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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

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Abstract

Two sensitive and selective methods based on solid phase microextraction (SPME) and liquid–liquid extraction (LLE) in 96-well format, in combination with high performance liquid chromatography (HPLC) with tandem mass spectrometric (MS/MS) detection have been developed to determine a model drug compound in human plasma. Both assays were performed on an Applied Biosystems-Sciex API 4000 tandem mass spectrometer interfaced with a turbo ion-spray probe and operated in the negative ionization mode. A lower limit of quantitation (LLOQ) of 1 ng/mL achieved when 0.25 mL of human plasma was processed. In both methods, a stable isotope labeled internal standard was utilized. The methods were validated in the concentration range of 1–500 ng/mL. The intraday precision (%C.V.) of the method using LLE was 0.8% at LLOQ, and was equal to or lower than 3.3% at all other concentrations, while the intraday precision (%C.V.) of the method using SPME was 6.9% at LLOQ, and was equal to or lower than 5.7% at all other concentrations. Based on the direct comparison of the two methods and their successful applications in clinical sample analysis, it may be concluded that SPME may be considered and used as an alternative approach for quantitative determination of drugs in pharmacokinetic studies.

Keywords: Liquid–liquid extraction; Solid phase microextraction; 96-Well format; HPLC-MS/MS

1. 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 [1–3]. 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 [4–7]. In addition, the co-eluting metabolites of a drug being analyzed may give a MS/MS response in the channel used for drug quantification [8–10]. Therefore, isolation of analytes from biological matrices using an effective sample clean up technique is often critical for achieving assay selectivity.

Liquid–liquid extraction (LLE) and solid phase extraction (SPE), either on-line or off-line, are two very commonly used approaches and are generally found to be sufficient for reducing or eliminating matrix effects and providing reliable HPLC-MS/

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MS data [11,12]. Although both LLE and SPE involve sometimes 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 pharmaceutical industry. With the development of new analytical instruments and techniques, high sensitivity of MS/MS detection may be achieved; therefore in drug analysis the trend is to use small sample volumes (usually less than 100 μL) and simplify sample extraction procedures to improve overall method efficiency with low cost. Extracting very small volume of samples using conventional methods, such as LLE and SPE may be challenging. As an alternative method, solid phase microextraction (SPME) has shown great potential as a highly efficient sample preparation technique. Since its invention in 1990 [13], SPME has been widely used in biomedical and pharmaceutical analysis, and several excellent reviews have been published on this topic [14–18]. 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 into one step. The method could save sample preparation time and disposal cost, and can be used with 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 pharmaceutical industry. Ulrich [15] listed some principal disadvantages of SPME in biomedical analysis preventing its applications in drug analysis in biological fluids. Also, limited selections of commercially available SPME fibers and difficulties with method automation for high-throughput sample analysis are also the 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 intra-day 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. Experimental

2.1. Materials

A drug compound ($C_{28}H_{24}F_3NO_6$) under clinical development and its deuterated internal standard (d_6-ISTD), were synthesized at Merck Research Laboratories (Rahway, NJ, USA). The form of the free carboxylic acid dehydrate drug was a highly crystalline, rod shape and needle like morphology with a pK_a, water solubility and melting point of 3.2, 0.06 mg/mL and 78.1 °C, respectively. The log *D* (pH 7.4) for the compound was calculated to be 3.56 using the ACD/log *D* software from ACD/Labs (Toronto, ON, Canada). 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). 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.

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 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 4 min. 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 quadruple 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 turbo ion spray 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 μL/min into a mobile phase pumped at 0.2 mL/min through the turbo ion spray interface. Multiple reaction monitoring of the precursor → product ion pairs at *m/z* 526 → 440 for drug compound and *m/z* 532 → 440 for d_6-ISTD was used for quantitation.

2.3. Preparation of standard solutions and quality control samples

A stock solution of drug compound (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 5, 25, 50, 250, 500, 1000 and 2500 ng/mL. The d_6-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 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 quality control (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.

2.4. Liquid–liquid extraction procedure

QC and subject plasma samples were thawed at room temperature. Two hundred and fifty microlitres of subject plasma samples and QC samples were added individually into a 2 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, 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 adding 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 min for LLE. The plate was then centrifuged 15 min at 3000 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 was injected into the HPLC-MS/MS system.

