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Determination of a β_3-agonist in human plasma by LC/MS/MS with semi-automated 48-well diatomaceous earth plate

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Abstract

Methods for the determination of a β_3-agonist (**A**) in human plasma were developed and compared based on high-performance liquid chromatography (HPLC) with tandem mass spectrometric (MS/MS) detection using a turbo ion spray (TIS) interface. Drug and internal standard were isolated from plasma by three sample preparation methods, liquid–liquid extraction, Chem Elut cartridges and 48-well diatomaceous earth plates, that successively improved sample throughput for LC/MS/MS. MS/MS detection was performed on a PE Sciex API 365 tandem mass spectrometer operated in positive ion mode and using multiple reaction monitoring (MRM). The precursor/product ion combinations of m/z 625/607 and 653/515 were used to quantify A and internal standard, respectively, after chromatographic separation of the analytes. Using liquid–liquid extraction and Chem Elut cartridges, the assay concentration range was 0.5–100 ng/ml. Using diatomaceous earth plates, the concentration range of the assay was extended to 0.5–200 ng/ml. For all three assays, the statistics for precision and accuracy is comparable. The assay accuracy ranged from 91–107% and intraday precision as measured by the coefficient of variation (CV) ranged 2–10%. The sample throughput was tripled when the diatomaceous earth plate method was compared with the original liquid–liquid extraction method.

Keywords: β3-Agonist; LC/MS/MS; Diatomaceous earth plate

1. Introduction

Compound **A** (Fig. 1a) ((*R*)-*N*-[4-[2-[[2-hydroxy-2-(3-pyridinyl)ethyl]amino]ethyl]phenyl]-4-[4-[4-(trifluoromethyl)phenyl]thiazol-2-yl]benzenesulfonamide) is a potent and selective β_3-adrenergic receptor agonist developed for the treatment of

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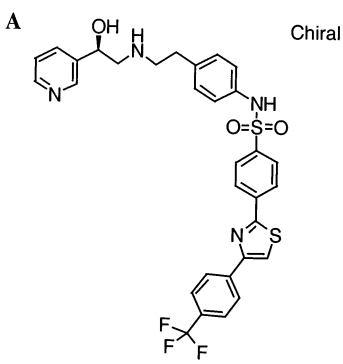
obesity [1]. Obesity is closely associated with the development of Type II Diabetes, coronary heart disease and hypertension. The morbidities associated with these diseases can be reversed by weight loss. Recent studies have shown that decrease in body weight in humans is accompanied by compensatory decrease in metabolic rate, so that weight loss is difficult to maintain by caloric restriction alone [2]. Therefore, an optimal therapeutic approach to obesity may include treatment to enhance energy expenditure by increasing metabolic rate. β_3-agonists, such as compound **A**, can stimulate lipolysis in white and brown adipose tissue, thus increasing energy utilization [3,4].

LC/MS/MS has been extensively used in the pharmaceutical industry as a quantitative bioanalytical technique with high sensitivity and selectivity. However, the sample preparation procedure often is the most labor intensive step in LC/MS/MS assay. To address this issue, automated liquid–liquid and solid phase extraction methods in a 96-well format have been developed to increase the sample throughput [5–8].



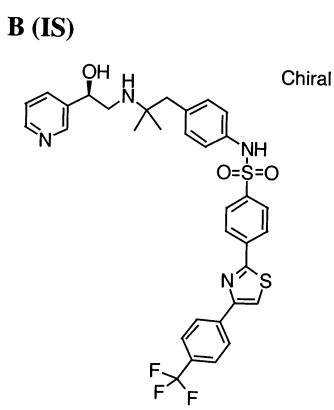


Fig. 1. Chemical structures of compounds **A** and **B**.

