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A simple 96-well liquid–liquid extraction with a mixture of acetonitrile and methyl *t*-butyl ether for the determination of a drug in human plasma by high-performance liquid chromatography with tandem mass spectrometry

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Abstract

A simple 96-well plate liquid–liquid extraction (LLE), liquid chromatography/tandem mass spectrometry (LC/MS/MS) method has been developed and validated for the determination of a basic drug candidate in human plasma. Against the wisdom of conventional approaches, an aqueous/organic miscible solvent, acetonitrile, was used for liquid–liquid extraction along with methyl *t*-butyl ether. The use of acetonitrile effectively eliminated the formation of the irregular emulsion between aqueous/organic interfaces and modulated the polarity of the extraction solvents to achieve the desired recovery. This approach, which solved the emulsion problem, permitted the method to be automated using standard 96-well plate technology. A practical application was demonstrated through the use of this technique in the measurement of a novel drug in human plasma samples by LC/MS/MS. Chromatographic separation was achieved isocratically on a Phenomenox C18(2) Luna column (2 mm × 50 mm, 5 μm). The mobile phase contained 60% of 0.1% formic acid and 40% acetonitrile. Detection was by positive ion electrospray tandem mass spectrometry. The standard curve, which ranged from 1.22 to 979 ng/ml, was fitted to a $1/x^2$ weighted quadratic regression model. The validation results show that this method was very rugged and had excellent precision and accuracy. The actual sample analysis results further demonstrated that this extraction procedure is well suited for real life applications. It is expected that with some modifications, this approach can be applied for the extraction of similar compounds from various biological fluids.

Keywords: LC/MS/MS; 96-Well liquid–liquid extraction; Human plasma

1. Introduction

During the past 10 years, liquid chromatography/tandem mass spectrometry (LC/MS/MS) has emerged as an effective tool in the quantitation of drugs and their metabolites in biological fluids

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because of its superior sensitivity and selectivity. Even with the tremendous selectivity of mass spectrometry, a good sample cleanup is still essential for a successful, rugged bioanalytical method. Currently, there are three routinely used sample preparation methods: protein precipitation, liquid–liquid extraction (LLE) and solid phase extraction (SPE). Among them, SPE has been most widely used because it is amenable to automation. SPE is now commercially available in the 96-well plate format and has also been adapted for direct injection [1–3].

LLE has long been popular as an alternative to SPE because it is easy to develop and capable of providing very clean sample extracts [4,5]. The cleaner sample extracts can greatly reduce the burden on the mass spectrometer ion source. The drawback of the technique is that it requires a large amount of organic solvent and is less amenable to automation [1]. In the past few years, efforts have been made to study the utility of semi-automated 96-well LLE for the quantitation of drugs and their metabolites in biological fluids. Jemal et al. [6] reported 96-well LLE for the determination of a carboxylic acid ($C_{23}H_{32}N_2O_6S$), which is structurally similar to omapatrilat, in human plasma with methyl *t*-butyl ether. Zhang et al. [7] reported 96-well LLE for the quantitation of diphenhydramine, desipramine, chlorpheniramine and trimipramine in rat plasma with a selection of organic solvents. Ramos et al. [8] reported a 96-well LLE method for the determination of methylphenidate in human plasma using cyclohexane. Steinborner and Henion [9] reported an innovative approach for the determination of methotrexate and its major metabolite in human plasma. They used excessive acetonitrile to precipitate proteins. To the acetonitrile/water supernatant, chloroform was added for further sample cleanup, and the aqueous layer was collected for the sample analysis.

In this paper, we describe a semi-automated 96-well LLE, LC/MS/MS method for a novel basic drug candidate in human plasma. In this method, a small amount of acetonitrile was first added for protein precipitation and then used to enhance the LLE with methyl *t*-butyl ether. Subsequently, the extracted samples were separated and monitored by an LC/MS/MS system. A full validation was conducted to assess accuracy, precision, linearity, and lower limit of quantitation (LLOQ), and the results are presented here to demonstrate the feasibility of this new approach.

