described the principle and practice of 2D SPE method development for determination of verapamil and its metabolite in plasma [21]. In this type of experiment, two main factors that control the analyte retention in reverse-phase SPE are optimized. One is the concentration of the organic modifier, the other is pH. Fig. 3 describes one such experiment for posaconazole using μ-SPE tips. Posaconazole is a highly lipophilic base ($\log P > 3$, pK_a 3.6 and 4.6). It is completely ionized when diluted in 2% formic acid in water but completely un-ionized when diluted in 3% ammonium hydroxide in water. In the 2% formic acid elution study, no posaconazole was eluted until the methanol composition reached 30% and the elution maximized at a composition of 60% methanol. These results indicated that under acidic conditions, up to 30% methanol can be used in a wash mixture to remove interferences and an elution solution containing at least 70% methanol would result in the maximum recovery of posaconazole from the μ-SPE tips. By using basic elution mixtures, the entire elution profile is shifted by approximately 10–15% toward higher organic compositions presumably because non-ionized analyte will interact more strongly with the reverse phase sorbent. For example, no posaconazole was eluted at a methanol composition of 30% and only a minimal amount of posaconazole was eluted at a methanol composition of 40%. Maximum recovery of posaconazole was observed at a methanol composition of approximately 80–90%. Of note is that slightly higher detector responses were observed when posaconazole is eluted under basic conditions compared to elution under acidic con-

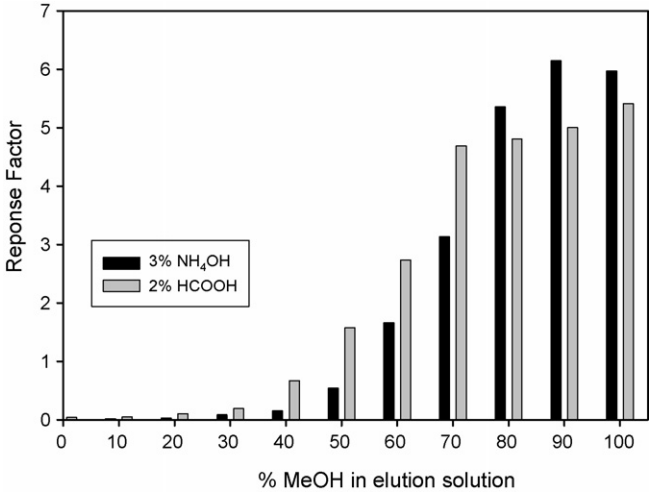


Fig. 3. 2D experiment for posaconazole under acidic and basic elution conditions.

ditions. In the final protocol, methanol–water (20:80, v:v) was used as the wash solution to remove endogenous matrix components and methanol–water (90:10, v:v) was used as the elution solution.

3.2. Capacity experiment

Because the μ-SPE tips have an extraction bed of 4.2 mg of monolithic C18 silica, it is important to calculate the sorbent bed capacity. In a toxicology study, a dynamic range for quantitation of 10 ng/mL → 10,000 ng/mL is typical. This routinely translates into a sample aliquot of up to 100 μL of serum, plasma, urine or bile for analysis. Fig. 4 describes an experiment examining the extraction capacity of the μ-SPE tips by comparing the extraction efficiency of posaconazole at four aliquot sizes between 25 μL and 100 μL. Each aliquot was diluted 1:1 (v:v) with 3% ammonium hydroxide solution and recoveries from $n = 3$ extractions were averaged for each aliquot size and compared against an extracted blank sample fortified with the same concentration of posaconazole. Two aliquots of 100 μL of elution solution was used for all studies to ensure that sufficient elution volume was used to completely release posaconazole from the sorbent. The results indicated there was a small decrease in recovery at the 25 μL and 100 μL aliquot sizes, where only 80% of posaconazole was recovered. At aliquot sizes of 50 μL and 75 μL, at least 90% of posaconazole was recovered. The slight decrease in recovery observed at the 25 μL aliquot size could be associated with sample loss on the walls of the extraction tips. Conversely, the decrease in recovery observed with a 100 μL aliquot was most likely the result of insufficient sorbent capacity. Thus, at a concentration of 10 μg/mL, the extraction capacity of the μ-SPE tips for posaconazole was calculated to be at least 1 μg in rat EDTA plasma; more than sufficient to support most toxicology studies for this NCE.

