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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

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ABSTRACT

Studies using in-tip solid phase microextraction (in-tip SPME) in a 96-well plate format are conducted to investigate the feasibility of SPME automation. The sample preparation process, including extraction and desorption, was fully automated and coupled with currently commercially available automated liquid handling systems. Several process parameters including extraction time and speed, and desorption time were investigated. An LC–MS/MS method has been developed and validated to determine the levels of a drug compound (MK-0533) in human plasma that demonstrates the suitability of this new approach. The developed method has a lower limit of quantitation (LLOQ) of 5 ng/mL when 0.25 mL of human plasma is processed and is validated in the concentration range of 5–2000 ng/mL. The successful application of the assay in clinical sample analysis indicates that in-tip SPME can be easily automated and has great potential to be used for high throughput quantitative determination of drugs in pharmaceutical industry.

1. Introduction

Solid-phase microextraction (SPME), as a solvent-free extraction technique which combines sampling, sample clean-up and pre-concentration into a single step, has been widely used in many areas of analytical chemistry [1–5]. Automation of the SPME process using a fiber configuration has gained great success primarily coupled with GC. However, there are great challenges with automation of SPME–LC because of the different desorption mechanism between GC and LC [3,6,7]. The requirement of solvent desorption for nonvolatile drug analytes has often resulted in poorly designed and cumbersome to use SPME–LC interfaces, that may damage the fibers or introduce chromatographic artifacts such as peak broadening during liquid phase separations.

As recently reviewed in the literature [8], interfacing of LC and SPME has included such strategies as manual injection interface tees, in-tube SPME and off-line desorption followed by conventional liquid injection. Unfortunately, none of these approaches allow for automated and parallel sample preparation. Recently, many studies have been done to investigate the feasibility of SPME automation coupled with LC–MS/MS in a 96-well plate format [9–11]. Although the concept of SPME automation to liquid chromatography has been illustrated; in reality, the goal has not been fully achieved and the use of this technique as an alternative approach for quantitative determination of analytes especially in pharmaceutical bioanalysis has not been realized.

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 a new approach of using pipette tip-based SPME in a 96-well plate format. Since automated liquid handling systems are widely used in pharmaceutical industry, we are trying to couple in-tip SPME with these commercially available systems, while maintaining the simplicity of SPME technique, which is the biggest advantage compared with other conventional extraction methods such as liquid–liquid extraction (LLE) and solid-phase extraction (SPE). Based on this new approach, we have developed an assay in human plasma to determine levels of a peroxisome proliferator activated receptor modulator drug compound (MK-0533) under going clinical investigation [12]. In this assay, sample extraction and desorption using in-tip SPME is fully automated, and several process parameters such as fiber preparation, extraction time, extraction speed, and desorption time are investigated. A head-to-head comparison of results obtained from healthy subjects after administration of 75 mg of drug using in-tip SPME and LLE extraction methods, demonstrate that this revolutionary concept of coupling in-tip SPME with automated liquid handling system is a suitable approach for SPME–LC automation.

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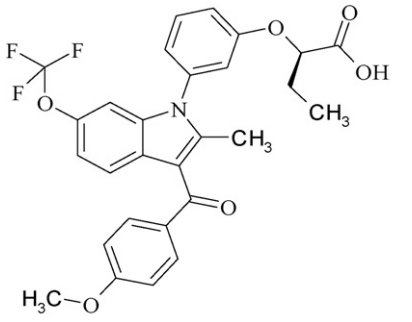
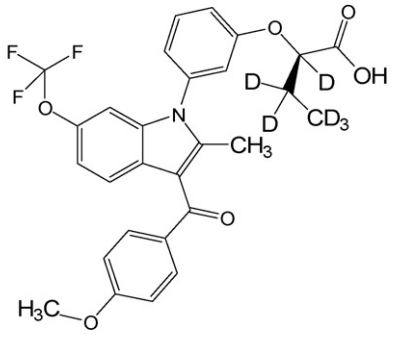


MK-0533



ISTD

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

2. Experimental

2.1. Materials

A peroxisome proliferator activated receptor modulator drug compound (MK-0533, Fig. 1) being investigated for the treatment of type 2 diabetes, and its deuterated internal standard (ISTD), 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). Polydimethylsiloxane (PDMS)–divinylbenzene (DVB) fibers (60 μm) were purchased from Supelco (Bellefonte, PA, USA). Deep 96-well 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.