Here we describe three separate sample preparation methods, which were evaluated to assay compound **A** with each successive method increasing sample throughput. The first of these methods is liquid–liquid extraction. The second method replaces time consuming liquid–liquid extraction with Chem Elut cartridges (diatomaceous earth packing). In the third method, Chem Elut cartridges are replaced with 48-well diatomaceous earth plates. Diatomaceous earth can be used in microtiter format as an automated approach to liquid–liquid extraction that triples sample throughput compared with liquid–liquid extraction without compromise in extract quality. Methods for plasma sample quantitation with Chem Elut cartridges have been widely used [9–12]. Diatomaceous earth in 96-well format has been used in purification of combinatorial libraries [13]. To date there are no reports of diatomaceous earth being used in a 96-well or 48-well format for high throughput clinical sample analysis.

2. Experimental

2.1. Materials

Compound **A** and internal standard (IS, **B**, an analogue of **A**) were synthesized at Merck Research Laboratories (Rahway, NJ) and used as received. Optima grade acetonitrile and methanol, high-performance liquid chromatography (HPLC) grade ammonium acetate, and ACS reagents sodium carbonate and sodium chloride were obtained from Fisher Scientific (Pittsburgh, PA). ACS reagent grade formic acid (96%), and Omnisolv methyl-*tert*-butyl ether (MtBE), were purchased from Aldrich (St. Louis, MO) and E.M. Science (Gibbstown, NJ). Drug-free heparinized human plasma was purchased from SeraTec biologicals (New Brunswick, NJ). Water was purified by Milli-Q from Millipore (Bedford, MA). Chem

Elute cartridges, size 1-ml, are from Varian (Harbor City, CA). RAPILUT-48-WELL diatomaceous earth plates are packed by Chemical Separation Corporation (Phoenixville, PA) using diatomaceous earth sorbent supplied by Varian and sized before use.

2.2. Instrumentation and chromatographic conditions

Liquid chromatography was performed on a Hewlett–Packard Series 1050 quaternary HPLC pump (Agilent, Wilmington, DE) with Hewlett–Packard Series 1050 autosampler or Varian ProStar 430 (Varian, Inc., Walnut Creek, CA). The mobile phase, consisting of 63% acetonitrile, 3% methanol and 34% 0.01 M ammonium formate/acetate buffer (pH 3), was filtered and degassed through a Rainin 0.2 μm filter. The flow rate was 0.16 ml/min. Chromatography was performed on a Hypersil BDS C8 column (3 μm, 100×2.0 mm, Phenomenex, Torrance, CA) at room temperature.

A PE-SCIEX (Toronto, Canada) API 365 triple quadrupole mass spectrometer equipped with a turbo ion spray (TIS) interface was used for all analyses. The TIS interface was heated to 450°C, the nebulizing gas flow (N_2) was approximately 8000 cm^3/min, and the collision gas was nitrogen (CGT = 2×10^{15} molecules per cm^2). Multiple reaction monitoring (MRM) was done in positive ion mode at m/z 625 → 607 for **A** and m/z 653 → 515 for **B**, with 250 ms dwell time and 5 ms pause time for each transition. Unit resolution (at half peak height) was used for both Q1 and Q3. Peak area ratios were utilized for construction of calibration curves and quantitation using McQuan version 1.6 (Sciex, Toronto, Canada) data analysis program.

2.3. Sample preparation

2.3.1. Liquid–liquid extraction

Aliquots of 0.5 ml of control plasma and plasma samples were mixed in 15-ml polypropylene culture tubes with the following, 50 μl of working standard **A** or 50% acetonitrile, 100 μl of 0.1 μg/ml working internal standard **B**, and 100 μl of 0.5 M sodium carbonate. MtBE (6 ml) was added to each tube. The tubes were mixed for 10 min on a rotator (Glas-Col, Terre Haute, IN) and centrifuged at 3000 rpm for 5 min. The bottom aqueous layer was frozen in acetone-dry ice bath. The organic layer was decanted into a 15-ml tube. The extract was mixed with 1.0 ml of 0.1 M NaCl, centrifuged, separated as before in acetone-dry ice, and decanted into a third 15-ml tube. The organic phase was evaporated under a stream of air in a 50°C Turbo-Vap (Zymark, Hopklnton, MA). The samples were reconstituted in 250 μl of 50% ACN/water, vortexed and sonicated.