2.5. Solid-phase microextraction procedure

QC and subject plasma samples were thawed at room temperature. Two hundred and fifty microlitres of subject plasma samples and QC samples were added individually into a 2 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, except to the well designated for the double blank plasma. After adding 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 home-made plastic module (Fig. 1) 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 had 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 (see Fig. 1). Secondly, after extraction, the fibers would be first withdrawn to the needles individually, and 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 min. 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 min, 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. When desorption of all the samples was completed, the collection plate was evaporated to dryness under heated N_2 stream and reconstituted in 150 μL of the same

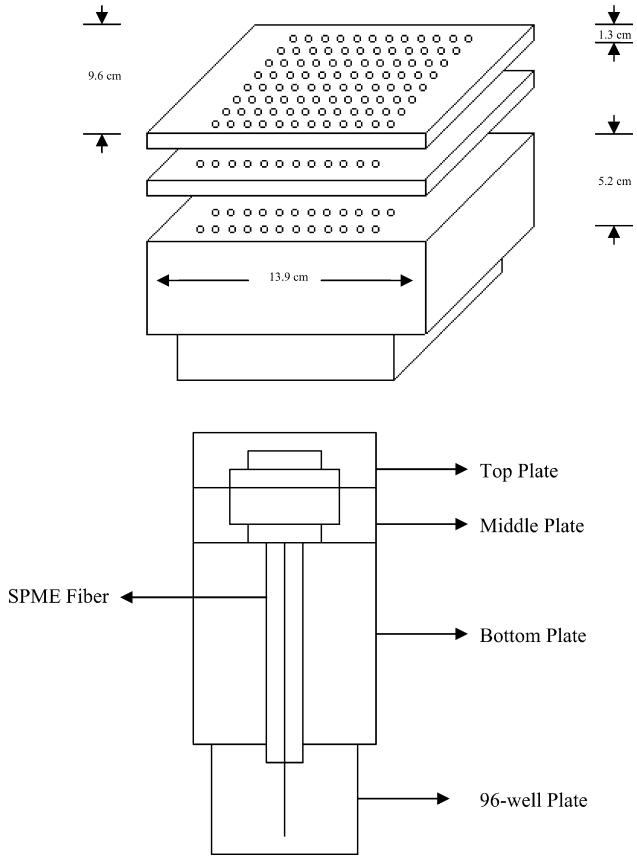

Fig. 1. A home-made plastic module for SPME in 96-well format.

solution as in LLE procedure, and 10 μL aliquots were injected into the HPLC-MS/MS system.

2.6. Precision and accuracy

The precision of the method was determined using 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 coefficient of variation (%C.V.) was required to be <15% at the LLOQ and <10% at other concentrations used for constructing the standard curve.

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, "cross-talk" between MS channels used for monitoring the analyte and the internal standard was evaluated.

2.8. Recovery and matrix effect

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 obtained from the standards added to and extracted from the control plasma to the peak areas of the standards added to the control plasma extract obtained from the same volume of the control plasma as used for analyte extraction. Since stable isotopically labeled compound was used as an internal standard, a potential "relative" matrix effect on ionization should not have any adverse effect on the quantitation of the drug compound in different plasma lots. The absence of "relative" matrix effect was illustrated by the examination of the slopes of the calibration curves in five different lots of control plasma.

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.

3. Results and discussion

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 current study, 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. 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 min. However, at room temperature, hydrolysis was observed in non-acidified heparinized human control plasma after 30 min and it became significant after 60 min. 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 freeze–thaw cycles. The determined drug concentrations in these samples practically did not change following up to three freeze–thaw cycles.

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 mm × 2.1 mm, 3 μm) produced the best results in terms of peak shape and retention of analytes. The acyl-glucuronide 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 pH 3. However, good reproducibility was also

observed when the extraction solvent was composed of 80% hexanes and 20% isopropanol. The solubility of isopropanol in aqueous media plays a critical role during the extraction, but its volume should not exceed 20%. 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 inter-molecular 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 analyte 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 that significantly reduces the diffusion coefficients within the structure and extraction occurring through adsorption on the surface of the fiber [19–22]. 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 was made, except 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 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 and 10 min 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 4–8 min to eliminate carry-over depending on analyte concentration. Further experiments, based on optimized temperature and increased shaking or sonication conditions, are needed to further reduce the time and to increase the efficiency of the desorption procedure. In the work presented 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.

Table 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%.

3.3. Method validation

The two methods based on LLE and SPME were validated in human plasma over the concentration range of 1–500 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 intraday precisions (%C.V.) 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 ±2% of the nominal concentration for all the standards evaluated. For SPME, the intraday precisions (%C.V.) 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 ±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
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%.