2.2. Instrumentation

A PerkinElmer (Norwalk, CT, USA) LC-200 micro-pump and a CTC PAL Leap autosampler (Carrboro, NC, USA) for 96-well plates were used in this work. The chromatographic separation of analytes was performed on a Restek BDS Hypersil C18 column (5 mm × 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 3 min. 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 quadruple mass spectrometer (Foster City, CA, USA) equipped with a turbo ion spray (TIS) source operating in the positive ion ionization mode was used for all HPLC–MS/MS analysis. Multiple reaction-monitoring (MRM) mode was utilized for quantitation. In TIS experiments, the turbo ion spray probe temperature was maintained at 450 °C, and the ion spray voltage was 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 μL/min into a mobile phase pumped at 0.2 mL/min through the turbo ion spray interface. Multiple reactions monitoring of the precursor → product ion pairs at *m/z* 528 → 135 for MK-0533 and *m/z* 534 → 135 for ISTD was used for quantitation.

2.3. Preparation of standard solutions and plasma samples

A stock solution of MK-0533 (100 μg/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 25, 50, 250, 500, 1000, 2500, 5000 and 10,000 ng/mL. The ISTD was also prepared as a stock solution (100 μg/mL) in ACN: water (50:50, v/v). A working standard solution of 1000 ng/mL of 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 was added to prevent potential hydrolysis of acyl-glucuronide metabolite). The resulting plasma standard concentrations ranged from 5 to 2000 ng/mL.

2.4. Preparation of in-tip SPME fibers

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. (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. Up to 24 in-tip SPME fibers were prepared in the same way and were used simultaneously for extraction and desorption of the samples.

2.5. SPME conditions

Subject plasma samples were thawed at room temperature. 250 μL of subject plasma samples were added individually into a 2.4 mL deep 96-well plate spiked with 50 μL of ACN:water (50:50, v/v). Standard curve samples were prepared by spiking 50 μL of appropriate standard into 250 μL of acidified human control plasma. Internal standard solution (50 μL) was added to each well of the plate. After adding 250 μL of water 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 min. A Packard MultiPROBE II liquid handling system (Meriden, CT, USA) was used for plasma sample and all liquid transfer from the above process. In-tip SPME extraction and desorption process

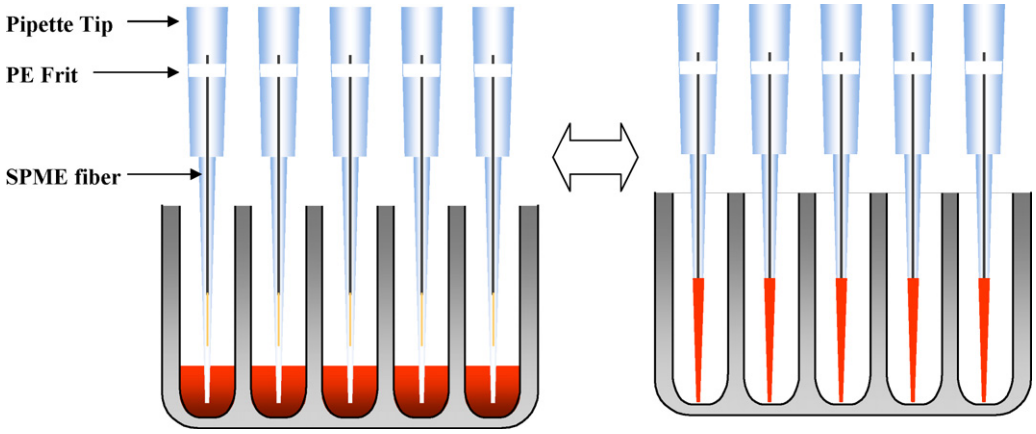


Fig. 2. In-tip SPME aspiration and dispense demo.