2.3.2. Chem Elut cartridge

Aliquots of 0.5 ml of control plasma and plasma samples were mixed in 4.5-ml polypropylene test tubes with the following, 50 μl of working standard **A** or 50% acetonitrile, 100 μl of 0.1 μg/ml working internal standard **B**, and 100 μl of 0.5 M sodium carbonate. Basified plasma samples were loaded directly onto a 1-ml Chem Elute extraction cartridge. After 5 min **A** and **B** were eluted twice (4 ml each) with MtBE under gravity. The eluates were collected into 15-ml polypropylene culture tubes and evaporated to dryness. The samples were reconstituted in 250 μl of 50% ACN/water, vortexed and sonicated.

2.3.3. Forty-eight-well diatomaceous earth plate

Aliquots of 0.5 ml of control plasma and plasma samples were mixed in 4.5-ml polypropylene test tubes with the following: 50 μl of working standard **A** or 50% acetonitrile/water, 50 μl of 0.2 μg/ml working internal standard **B** and 50 μl of 1 M sodium carbonate. Basified plasma was loaded onto a 48-well diatomaceous earth plate using Matrix Impact eight-channel expandable electronic pipette (Hudson, NH). After 5 min, **A** and **B** were eluted ten times (0.5 ml each) with MtBE under gravity using an eight channel stream splitting pipetter to deliver 0.5 ml aliquot in each channel (Horizon Specialty, Inc., Yardley, PA). MtBE eluate, about 4 ml, was collected into a 48-well collection plate (5-ml per well) and taken to dryness under nitrogen at 50°C in an SPE dry 96 evaporator (Jones Chromatography). An additional 4 ml of MtBE was eluted from the