2. Experimental

2.1. Chemicals and reagents

Compounds **I** (the analyte) and **IS** (the internal standard), which are closely related chemical analogs, are characterized products of Bristol–Myers Squibb Pharmaceutical Research Institute. Their chemical structures cannot be shown for proprietary reasons. Compound **I** contains tertiary amines, ketone, pyrrole, pyridine, piperazine and benzamine. Its solubility in water is 0.17 mg/ml at 25 °C, and the pH of a saturated solution in water is 8.4. It exhibits two ionizations with pK_a values of 2.9 and 9.6. The **IS** contains the same functional groups as compound **I** does, and it also exhibits two ionizations with pK_a values of 2.2 and 9.3. Its solubility in water is 0.2 mg/ml at pH ~8.0 and 4 mg/ml at pH 1.0. The functional groups of compound **I** and **IS** may be protonated or deprotonated in the electrospray ion source. Acetonitrile (HPLC grade), formic acid (98%), and methyl *t*-butyl ether (HPLC grade) were purchased from EM Science (Gibbstown, NJ, USA). House deionized water, further purified with a Milli-Q water purifying system (Millipore Corporation, Bedford, MA, USA), was used. Drug-free human plasma was purchased from Biological Specialty Corp (Colmer, PA, USA).

2.2. Instrumentation

The robotic liquid handling system used was a Tecan Genesis RSP 150 Series with Gemini Software (Tecan US, Research Triangle Park, NC). The collection microtubes racked in 96-well format and the microtube caps were purchased from US Scientific (Ocala, FL). The 96 deep well collection plate was from Varian (Harbor City, CA). A Savant evaporator, Model SpeedVac AES-2000, was used for evaporation of extracts (Farmingdale, NY, USA). The shaker used was an Eberbach two-speed shaker, purchased from Baxter Scientific (Ann Arbor, MI, USA).

Compound **I** and **IS** were separated isocratically, using a mixture of aqueous 0.1% formic acid and acetonitrile (60:40) as mobile phase. The flow rate was 200 μl/min at room temperature. The separation column was a Phenomenex C-18 (2) Luna (2 mm × 50 mm, 5 μm, Torrance, CA). The liquid chromatography separation system consisted of two

Shimadzu LC-10AD pumps (Columbia, MD, USA), a Perkin-Elmer Series 200 LC autosampler (Norwalk, CT, USA). The injection volume was 10 μl and the run time was 1.8 min.

A Micromass Quattro LC MS/MS system (Beverly, MA, USA) operating under MassLynx 3.4 software was used. The electrospray ion source was run in a positive ionization mode for all experiments. The typical ion source parameters were: Capillary 3.5 kV, Core 40 V, RF lens 2 V, source temperature 100 °C, desolvation temperature 300 °C, nebuliser 75 l/h and dry gas 900 l/h. Quadrupole 1 (Q1) parameters were: low mass resolution 12 V, high mass resolution 12 V, and ion energy 2 V. Quadrupole 2 (Q2) parameters were: collision gas 2.5 e-3 mbar, collision energy 30 V. Quadrupole 3 (Q3) parameters were: low mass resolution 12 V, high mass resolution 12 V, and ion energy 2 V. The multiplier was set at 650 V. The samples were analyzed via selected reaction monitoring (SRM). The monitoring ions were set to *m*/*z* 406.8 to 174.5 for compound **I** and *m*/*z* 370.0 to 144.5 for compound **IS**. The scan dwell time was set 0.2 s for both channels.

2.3. Standard and QC preparations

Initially, the standard stock solution was prepared at the expected concentration, 0.500 mg/ml in acetonitrile. Subsequent to the validation, the purity was determined to be less than 100%, and with the adjustment of the purity, the actual concentration was 0.490 mg/ml. All concentrations reported here reflected the adjusted concentration. A standard working stock solution of 9790 ng/ml was prepared by appropriate dilution of the 0.490 mg/ml stock solution with drug-free human plasma. The final standard concentrations in human plasma were 979, 734, 490, 245, 49.0, 24.5, 12.2, 2.45, and 1.22 ng/ml. Standard curves were prepared fresh daily.

A 0.490 mg/ml of the QC stock solution was prepared from a separate weighing. Dilutions were used to prepare four levels of QCs in human plasma, 15664 (Dilution QC), 783 (High QC), 392 (Mid QC), and 3.67 (Low QC) ng/ml. QCs were stored at −30 °C.