3.3. Exhaustive extraction experiment

Serial extraction is often used in liquid/liquid extraction protocols because the extraction efficiency is dictated by the partition coefficient of the analyte between the aqueous and organic phases. Repeated extractions of pH adjusted plasma with nonpolar solvents such as hexanes or ethyl acetate can often improve recovery. Although similar in principle, serial extraction is difficult to perform when using SPE because of the various steps involved such as conditioning, washing and elution.

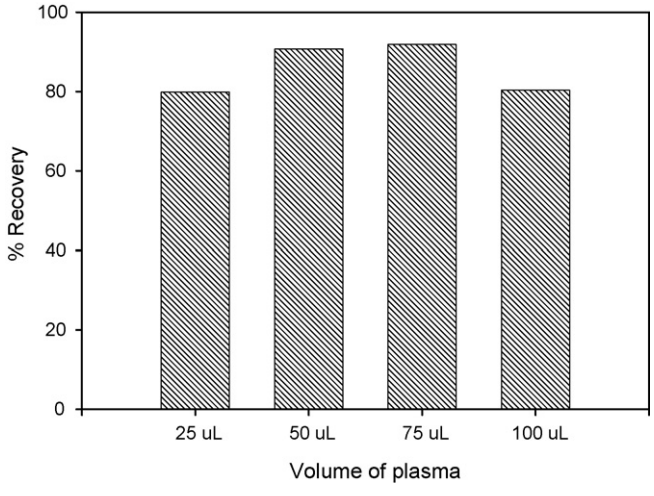


Fig. 4. Effect of an increased loading of plasma matrix on recovery.

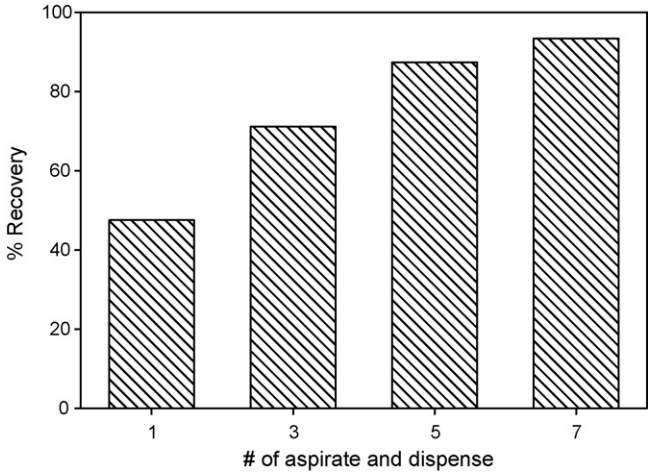


Fig. 5. Comparisons of posaconazole recovery on number of aspirate/dispense steps.

μ-SPE tips are constructed principally as automated pipette tips. Compared to the traditional 96-well SPE block, the μ-SPE tips merely require the repetition of the aspirate and dispense functions of an automated liquid handler. The advantages of this are two-fold, it is simple to perform and there are multiple interactions of the sample with the extraction medium. Fig. 5 illustrates an experiment where the recovery of posaconazole was compared against an extracted blank sample fortified with an identical concentration of posaconazole. The results of this experiment indicate that recovery increased linearly with the number of aspirates/dispenses performed. For example, when a single aspirate/dispense was performed, the average observed extraction recovery for $n = 3$ tips was slightly higher than 45%. With seven aspirate/dispense steps performed, the average observed extraction recovery for $n = 3$ tips was greater than 95%. These data highlight the ease by which the recovery of difficult to extract analytes can be improved by simply using μ-SPE tips and multiple serial extractions.