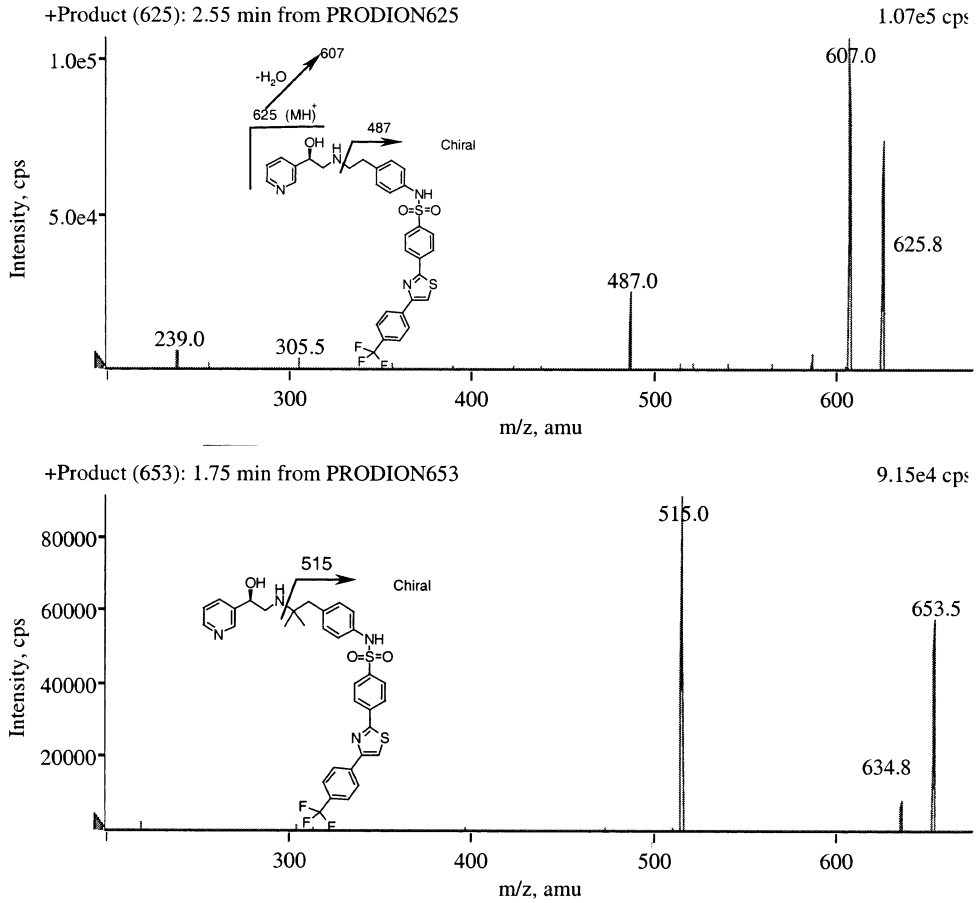


Fig. 2. Positive ion product mass spectra of protonated molecules of compounds **A** and **B**.

diatomaceous earth plate into the same collection plate and again taken to dryness. The samples were reconstituted with 250 μl of 50% ACN/water and then transferred to a 96-well plate for HPLC/MS/MS analysis.

3. Results

3.1. Method validation

Compound **A** and **B** are structural analogs that differ by two methyl groups that are adjacent to a secondary amine and methylene (Fig. 1). Precursor ions for **A** and **B** were determined from a Q1 scan during the infusion of neat solutions of these compounds in the positive ionization mode. Under these conditions, the analytes yielded predominantly protonated molecular ions at m/z 625 for **A** and 653 for **B**. Each of the precursor ions was subjected to collision induced dissociation in order to determine the resulting product ions. Product ion spectra for **A** and **B** are shown in Fig. 2. Product ions corresponding to the loss of water in the case of **A** and loss of the 3-(2-hydroxy-ethylamine)-pyridine in the case of **B** were chosen for the analysis. MRM chromatograms obtained from the extracted patient plasma samples of predose (double blank and single blank) and 120 h after an oral administration of **A** and an extracted plasma standard are shown in Fig. 3. Retention time and chromatographic profile were reproducible throughout the entire study.