The stock solution of compound **IS** was prepared in acetonitrile, and subsequently it was diluted with acetonitrile to 500 ng/ml as the working internal standard solution.

2.4. Liquid–liquid extraction procedure

The Tecan was used to pipette an aliquot (200 μl) of each standard or QC to a rack containing 96 microtubes (1.2 ml). To each standard and QC sample, 80 μl of the working internal standard solution in acetonitrile (200 ng/ml **IS** in plasma and 40 ng/microtube) and 650 μl of methyl *t*-butyl ether were added. The microtubes were capped with collection caps and then shaken for 10 min. The top layer (acetonitrile and methyl *t*-butyl ether) was then transferred via the Tecan to a clean 96-well plate, and the samples in the 96-well plate were placed in a Savant SpeedVac vacuum dryer to remove organic solvent. Finally, the samples were reconstituted to 100 μl of the mobile phase. A fully processed plate was transferred to the LC/MS/MS and analyzed.

3. Results and discussions

3.1. Extraction procedure

Traditionally, LLE involves mixing an aqueous sample solution with an immiscible organic solvent for a period of time and allows the two immiscible liquid phases to interact with the intent that the analyte(s) will be extracted from the aqueous layer into the organic layer, where the extraction efficiency for the analyte of interest depends on the nature of the compound and the pH of the matrix. In our experiment, a small amount (50–100 μl) of acetonitrile, an aqueous/organic miscible solvent, was added to the sample (200 μl). Here acetonitrile served as both the **IS** solution and the protein precipitation reagent; therefore, no separate **IS** solution was added. This was followed by the addition of 650 μl of methyl *t*-butyl ether, an aqueous immiscible organic solvent. The small amount of acetonitrile added effectively precipitated proteins in the plasma. As a result, during LLE, the formation of the irregular emulsion by the proteins was eliminated. This emulsion has been the major drawback when methyl *t*-butyl ether was used alone as the extract solvent for 96-well LLE. Consequently, freezing the aqueous layer or centrifuging 96-well plates prior to transferring the organic layer became unnecessary. Initially, between the addition of acetonitrile and methyl *t*-butyl ether, the microtubes

were vortexed. The further experimental results (results not shown) revealed that the vortex step was unnecessary, since both approaches provided the same extraction results. It was noticed that when less than 50 μl of acetonitrile was used, the irregular emulsion still existed to some plasma samples tested. More acetonitrile gave better recovery but less selectivity, since some endogenous materials in plasma were also extracted to the organic phase. During the validation, 80 μl of acetonitrile was used. With only a limited amount of acetonitrile being used for the extraction, the extracts were still very clean. Furthermore, the addition of acetonitrile did not significantly increase the evaporation time. It should be noted that acetonitrile contribution from the standard or QC stock solutions was very low, being less than 0.5% (0.4 μl acetonitrile in 200 μl plasma/80 μl acetonitrile used for the extraction) for the highest standard, 979 ng/ml, and even less for other standard or QC samples. Such small contribution should have no impact to the method performance, since the variability in the amount of acetonitrile in the **IS** solution added by Tecan was normally greater than 0.5%. The consistent day to day performance indicated that slight differences in the amount of acetonitrile in the samples did not inadvertently affect the performance of the method. Therefore, it was confirmed that an appropriate combination of acetonitrile and methyl *t*-butyl ether was well suited for 96-well plate LLE, especially for blood, plasma, or serum samples.

During experiments examining the acetonitrile: methyl *t*-butyl ether ratio, it was noticed that as long as the ratio was less than 1:3, the acetonitrile was still part of the organic layer. Here, the ratio was defined as acetonitrile volume/methyl *t*-butyl ether volume. For example, 80 μl of acetonitrile/650 μl of methyl *t*-butyl ether equals to approximately 1:8, which is less than 1:3. Therefore, by properly adjusting the amount of acetonitrile added, the system could potentially extract other compounds from biological fluids with a good balance of selectivity and recovery. When the acetonitrile:methyl *t*-butyl ether ratio is greater than 1:3, the acetonitrile separated from the methyl *t*-butyl ether phase and became part of the aqueous phase again. In this case, the benefit of protein precipitation still remained; however, the acetonitrile would not modulate the polarity of the organic phase and may actually be detrimental to the extraction by changing the lipophylicity of the aqueous phase.