Table 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.

3.4. Selectivity

Assessment of the selectivity of a method is critical and needs to be confirmed also 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 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.

Fig. 2 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.

3.5. Recovery and assessment of the matrix effect

In 96-well LLE procedure, extraction recovery and the effect of the plasma matrix on ionization were evaluated for drug compound using standards spiked at concentrations of 5, 50, and 200 ng/mL and for d_6-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 3. Based on the intraday precision and accuracy results (Table 1) and the slope data (Table 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 effect and/or recovery between the different lots of human control plasma. Therefore, "relative" matrix effect [9] on ionization from five different lots of human control plasma was negligible. A general practice in determination of the relative 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. Because of the relatively small surface area of the stationary phase of the SPME fiber and the use of different extraction mechanism compared with LLE, the relative recoveries observed in SPME are generally 1 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 effect. It was expected that variation in the "absolute" matrix effect 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) and the slope data (Table 4) that were obtained using five different lots of human control plasma, it was found that the "relative" matrix effect was 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 effect 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, careful

Table 4
Standard curve slopes in five different lots of acidified human control plasma

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, $n = 5$.

assessment of the "relative" matrix effect is necessary when LLE, SPME, or other sample preparation procedures are utilized.

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

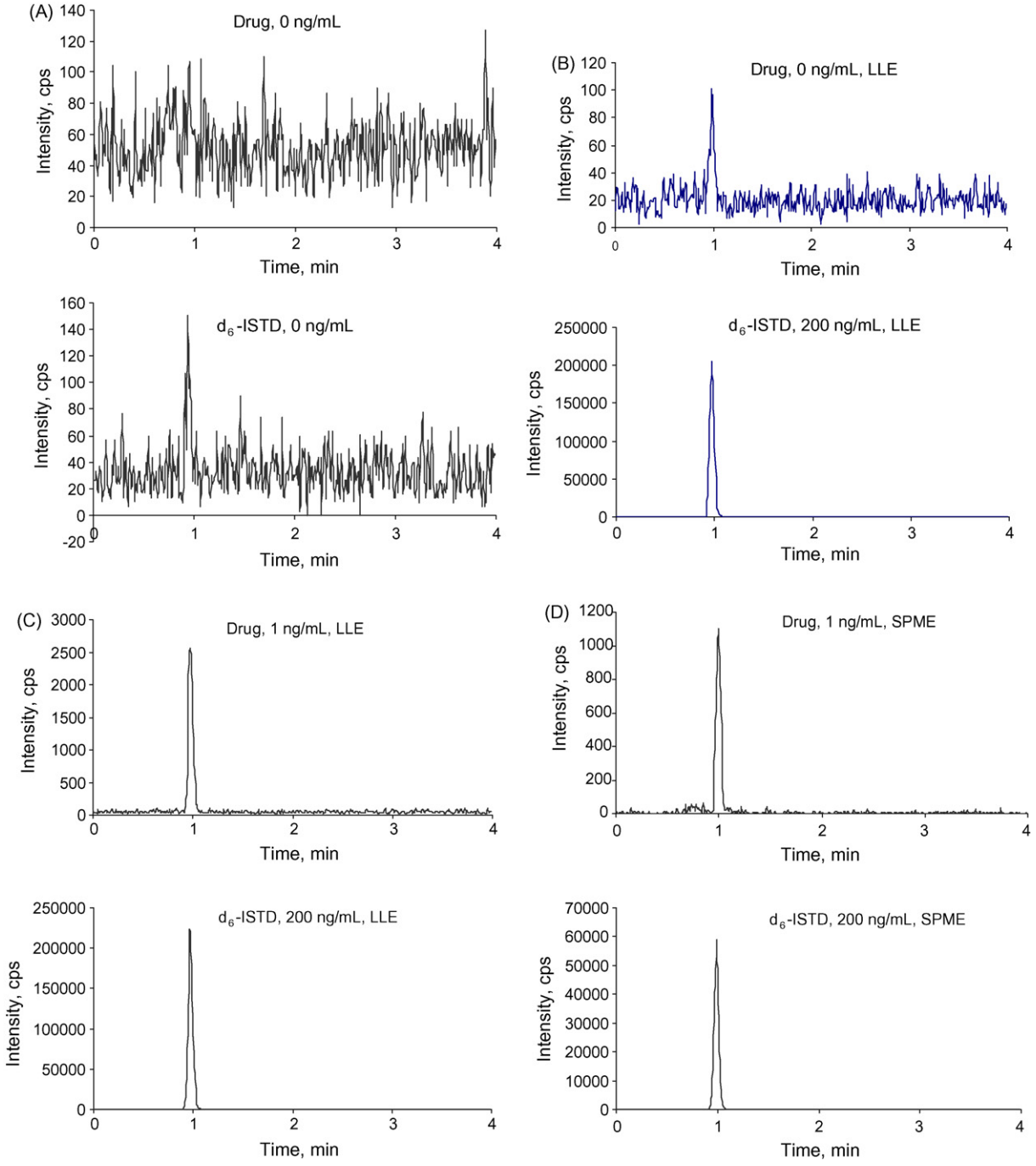

Fig. 2. Representative extracted ion chromatograms of (A) double blank; (B) single blank, spiked with 200 ng/mL of d_6-ISTD; (C) lower limit of quantification (LLOQ), 1 ng/mL of drug with 200 ng/mL of d_6-ISTD using LLE; (D) LLOQ, 1 ng/mL of drug with 200 ng/mL of d_6-ISTD using SPME; (E) 500 ng/mL of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