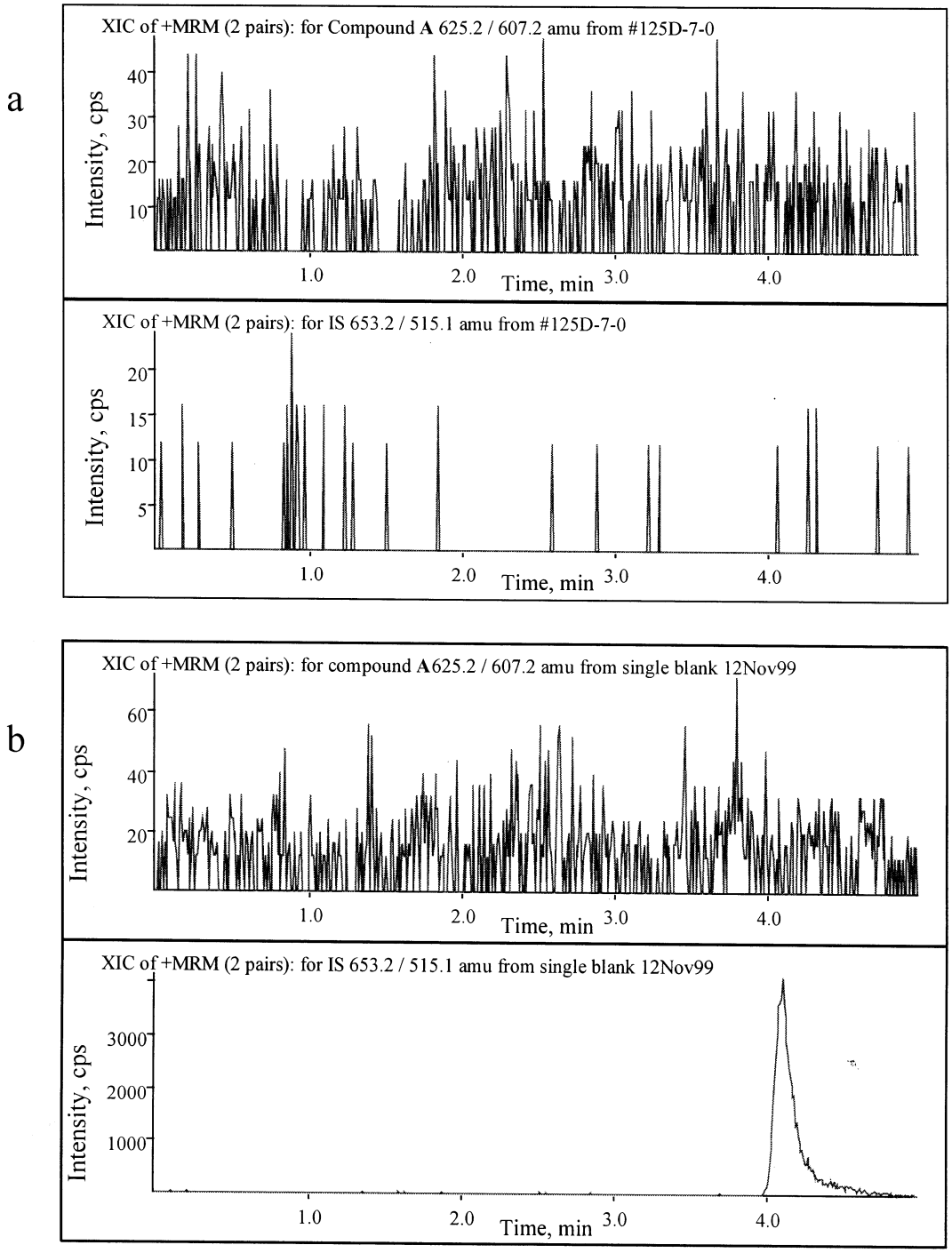
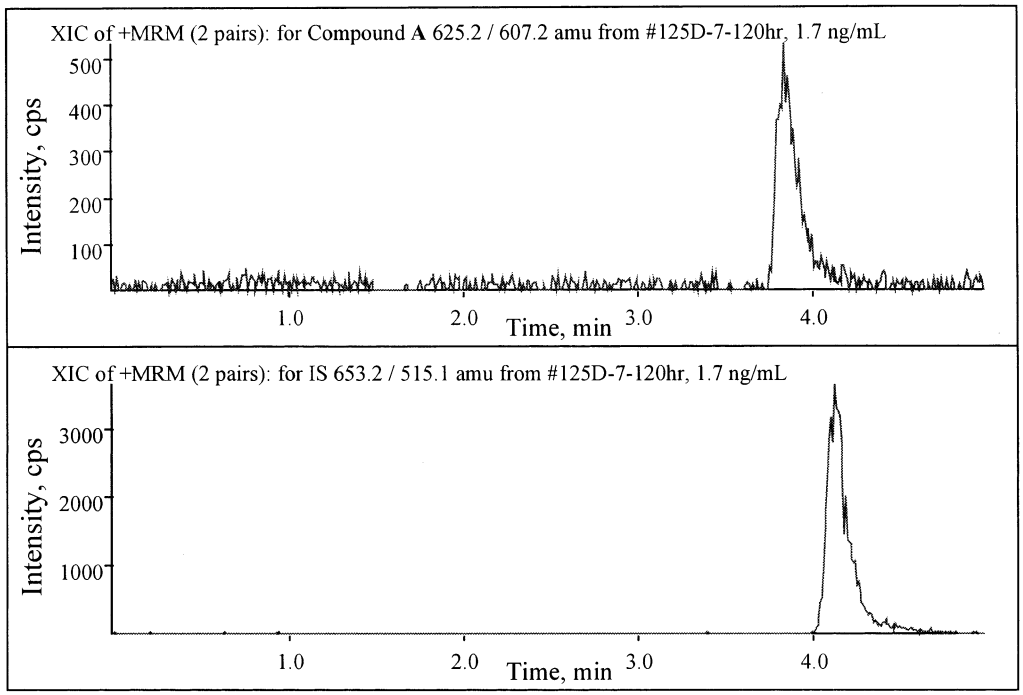