3.2. LC/MS/MS

Along with this 96-well LLE procedure, an LC/MS/MS system was used to separate and monitor compound **I** and its internal standard in the extracted samples. Figs. 1 and 2 show the electrospray positive ion MS and MS/MS product ion spectra of compounds **I** and **IS**. The MS spectra for both compounds are dominated by the $[M + H]^+$ ions: *m*/*z* 406.8 for compound **I** and *m*/*z* 377.0 for compound **IS**. The MS/MS product ion spectra of the $[M + H]^+$ for both compounds produced major product ions at *m*/*z* 174.5 and 144.5, respectively. Thus, the SRM used was *m*/*z* 406.8 → 174.5 for compound **I** and *m*/*z* 377.0 → 144.5 for the internal standard. The retention times of compound **I** and the internal standard were 1.0 and 1.3 min and the chromatographic run time was 1.8 min (Fig. 3).

3.3. Standard curves

After the 96-well LLE procedure and the LC/MS/MS conditions were defined, a full validation was performed to assess the performance of the extraction procedure as well as the method as a whole. A nine-point calibration curve ranging from 1.22 to 979 ng/ml of compound **I** in human plasma was used in duplicate in each analytical run. Peak area ratios of compound **I** to internal standard were used for regression analysis. A linear regression model was evaluated first; however, a weighted $(1/x^2)$ quadratic regression, where x is the concentration of compound **I**, provided a better fit for the validation data at the higher end of the calibration curve due to nonlinear response from mass spectrometry. Therefore, the weighted $(1/x^2)$ quadratic regression model was used for this validation. The individual standard curve data obtained from four runs on four separate days during the method validation showed that only one out of 72 standards had the deviation of its back-calculated concentration from its spiked value greater than 15%. The regression coefficients (*R*-squared) for the four runs were greater than 0.9925, linear slopes ranged from 0.003964 to 0.0088300, intercepts were from 0.000000 to 0.000001, and quadratic slopes were

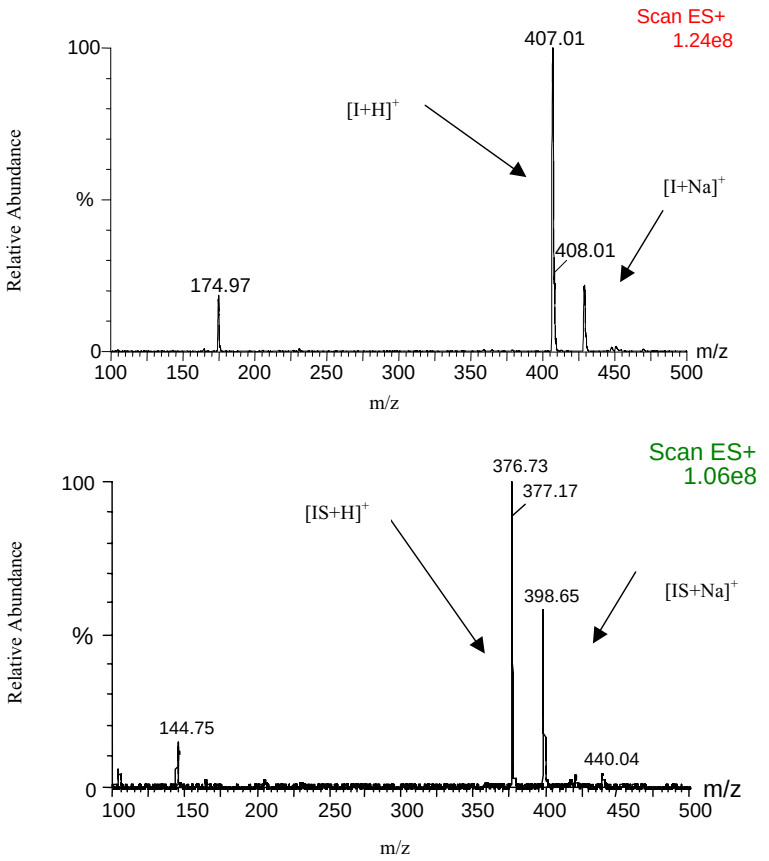

Fig. 1. Electrospray positive ion Q1 mass spectra of compound **I** (top) and its internal standard (**IS**, bottom). Relative abundance–mass spectrometry response, *m*/*z*—mass to charge ratio.

from 0.0006877 to 0.0029089. The **IS** compensated for these changes. Based on the presented data, it was concluded that the calibration curves used in this method were precise and accurate for the measurement of compound **I** in human plasma, and the 96-well LLE procedure used in this method was capable of producing satisfactory standard concentration data for compound **I**.