was fully automated using a Tomtec Quadra 96 workstation (Hamden, CT, USA) as controlled by the instrument's software. Briefly, 24 in-tip SPME fibers were loaded at position 1 (tip plate) on the deck of the Tomtec Quadra 96 workstation. Water, ACN and fiber cleaning solvent (80% ACN:20% water, v:v) reservoirs were placed at position 2 (washing plate), position 3 (ACN plate), and position 5 (cleaning plate), respectively. An empty 1.2 mL deep 96-well plate was placed at position 4 (desorption plate) for desorption. The mat was carefully removed from the 2.4 mL sample plate and placed the plate at position 6 (extraction plate) for extraction. The Tomtec Quadra 96 workstation was programmed as follows: After 24 tips were picked up, 100 μL of plasma was repeatedly aspirated and dispensed for 40 min from extraction plate at position 6. When extraction was completed, 24 in-tip SPME fibers were washed once with 100 μL water at washing plate, then 200 μL of ACN was aspirated from ACN reservoir with 50 μL air gap and dispensed into the empty 1.2 mL desorption plate, followed by 100 μL of ACN repeatedly aspirated and dispensed in the same plate for 120 cycles. Finally, the 24 in-tip SPME fibers were moved to cleaning plate at position 5 for fiber cleaning, and the aspiration/dispense cycles depended on an acceptable carry-over level after cleaning. The 1.2 mL deep 96-well plate was evaporated to dryness under heated N_2 stream and reconstituted in 150 μL of ACN:10 mM ammonium formate (80:20, v/v, adjusted pH to 3.3 using formic acid) solution, and 10 μL aliquots were injected into the HPLC–MS/MS system.

2.6. Method validation (precision, accuracy, selective, stability, and recovery)

The precision of the method was determined using triplicate analysis ($n = 3$) of drug compound in three 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 coefficient of variation (% C.V.) was required to be <15% at the LLOQ and <10% at other concentrations used for constructing the standard curve.

The selectivity of the method, extraction recovery and matrix effect, and the stability of the MK-0533 in the same matrix (human plasma), were investigated and reported previously [11]. Only slight modifications in instrumentation and calibration range were performed to adjust for dose increases and were compensated with addition of an internal standard.

3. Results and discussion

3.1. Optimization of extraction time (aspiration/dispense cycle)

In order to decrease SPME extraction time, different agitation methods are often investigated during a SPME method development to determine the faster method of reaching equilibrium between the SPME phase and the analyte extracted. These methods include magnetic stirring, sonication, orbital shaking, etc., which can bring great challenges to SPME automation in terms of system configurations and other related issues. In-tip SPME automation using Tomtec Quadra 96 workstation greatly minimizes the efforts of agitation by simply utilizing aspiration and dispense functions of the system, such that equilibrium could be established between the SPME phase and the analyte extracted through a certain number of aspiration and dispense cycles (Fig. 2).

In this particular study, 60, 120, 240, 480, and 720 aspiration/dispense cycles were evaluated while the aspiration/dispense volume and speed were kept constant at 100 μL and setting 3, respectively. As described in Section 2, 250 μL of water was added to reduce the viscosity of the plasma sample caused by the presence of a concentrated acid. Under the current experimental conditions, 60 cycles were approximately equal to 5 min. Based on previous studies [10], PDMS-DVB fibers were chosen for extraction of the analyte. Three different PDMS-DVB fibers were used at three different well locations for each aspiration/dispense cycle point. It was found that the equilibrium was nearly reached at 60 min (Fig. 3) and the % C.V. from each cycle point ranged from 8.7% to 11.6%. The in-tip SPME extraction process was based on repeatedly aspirating the plasma sample into the pipette tips and dispensing into a well plate; approaching or reaching between the SPME phase and the analyte in the plasma sample. Although this process is simple, there are several issues to consider. First, the positions of in-tip SPME fibers after loading by Tomtec could be kept stationary during the aspiration/dispense cycles in order to minimize the time required for extraction. However because of the viscosity of the plasma, even after the addition of 250 μL of water, residues of plasma were found inside some of the pipette tips after several aspiration/dispense cycles, which may increase extraction variation across different SPME fibers. Therefore, it is highly recommended to lower the tips to the bottom of the well during aspiration and to raise the tips to the top of the well after aspiration such that plasma samples can be completed dispensed into the well plate before the next cycle. The trade off is that the extraction time could