3.4. Elution volume experiment

The small bed volume of the μ-SPE tips allows for efficient release of analytes from sorbent with minimal elution volumes. The elution data in Fig. 6 describes an experiment where the recovery of posaconazole was determined as a function of elution volume. For this experiment, $n = 2$ tips were used for each elution condition. The average peak response measured was compared to an extracted blank sample fortified with an identical concentration of posaconazole. The data reveal that as much as 60% of the analyte was recovered with duplicate aliquots of 20 μL of elution solvent. Although reasonable, working with such small volumes was difficult for the Tomtec Quadra liquid

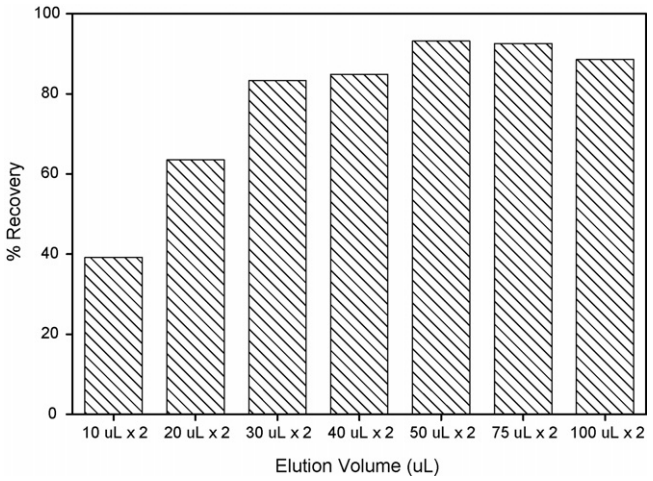


Fig. 6. Comparisons of posaconazole recovery with various elution volumes.

handler. A more reproducible elution scheme used $2 \times 50\ \mu L$ aliquots of elution solvent. This scheme provided efficient recovery of posaconazole and the potential for in-well dilution thereby eliminating the need for evaporation and reconstitution steps.

3.5. Comparison of μ-SPE tips and 96-well extraction plates

To complete the evaluation of these μ-SPE tips, a comparison was made to traditional 96-well SPE discs prepared from the same material. Triplicate extractions were performed using a 15 mg Varian SPEC® C18 plate. The same volumes of solutions used in the tip extractions were used for the plate extractions. The extraction recoveries were calculated by comparing the peak responses measured following extraction of 50 μL of 10 μg/mL posaconazole in rat EDTA plasma with those from extracted blank samples fortified with an identical concentration of posaconazole. The recoveries for this experiment were approximately 70% which is comparable to the recoveries obtained when using μ-SPE tips with triplicate aspirate/dispense steps.

3.6. Assay validation

To better evaluate the use of μ-SPE tips for routine bioanalysis, a method validation was performed. Three separate analytical batch runs were assayed. Each batch run consisted of ten non-zero calibration standards ($n = 2$ at each concentration), quality control samples prepared at three different concentrations ($n = 6$ at each concentration), two zero standards (blank rat EDTA plasma with internal standard) and two control blanks (blank rat EDTA plasma without internal standard). Assay acceptance criteria were those defined in the latest FDA guidance document for bioanalytical method validation.