3.4. Accuracy and precision

During the method validation, the accuracy and precision was also assessed by analyzing QC samples at concentrations within the lower (3.67 ng/ml), the second (392 ng/ml), and the upper quartile (783 ng/ml) of the standard curve. A fourth QC sample (15,664 ng/ml), with a concentration higher than the upper limit of the standard curve range, was also analyzed. This QC sample, known as the dilution QC, was diluted 1:20 with control human plasma, and the concentration after dilution was 783 ng/ml, which was within the calibration range. A portion of the diluted QC sample was processed and analyzed. The QC samples at each concentration were analyzed in four runs on four separate days. The accuracy was determined by calculating the deviations of the predicted concentrations from their spiked values. The intra- and inter-day precision was determined by calculating the %CV values.

Only one out of the 90 QC samples within the four runs had a measured concentration with deviation greater than 15% from the spiked concentration. To further assess the method accuracy and precision, a one-way ANOVA analysis was performed for the four

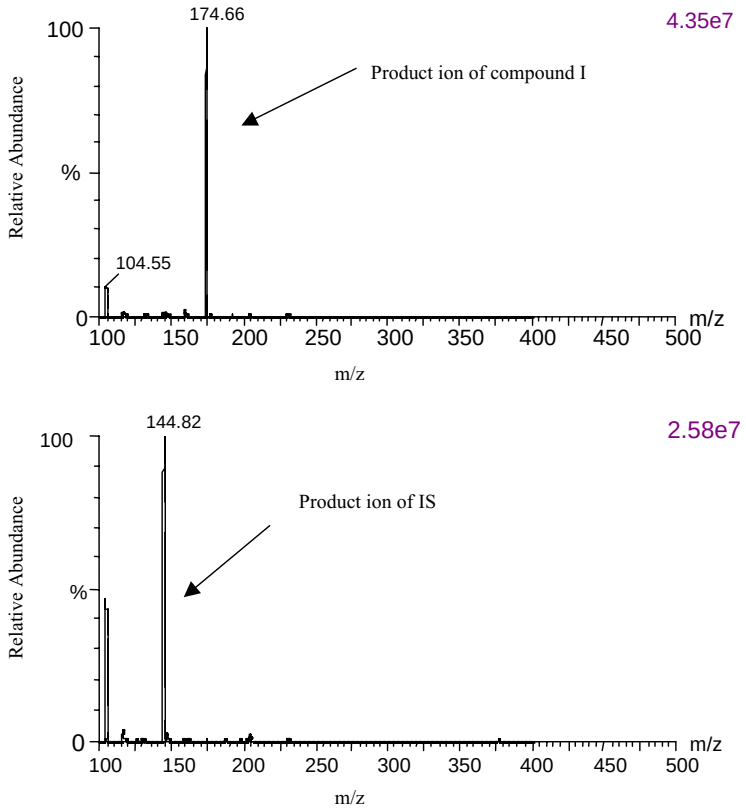

Fig. 2. Electrospray positive ion MS/MS product ion spectra of $[M + H]^+$ for compound **I** (top) and its internal standard (**IS**, bottom). Relative abundance–mass spectrometry response, *m*/*z*—mass to charge ratio.