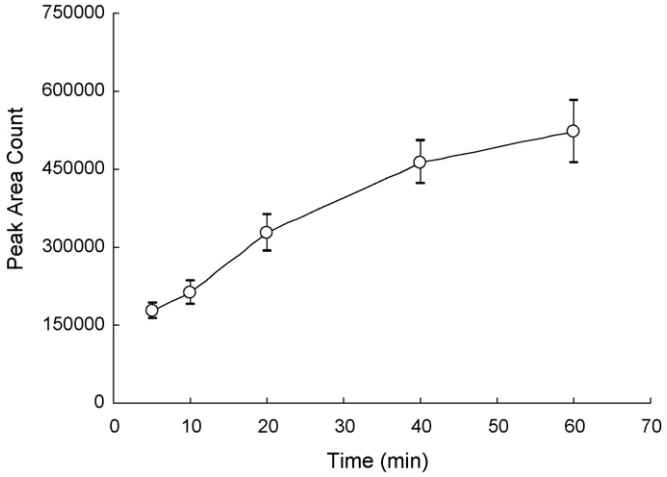


Fig. 3. In-tip SPME extraction time profile. Conditions: in-tip SPME was performed in accordance to the method outlined in Section 2. Three in-tip SPME fibers were extracted simultaneously in three different wells with aspiration/dispense volume at 100 μL and speed at 3. Desorption conditions for all time points were the same.

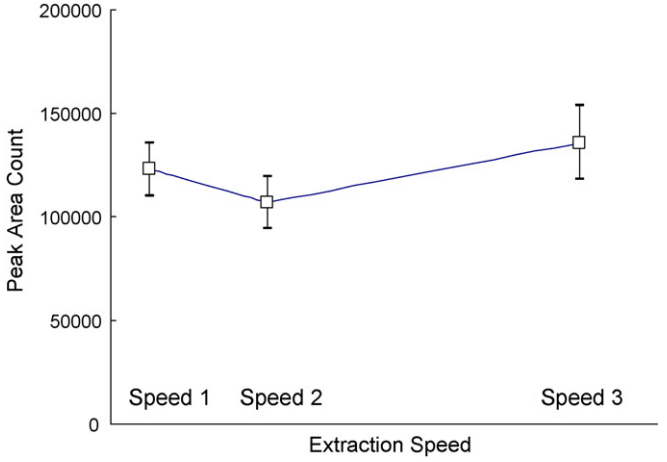


Fig. 4. In-tip SPME extraction speed profile. Conditions: in-tip SPME was performed in accordance to the method outlined in Section 2. Three in-tip SPME fibers were extracted simultaneously in three different wells with aspiration/dispense volume at 100 μL. Desorption conditions for all time points were the same.

be slightly extended. Secondly, the PE frit positions are also very important while preparing in-tip SPME fibers (Fig. 2). There should be enough room between the PE frit and narrow part of the pipette tip such that plasma can be easily aspirated without blocked of the tip. Thirdly, the aspiration volume can be increased to allow longer exposure time of analyte to SPME fibers. The maximum aspiration volume from the Tomtec Quadra 96 workstation using in-tip SPME is 400 μL; however, this would dramatically increase extraction time, yielding comparable sample preparation times as increasing aspiration and dispense cycles.

3.2. Assessing aspiration/dispense speed

Besides aspiration/dispense volume and cycle, aspiration and dispense speed is another parameter that can be evaluated and optimized with the Tomtec Quadra 96 workstation. There are three types of aspiration and dispense speed available, from low (speed 1), 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, dispense at speed 2, etc. No significant differences among the different speed were observed with % C.V. from 10.4% to 13.1% (Fig. 4). 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.

3.3. Investigation of desorption time

Solvent desorption is the only choice for SPME technique when coupled with liquid chromatography. Two typical off-line approaches are commonly used for SPME–LC desorption. One is to use a small amount of desorption solvent, such as ACN or methanol; a small portion of this solution is directly injected into a HPLC system for analysis. The other approach is to evaporate the desorption solvent under an inert gas such as nitrogen and to reconstitute the sample into an appropriate solution to increase sensitivity or obtain better chromatographic peak shape. Since ACN was demonstrated as a suitable solvent from a previous study [11], 200 μL of ACN was used for analyte desorption in this experiment. It is very important to rinse the in-tip SPME fibers with water right after extraction process. Since small plasma residues were retained at the bottom of the pipette tips even after the final blow out, protein precipitation was observed if the tips were used for transferring ACN directly, and this could potentially block the SPME tips for further solvent desorption. In contrast to positioning the pipette tip at an elevated height during the extraction process to ensure complete dispensing of the plasma into the well plate, pipette tips could be kept immobilized through the whole desorption process without any resistance from ACN solvent. Three different PDMS-DVB fibers were used at three different well locations for the desorption test, and aspiration/dispense cycles were selected at 60, 120, and 240 times. It was found that the amounts of analytes desorbed into the solvent slightly increased as aspiration/dispense cycle times increased (Fig. 5). The reproducibility of the three simultaneous desorption at each point ranged from 10.5% to 15.7%.