Fig. 3. Chromatograms obtained through MRM from the extracted plasma samples of a subject by 48-well diatomaceous earth plate extraction (a) predose (b) single blank (c) 120 h after an oral administration of compound **A** and (d) an extracted 0.5 ng/ml plasma standard.

c



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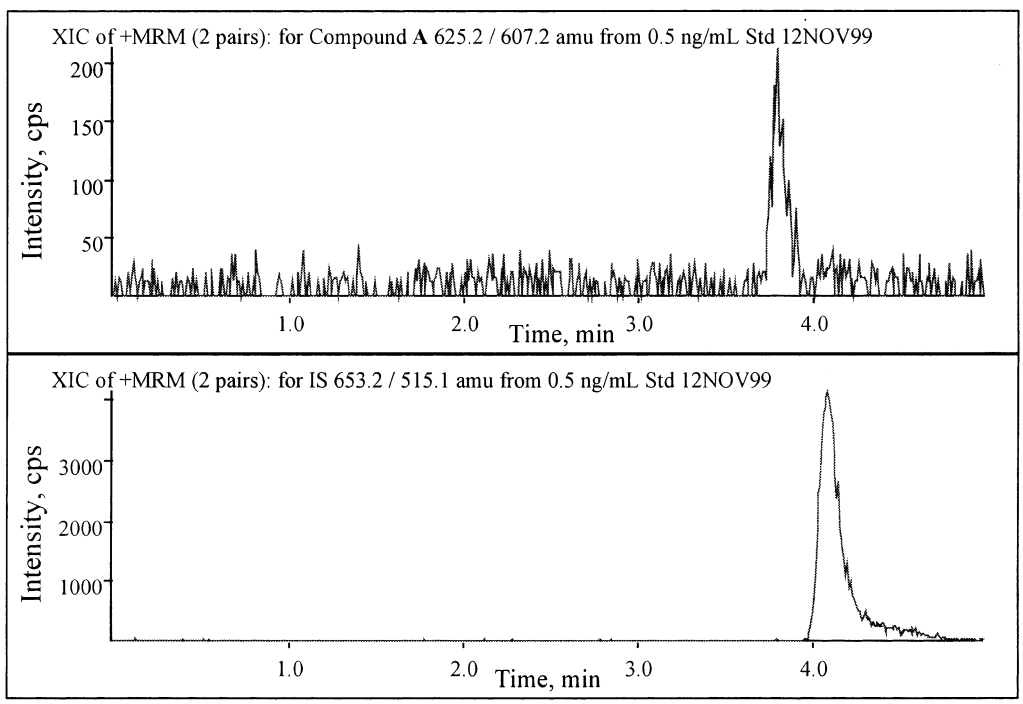

Fig. 3. (*Continued*)

Table 1
Intraday accuracy and precision for the determination of **A** in human control plasma ($n = 5$) by liquid–liquid extraction, Chem Elut and 48-well diatomaceous plate method

Nominal concentration (ng/ml)	Liquid–liquid extraction		Chem Elut		Forty-eight-well diatomaceous plate	
	Accuracy[a]	Precision[b]	Accuracy	Precision	Accuracy	Precision
0.5	103	9.8	106	11	97.7	6.5
1	94.3	7.4	97.6	5.8	97.0	7.7
2.5	102	3.5	93.5	8.2		
5	97.4	3.4	101	5.9	104	5.3
10	90.7	3.5	96.6	3.5		
20	97.1	3.3	103	6.5	103	3.2
50	107	2.1	98.5	3.1	97.8	2.8
100	107	2.1	101	2.2	102	2.4
200					99.3	2.4
Regression equation	$R = 0.998$		$R = 0.996$		$R = 1.000$	
	slope = 0.0051		slope = 0.052		slope = 0.048	
	intercept = −0.008		intercept = −0.001		intercept = −0.002	

[a] Expressed as [(mean observed concentration/nominal concentration)100].
[b] CV% of peak area ratios ($n = 5$).