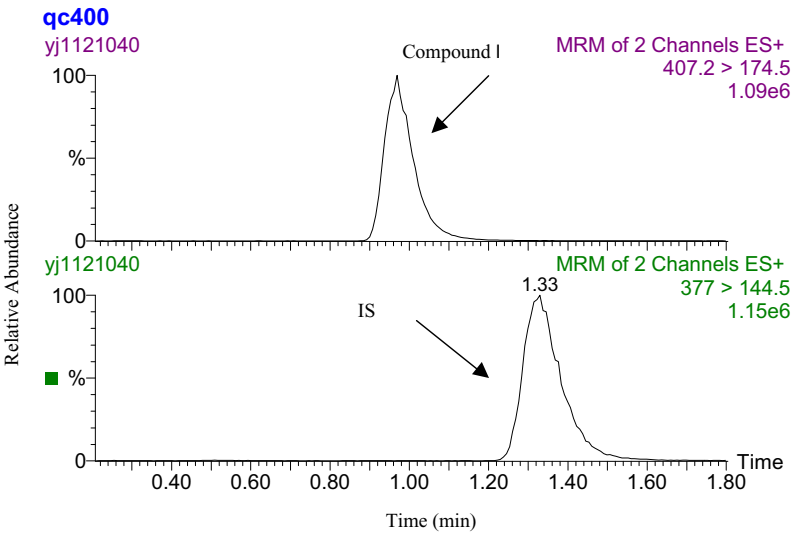

Fig. 3. Selected reaction monitoring chromatograms of a QC sample for compound **I** (tope) at 392 ng/ml and its internal standard (bottom) at 200 ng/ml. Relative abundance–mass spectrometry response, time (min)—run time in minute.

Table 1
Accuracy and precision for compound **I** in human plasma from the method validation (top) and during study sample analysis (bottom)

	Spiked concentration (ng/ml) used in method validation				Spiked concentration (ng/ml) used during sample analysis			
	3.67	392	783	15664	3.75	400	800	16000
Mean observed concentration	3.96	395	829	16993	3.80	420.69	826.58	16674.06
%Deviation	7.9	1.0	5.8	8.5	1.3	5.2	3.3	4.2
Inter-day precision (%CV)	4.5	0.7	0.0	0.0	8.5	4.3	3.8	5.2
Intra-day precision (%CV)	4.1	4.7	4.1	4.3	3.0	3.6	4.0	3.2
Total variation (%CV)	6.1	4.7	4.1	4.0	9.0	5.6	5.5	6.1
n	18	18	18	18	36	36	36	24
Number of runs	4	4	4	4	12	12	12	8

runs, and the results are shown in Table 1 (left). The intra-day precision was within 4.7% CV and inter-day precision was within 4.5% CV. The assay accuracy was within ±8.5% of the spiked values. Since QC samples are expected to be a good representation of unknown samples, similar precision and accuracy is expected from unknown samples. The precision and accuracy data provided further evidence that the 96-well LLE procedure employed in this method was well suited for measuring compound **I** in human plasma.

3.5. Lower limit of quantitation

To establish the lower limit of quantitation (LLOQ) of the calibration curve for this 96-well LLE, LC/MS/MS method, six different lots of control plasma were spiked at 1.22 ng/ml to obtain the six LLOQ samples. The LLOQ samples were processed and analyzed with a standard curve and QC samples, and their predicted concentrations were determined. The deviations of the predicted concentrations from the spiked value were within 10.0% for all six LLOQ samples. The relative small %deviations (≤10.0%) suggested that endogenous materials present in varying amounts in different plasma lots were effectively removed by the 96-well LLE extraction procedure. In addition, the void time of the LC system was around 0.4 min with the analyte and its internal standard being eluted later with retention times 1.0 and 1.3 min, which separated the analyte and **IS** from the majority of the polar endogenous materials. With the combination of LLE extraction and chromatographic separation, the matrix effect should be minimal [10–13]. A typical SRM chromatogram at the LLOQ concentration is shown in Fig. 4. The data demonstrated that the 96-well LLE, LC/MS/MS method was able to quantitatively measure compound **I** in human plasma as low as 1.22 ng/ml. Since 1.22 ng/ml was sufficient for our specific applications, no concentration lower than 1.22 ng/ml was tested. However, the LLOQ chromatogram indicated that LLOQ could go even lower than 1.22 ng/ml, as needed. In addition, it was expected that by increasing plasma volume used in the extraction, an even lower LLOQ can be achieved.