The data shows that the peak area ratios of calibration standards were proportional to the concentration of analytes in each assay over the nominal concentration range of 10.0–10,000 ng/mL. The calibration curves were quadratic and were well described by a least-squares regression. A weighting factor of 1/concentration^2 was chosen to achieve homo-

Table 2
Back-calculated posaconazole concentrations in rat plasma calibration standards and calibration curve parameters[a]

	STD1 10.0 ng/mL	STD2 20.0 ng/mL	STD3 40.0 ng/mL	STD4 100 ng/mL	STD5 200 ng/mL	STD6 500 ng/mL	STD7 1000 ng/mL	STD8 4000 ng/mL	STD9 8500 ng/mL	STD10 10000 ng/mL	*A*	*B*	*C*	R-squared
Batch 1	10.7	20.4	34.8	104	186	470	1010	3840	8930	9770	0.0000000287	0.00270	0.000967	0.9936
	9.66	20.3	35.9	99.0	222	497	1080	4530	8710	9220				
Batch 2	8.61	20.4	40.4	91.6	194	501	1020	4160	7930	10000	0.0000000380	0.00291	0.00332	0.9960
	11.3	20.3	39.4	100	207	499	1000	4070	9010	9820				
Batch 3	10.1	19.8	40.9	93.4	149[b]	496	1060	3170[b]	8610	10600	0.0000000348	0.00289	0.00230	0.9972
	9.91	20.2	40.3	90.4	205	521	1000	4120	7830	9780				
n	6	6	6	6	5	6	6	5	6	6	3	3	3	3
Overall mean	10.0	20.2	38.6	96.4	203	497	1030	4140	8500	9870	0.0000000338	0.00283	0.00220	0.9956
S.D.	0.920	0.225	2.60	5.39	13.7	16.3	33.7	249	505	446	0.0000000472	0.000116	0.00118	0.0018
%CV	9.2	1.1	6.7	5.6	6.7	3.3	3.3	6.0	5.9	4.5	14.0	4.1	53.6	0.2
%DIFF	0.0	1.0	−3.5	−3.6	1.5	−0.6	3.0	3.5	0.0	−1.3				

[a] $y = Ax^2 + Bx + C$, weighted 1/concentration^2, where y is the peak area ratio of posaconazole to IS, x is the concentration of posaconazole and A, B and C are curve parameters of the calibration curve.
[b] Deactivated: %DIFF >15%, not included in statistical calculations.