For the three methods, intraday accuracy and precision was determined by analyzing replicate calibration curves ($n = 5$). The peak areas of **A**, generated by MRM, were used to determine a response at each standard concentration of **A**. The calibration curves were calculated from weighted $(1/x)$ least-squares, linear-regression analysis of peak area ratios versus nominal concentrations.

The liquid–liquid extraction assay and Chem Elut cartridge assay were successfully validated over the sample concentration range of 0.5–100 ng/ml for **A** with the lower limit of quantitation (LLOQ) of 0.5 ng/ml. The diatomaceous plate assay was successfully validated over a range of 0.5–200 ng/ml. The curve range was extended so that samples for the multiple dose study would not have to be diluted to stay in the calibration range. For each assay, an assessment of the intraday variability of the assay was conducted in five different lots of human plasma spiked with both analytes over the calibration range of 0.5–100 ng/ml or in the case of the plate assay from 0.5 to 200 ng/ml. The resulting assay precision and accuracy data for the three asssays are presented in Table 1. The intraday precision of the liquid–liquid assay, as measured by the CV% ranged from 2.1 to 9.8% for all points on the calibration curves. The assay accuracy for the liquid–liquid assay was within 90.7 and 107% of nominal for all standards. For the Chem Elut assay the intraday precision and accuracy ranged from 2.2 to 11% and 93.5–106%, respectively. For the 48-well diatomaceous earth plate assay the intraday precision and accuracy ranged from 2.4 to 7.7% and 97.0–104%, respectively. Basically the statistics for precision and accuracy was comparable for all three assays.

No peaks eluting at the retention times of the analyte and internal standard were detected in samples from five lots of human control plasma that were processed in accordance with any of the three methods. The signal-to-noise ratios (mean, $n = 5$) at the LLOQ from the validation data are 13, 12, 22 for the liquid–liquid, cartridge and plate assays, respectively.

Matrix effect (ion-suppression or accentuation) was determined based on the difference in peak areas between the neat standards and the processed control plasma residues spiked with the standards post sample preparation. No significant matrix effect was observed. Intraday validation precision data from five different lots of plasma also supports a lack of significant matrix effect.

However, a substantial matrix effect occurred when ethyl acetate, ethyl acetate/pentane (9:1 or 8:2) or ethyl acetate/hexanes (8:2) were used as extraction solvents instead of MtBE.

Quality control (QC) samples were used to determine interday assay variability. A set of QCs at low, medium and high concentrations ($n = 3$) were analyzed daily with a standard curve and clinical samples. Acceptance of sample concentration data was based on the QC results. QC samples containing compound **A** were prepared at 2.5, 10, and 50 ng/ml in the liquid–liquid extraction and Chem Elut assays and 2.5, 50, and 100 ng/ml in the 48-well diatomaceous earth plate assay. Interday QC results for all three assays are shown in Table 2.

3.2. Application to clinical studies

Methods have been successfully applied to the determination of plasma concentration levels of **A** in support of pharmacokinetic analysis in four Phase I clinical studies. A plot of plasma concentration versus sampling time obtained from a subject following daily oral administration of 125 mg for 9 days is shown in Fig. 4.

4. Discussion

Diatomaceous earth is microamorphous silica with small amounts of alumina and other metallic oxide impurities. It has a very high surface area to weight ratio. The diatomaceous earth plate assay is robust and it produces clean sample extracts for injection onto LC/MS/MS. Results from intraday precision and accuracy (Table 1), obtained by using five different lots of human plasma, suggest that variations in the matrix between different sources of plasma did not effect the precision or accuracy of any of these assays in any significant fashion. Additionally, for 62 healthy volunteers enrolled in the Phase I clinical studies, the concentration versus time profiles and trends observed per treatment group did not suggest any significant matrix effects were occurring. Signal-to-noise ratio, sensitivity, precision and accuracy were comparable between all three methods.