3.6. Specificity

In additional to the LLOQ assessment, six different lots of control human plasma were analyzed with and without **IS** in order to determine whether any endogenous plasma constituents interfered with compound **I** or the internal standard. The degree of interference was assessed by inspection of SRM chromatograms. No significant interfering peaks from the plasma were found at the retention time and in the ion channel of either compound **I** or the internal standard (Figs. 4 and 5). Thus, the addition of acetonitrile during the 96-well LLE did not compromise the cleanness of the sample extracts. A control plasma blank was analyzed immediately following the highest concentration standard in each run to monitor the carry-over of compound **I** or the internal standard by the robot's probes. No carry-over peaks were observed at the retention times and the ion channels of either compound **I** or the internal standard. The absence of carry-over was achieved by thorough washing of the probes with the system liquid between sample transfers.

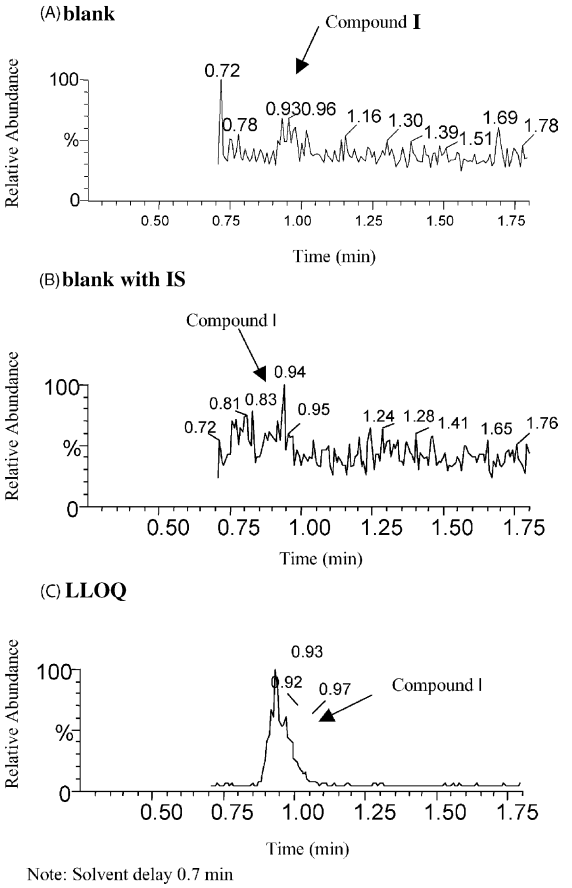

Fig. 4. Selected reaction monitoring chromatograms for compound **I** obtained from: (A) blank human plasma; (B) human plasma containing only internal standard (200 ng/ml); (C) human plasma containing compound **I** at lower limit of quantitation (1.22 ng/ml) and its internal standard at 200 ng/ml. Relative abundance–mass spectrometry response, time (min)—run time in minute.

3.7. Stability

As part of the method validation, the data were also generated to make sure compound **I** was stable under its storage conditions. Plasma samples containing two levels of compound **I** were used for the stability experiments. In plasma, compound **I** was found to be stable for at least 2 weeks at −30 °C, for at least 48 h at room temperature, and during three freeze-thaw cycles. The processed samples were stable for at least 48 h at room temperature.

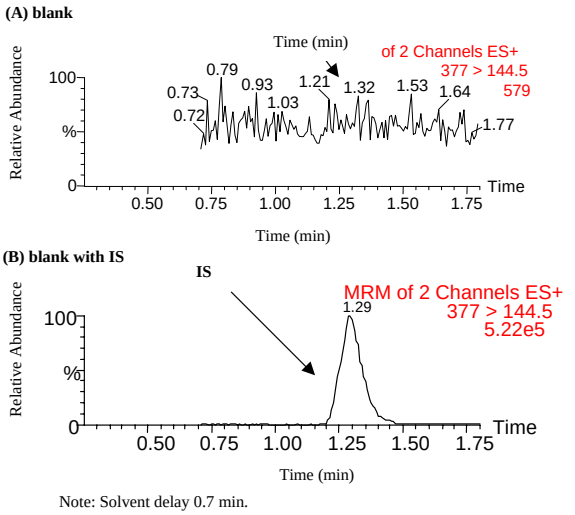

Fig. 5. Selected reaction monitoring chromatograms for the internal standard (**IS**) of compound **I** obtained from: (A) blank human plasma; (B) human plasma containing only the internal standard at 200 ng/ml. Relative abundance–mass spectrometry response, time (min)—run time in minute.