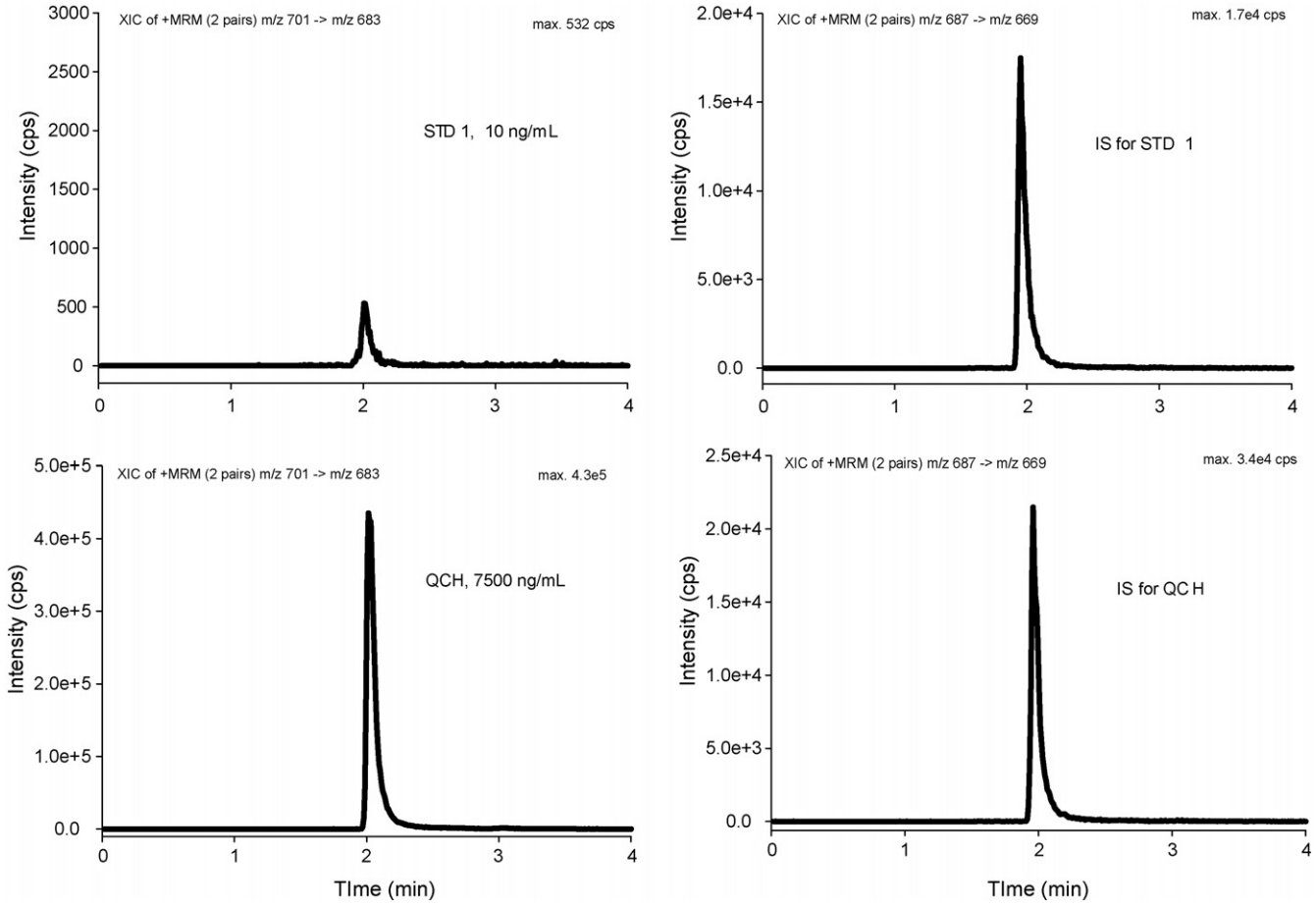


Fig. 7. MRM ion chromatograms of μ-SPE extracted calibration standard at the concentration of 10 ng/mL (top 2 traces) and MRM ion chromatograms of μ-SPE extracted quality control samples at the concentration of 7500 ng/mL (bottom 2 traces).

geneity of variance. The slopes, intercepts, and coefficients of determination from the validation analyses are summarized in Table 2.

For calibration standards, the between-run precision and accuracy results from the three analytical core runs are listed in Table 2. The intra-run accuracy (%DIFF) ranged from −3.6% to 3.5% and between run precision (%CV) ranged from 1.1% to 9.2%.

For QC samples, the between-run precision and accuracy results from three analytical runs are listed in Table 3. The inter-run accuracies were +7.7%, +1.3%, +0% for QCL, QCM and QCH ($n = 18$), respectively. The between run precision was 5.7%, 5.1% and 5.7% for QCL, QCM and QCH ($n = 18$), respectively.

Fig. 7 represents the typical response observed following an injection of a 10-μL aliquot of the reconstituted posacona-