3.4. Method validation

The in-tip SPME method was validated for human plasma analysis using calibration standards over the concentration range of

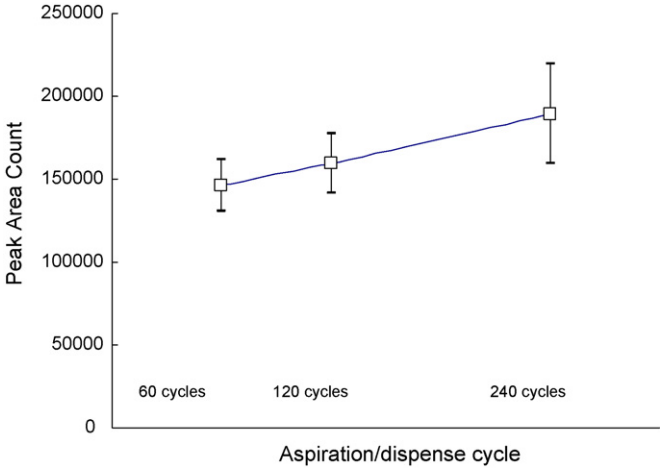


Fig. 5. In-tip SPME desorption profile. Conditions: in-tip SPME was performed in accordance to the method outlined in Section 2. Three in-tip SPME fibers were extracted and desorbed simultaneously in three different wells with aspiration/dispense volume at 100 μL and speed at 3.

Table 1
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 concentration (ng/mL)	Mean[a] calculated concentration (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%.

5–2000 ng/mL of MK-0533. Assessment of the intraday variability of the method was conducted using three different lots of acidified human control plasma spiked with MK-0533. The resulting method precision and accuracy data are presented in Table 1. The intraday precisions (% C.V.) 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. Representative extracted ion chromatograms of lower limit of quantification (LLOQ) at 5 ng/mL of drug with 200 ng/mL of internal standard are shown in Fig. 6. 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.

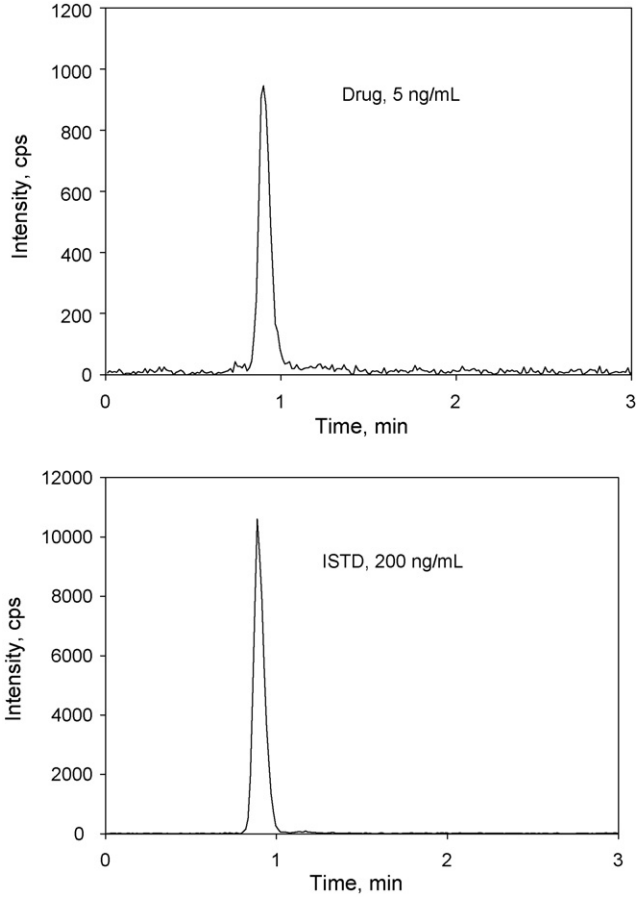


Fig. 6. Representative extracted ion chromatograms of lower limit of quantification (LLOQ) at 5 ng/mL of drug with 200 ng/mL of internal standard.

3.5. Clinical sample analysis

In order to demonstrate the feasibility of using the automated in-tip SPME technique for clinical sample analysis, pooled clinical samples from post-dose subjects were analyzed using the current assay and a conventional LLE approach (as previously described [11]) for comparison purposes. Concentration–time profiles of MK-0533 in plasma of these subjects after administration of 75 mg of drug obtained using in-tip SPME and LLE methods are presented in Fig. 7. The two data sets obtained using two widely different extraction methods are in excellent agreement, clearly demonstrating that in-tip SPME could be used in this case as an alternative approach for multi-sample analysis in pharmacokinetic studies.