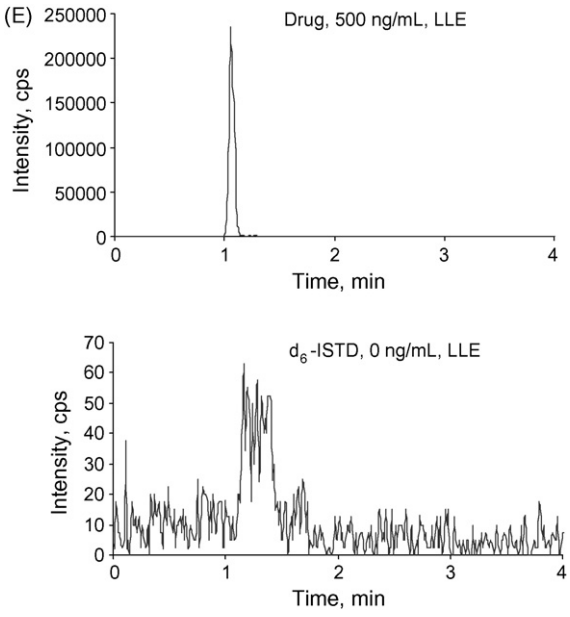

Fig. 2. (*Continued*).

that were subjected to additional freeze–thaw cycles were within ±8% of the nominal value. The results are shown in Table 5.

3.7. Clinical sample analysis

The method using LLE approach has been implemented in a clinical study, and thus far, more than 2000 plasma samples have been analyzed. Interday precision and accuracy of the method for the clinical samples analysis were determined by analyzing QC samples at low, medium, and high concentrations. Table 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 (%C.V., $n = 84$) was less than 4.5% with accuracy ranging from 97.9 to 99.6%.

In order to compare the clinical data obtained using LLE *versus* 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 this subject after single-dose administration of 25 mg of drug obtained using LLE and SPME methods are presented in Fig. 3. 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.

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 intraday precision and accuracy were all satisfactory in both methods. Recoveries of analytes using LLE were at least 10 times higher than the relative recoveries obtained by SPME and

Table 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)] × 100.

Table 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)] × 100%.
[b] Coefficient of variation.

overall sensitivity of detection was also higher for LLE than SPME with same injection volume. In the reported study, the overall sample preparation time for LLE was less than half of that required for SPME and the same number of clinical samples. However, the advantages of using SPME were also very evident. First of all, 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 its relative 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.

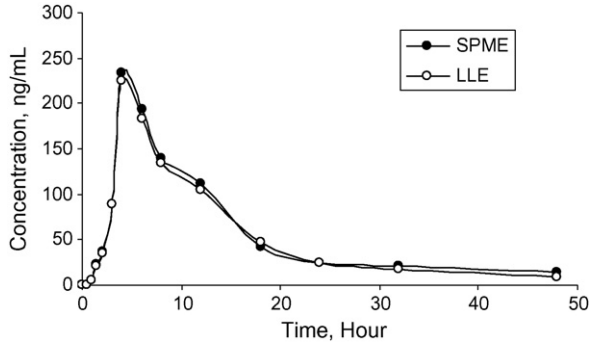

Fig. 3. Concentration–time profile of drug compound in plasma of a healthy subject after single-dose administration of 25 mg of drug using LLE and SPME techniques.

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 liquid–liquid extraction method was demonstrated by analysis of a drug compound in more than 2000 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.

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