The plate method was used to keep up with the demand of real time analysis of large multiple dose studies with 45 time points per subject. Using the plate method about 120 samples were processed in about 4 h (30 samples per h) as compared with the cartridge method where about 90 samples were processed in about 6 h (15 samples per h). For the liquid–liquid extraction method about 80 analysis could be performed in about 9 h (about 9 samples per h). The total time required for liquid–liquid extraction was three times longer than that for plate method because liquid–liquid extraction requires tube labeling, single channel pipetting, and capping of tubes and autosampler vials, which is time consuming. Both plate and Chem Elut methods are comparable to the liquid–liquid extraction method in extract cleanness, but liquid–liquid extraction includes the extra step of washing the organic layer to eliminate traces of base that partitions there. The final MtBE extract from pH 10 plasma sample loaded onto the diatomaceous earth sorbent was

Table 2
Interday quality control results for compound **A** by liquid–liquid extraction, Chem Elut and 48-well diatomaceous plate method

Nominal concentration (ng/ml)	Liquid–liquid extraction ($n = 36$)			Chem Elut ($n = 27$)			Forty-eight-well diatomaceous plate ($n = 18$)		
	2.5	10	50	2.5	10	50	2.5	50	150
Mean (ng/ml)	2.4	9.2	48.8	2.3	8.9	46.0	2.6	52.1	157.6
S.D.[a]	0.17	0.64	3.6	0.16	0.66	4.4	0.30	4.6	17.6
CV%[b]	7.2	7.0	7.4	6.9	7.5	9.7	12	8.8	11

[a] Standard deviation.
[b] Coefficient of variation.

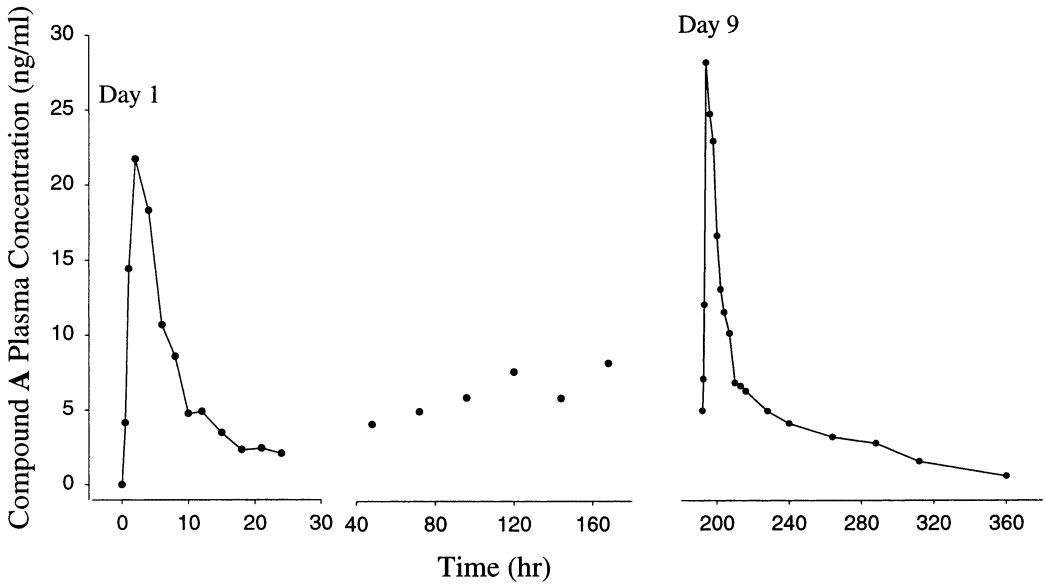

Fig. 4. Plasma concentrations of compound **A** of a subject following daily oral administration of 125 mg dose for 9 days.

neutral, thus, no washing of the organic was required.

The plate method is superior to liquid–liquid extraction as sample quality is identical to a 'clean' liquid–liquid extraction and sample throughput is substantially improved. Based on this work and the work of Burton [10] the diatomaceous plate method can be considered an alternative and in some cases an advantage to microtiter format solid phase extraction.

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