3.6. Advantages of in-tip SPME technique

In-tip SPME technique took advantage of widely used commercially available automated liquid handling system, and coupled it with fiber SPME in a very unique configuration. In-tip SPME is simple and easy for automation without introducing additional devices. More importantly, the approach is amendable to all fibers types possessing a wide range of different coating materials, which will overcome the drawback of limited selection of commercially available fibers and broaden its use with HPLC. This is achievable since many fiber coating techniques [13] are now available in the literature including sol–gel coating technology [14], electrochemical [15,16] and chemical procedures [17], and physical deposition of biocompatible materials [18]. In this regard, the automated in-tip

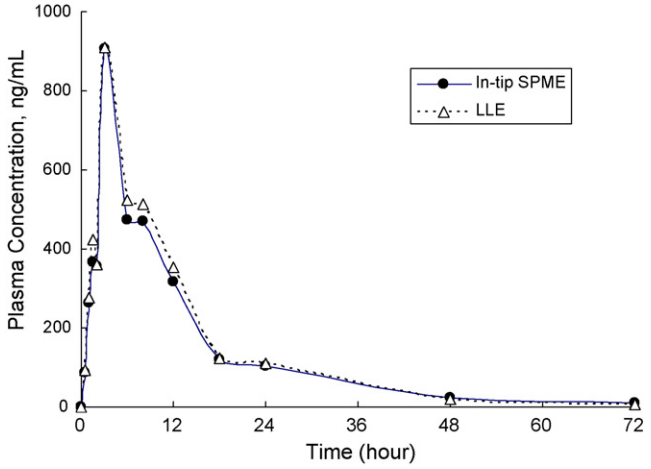


Fig. 7. Concentration-time profile of MK-0533 in plasma of pooled healthy subjects after administration of 75 mg of drug using in-tip SPME and LLE techniques.

SPME approach will also enable rapid and simultaneously screening of up to 96 different fiber chemistries during analyte method development for the determination of optimal extraction efficiency. Previous literature work [11] confirmed the utilization of a 96-well SPME format for clinical sample analysis. However the highly manual approach of this method introduced additional time and variation in the sample preparation process and is therefore unacceptable to meet the high throughput demands of clinical sample analysis. In contrast, the in-tip SPME approach enables complete automation of the sample preparation process with a commercially available Tomtec Workstation. This automated and parallel processing 96 samples will be highly advantageous for clinical simple analysis.

Although, the present study utilized 40 min for extraction, which is longer than typical protein precipitation or liquid–liquid extraction techniques; this time can be significantly reduced as it not necessary to reach the equilibrium extraction value. Since the extraction procedure is highly automated, good precision can be achieved at shorter times, however sensitivity will be compromised. Additional work is ongoing in our laboratories to investigate faster in-tip SPME extraction kinetics using turbulent flow conditions that minimize the extraction diffusion layer between the fiber coating and the bulk sample. More importantly, the use of protein precipitation and liquid–liquid extraction techniques provide poor selectivity for a chosen analyte and the subsequent extract can contain assay interferences and exaggerate mass spec ionization effects. In contrast, the SPME fiber extraction chemistry can be optimized for the target analyte; providing higher selectivity, cleaner biological extracts and higher quality LC–MS/MS data. Lastly, a very promising feature of in-tip SPME is that it will eliminate carryover and reduce costs, if the fibers are designed to be inexpensive and consequently disposable after single use. In addition, the use of a disposable microextraction device such as Ziptip®, could be combined with this automated approach for the isolation of proteins and peptides.

4. Conclusions

Preliminary results from the novel concept of coupling in-tip SPME with automated liquid handling system have indicated that it is a suitable direction for SPME automation in liquid chromatography. The approach was fully automated and allowed for parallel processing of up to 96 samples. Automation of the extraction and desorption processes for the technique provided high levels of precision, accuracy and linearity. The success of the developed method was confirmed with comparison to a liquid–liquid extraction of clinical pharmacokinetic samples, providing excellent agreement. The versatility of in-tip SPME can also be easily extended to a range of extraction coatings providing analysis of a wide range of analytes.

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