4. Application

This method has been successfully applied for the determination of compound **I** plasma concentrations for samples obtained from a first-in-man study, where new standards (1.25–1000 ng/ml) and QC samples (3.75, 400, 800, and 16,000 ng/ml) were prepared. With twelve analytical runs performed, all of them passed the batch acceptance criteria: three-fourths of standards within 15% of the spiked concentrations (20% for the LLOQ) and two-thirds of QCs within 15% of the spiked concentrations. A one-way ANOVA analysis was performed for the twelve runs, and the results are shown in Table 1 (right). The intra-day precision was within 4.0% CV and inter-day precision was within 8.5% CV. The assay accuracy was within ±5.2% of the spiked values. These numbers were comparable to what we got during the method validation (refer to Section 3.4). This indicated that the method behaved equally well during the sample analysis. Out of 1500 sample analyzed, a total of 36 samples from two subjects were reassayed in duplicate as pharmacokinetic outliers, and all the original values were confirmed. These actual sample analysis results further demonstrated that the 96-well LLE

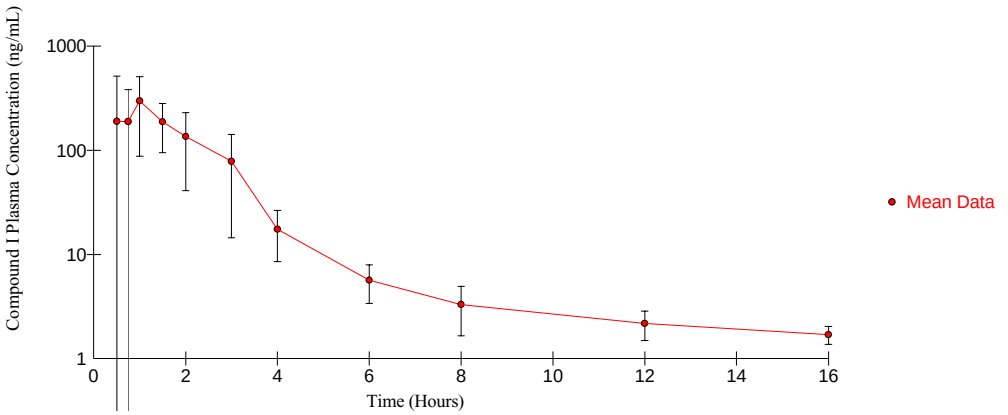

Fig. 6. Average plasma concentration vs. time profile for four subjects administered a 400 mg oral tablet of compound **I**. Compound **I** plasma concentration (ng/ml), time (hours)—time after dose.

method was rugged and well suited for real life applications. Representative plasma concentration versus time profiles following oral (400 mg) administration of compound **I** to four subjects are presented in Fig. 6. Characteristic pharmacokinetic information for compound **I**, such as t1/2, Cmax and AUC, was obtained for the whole study.

5. Conclusions

A 96-well LLE, LC/MS/MS method for the quantitation of a basic compound (compound **I**) in 0.2 ml of human EDTA plasma has been developed and validated over the concentration range of 1.22–979 ng/ml. A combination of acetonitrile and methyl *t*-butyl ether was used to extract compound **I** and its internal standard in 96-well plate format. It was found that the addition of acetonitrile during 96-well LLE effectively eliminated the formation of the irregular emulsion, which had been a major obstacle for 96-well LLE. In addition, a proper adjustment of acetonitrile and methyl *t*-butyl ether ratio was able to modulate the polarity of the organic solvent to achieve the desired extraction. The validation results demonstrated that this approach worked well in an automated 96-well plate format. The method possessed excellent precision and accuracy, and was proved to be rugged and reliable. The actual sample analysis results further demonstrated that this approach was well suited for real life applications. It is expected that this approach can be applied to the extraction for other compounds from biological fluids. A higher acetonitrile:methyl *t*-butyl ether ratio is expected to give better recovery but less selectivity, since some endogenous materials will be extracted as well.

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