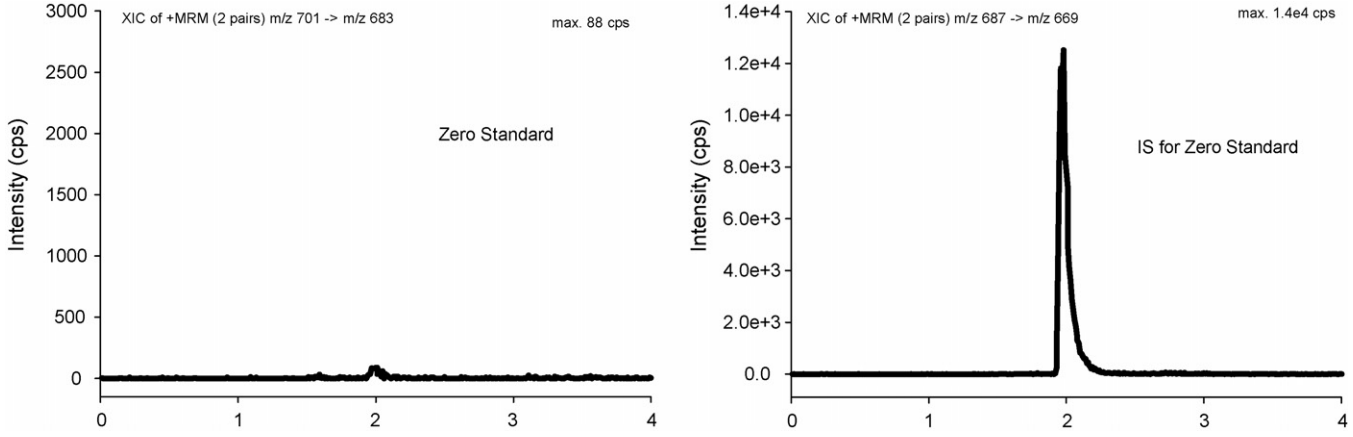


Fig. 8. MRM ion chromatograms of μ-SPE extracted zero calibration standard.

Table 3
Analytical performance of posaconazole quality control samples in rat plasma extracted using μ-SPE tips

Batch number	QCL 30.0 ng/mL	QCM 1500 ng/mL	QCH 7500 ng/mL
1	36.4	1560	7770
	33.3	1610	7740
	34.6	1570	8090
	31.6	1580	7850
	31.3	1550	8090
	31.2	1500	7050
2	35.7	1520	7120
	30.9	1490	7600
	31.5	1520	7310
	30.8	1550	7240
	31.0	1420	6990
	33.1	1630	7070
3	31.2	1520	7250
	31.6	1280	7640
	34.2	1540	7360
	29.7	1490	7600
	31.1	1470	8360
	31.7	1550	6890
Mean	32.3	1520	7500
S.D.	1.85	77.9	426
%CV	5.7	5.1	5.7
%Theoretical	107.7	101.3	100.0
%DIFF	7.7	1.3	0.0
n	18	18	18
Overall %CV	5.5		

zole LLOQ extract at 10.0 ng/mL using μ-SPE technology. As depicted, the signal/noise ratio is excellent at the LLOQ level and MRM trace is free of chromatographic interferences. As a comparison, the typical response observed following an injection of a 10-μL aliquot of the reconstituted posaconazole QCH is also shown in the same figure. Fig. 8 represents the typical response observed following injection of a 10-μL aliquot the reconstituted posaconazole zero standard (Blank with IS) sample. Typical response at the retention time of posaconazole was considered selectivity (<20% signal response of LLOQ at the same retention time). Cross-well contamination was not observed in these validation runs as all QCs back calculated with consistent accuracy and precision.

4. Conclusion

Automated μ-SPE tips were successfully utilized for the determination of posaconazole in a plasma matrix. The low bed mass of the μ-SPE tips allows for a significant reduction in conditioning/washing/elution volumes. Near complete recovery was demonstrated using as little as 60 μL of elution solvent. The capacity of the μ-SPE tips is sufficient to handle up to 100 μL of rat EDTA plasma when fortified with 10 μg/mL of posaconazole. Of particular note is the unique ability of these μ-SPE tips to perform exhaustive solid phase extraction more commonly performed when using liquid/liquid extraction. By employing exhaustive solid phase extraction, greater than 95% recovery for posaconazole was obtained. A bioanalytical method qualification demonstrated that μ-SPE tips are suitable for routine quantitation of posaconazole over the concentration range of 10.0–10,000 ng/mL in rat EDTA plasma. Inter-assay precision, based on percent relative deviation for $n = 18$ replicate quality controls, was ≤5.7%. Inter-assay accuracy based on $n = 18$ replicate quality controls was ±7.7%.

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