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LC/MS/MS plasma assay for the peptidomimetic VLA4 antagonist **I** and its major active metabolite **II**: for treatment of asthma by inhalation

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Abstract

In vitro and in animals, **I** is a potent and specific peptidomimetic for the potential treatment of airway inflammation in the pathogenesis of asthma. Preclinical studies indicated extensive conversion of **I** to an active metabolite **II**, and thus, a very sensitive assay for **I** and **II** was needed to support an inhalation ascending-dose study in man. The LC/MS/MS plasma/urine assay method (1.0 ml of sample) involves the following: liquid–liquid extraction of acidified plasma into pentane–ethyl acetate (90:10 v/v); evaporation of the organic extract, reconstitution into methanol; addition of water to the methanolic extract and freezing. After thawing, the extract is centrifuged and the clear supernatant injected for chromatography. Extract is chromatographed on a YMC ODS-AM column (50 × 2.0 mm). For detection, a Sciex 365 LC/MS/MS with an electrospray inlet and used in the positive ion, multiple reaction monitoring mode was used to monitor precursor → fragment ions of m/z 709 → 594 for **I** and m/z 513 → 380 for **II**. The plasma assay was linear over the concentration range of 0.1–100 ng/ml in plasma for **I** and **II**. Accuracy and precision for **I** ranged from 97.9 to 102.1% of nominal with a 0.84–10.65% CV; similarly for **II**, 98.0–101.7% and 1.39–9.28% CV, respectively. Extraction recovery averaged 63.7% for **I** and 64.9% for **II**. This general assay methodology may be applied to assay small acidic peptides and peptidomimetics from biological fluids by LC/MS/MS.

Keywords: VLA4 antagonist; Peptide LC/MS/MS; Protein precipitation

1. Introduction

Compound **I** and its major metabolite **II** (Fig. 1) are antigen 4: alpha-4 beta-1 integrin (VLA-4) antagonists developed as potent peptidomimetics

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for treating airway inflammation in the pathogenesis of asthma [1]. **I** and **II** appear to have a mechanism of action, VLA-4 antagonism, distinct from currently marketed anti-asthmatic products. VLA-4 is expressed on cells thought to be involved in pulmonary inflammation. Blocking activation and or recruitment of immune cells may possibly interrupt pathological processes resulting in amelioration of asthma [1–3].

Pre-clinical studies in different species revealed extensive conversion of **I** to **II**. Thus, a very sensitive clinical assay was needed for **I** to provide pharmacokinetic support for the first safety and tolerability study of inhaled **I** in humans. The LC/MS/MS assay for quantitation of **I** and its major metabolite of **II** in plasma and urine is described herein using liquid–liquid extraction of 1.0 ml of sample to attain a lower limit of quantitation (LLOQ) at 0.1 ng/ml for **I** and **II**. Deuterated internal standards are used for **I** and **II**.

2. Experimental

2.1. Materials and reagents

Parent drug (**I**), and metabolites (**II** and **III**) were synthesized at Biogen Inc. (Cambridge, MA, USA[1]). Methanol (Optima grade), ammonium formate and concentrated formic acid (ACS reagent grade), *n*-pentane, methyl-*t*-butyl-ether (MTBE), and ethyl acetate (HPLC grade) were all obtained from Fisher Scientific (Pittsburgh, PA, USA). Milli-Q water was generated at 18 MΩ (Millipore, Bedford, MA, USA). *n*-pentane was mixed with ethylacetate (1:9 v/v). Ammonium formate buffer (5 mM, pH 3.0) was prepared with addition of concentrated formic acid. Human control plasma containing 1.4–1.8 mg of tripotassium ethylenediamine tetraacetate acid (K_3EDTA) was purchased from Biological Specialties Corporation (Lansdale, PA, USA) and $K_3EDTA \cdot 2H_2O$ (98% pure) from Aldrich (St. Louis, MO, USA).

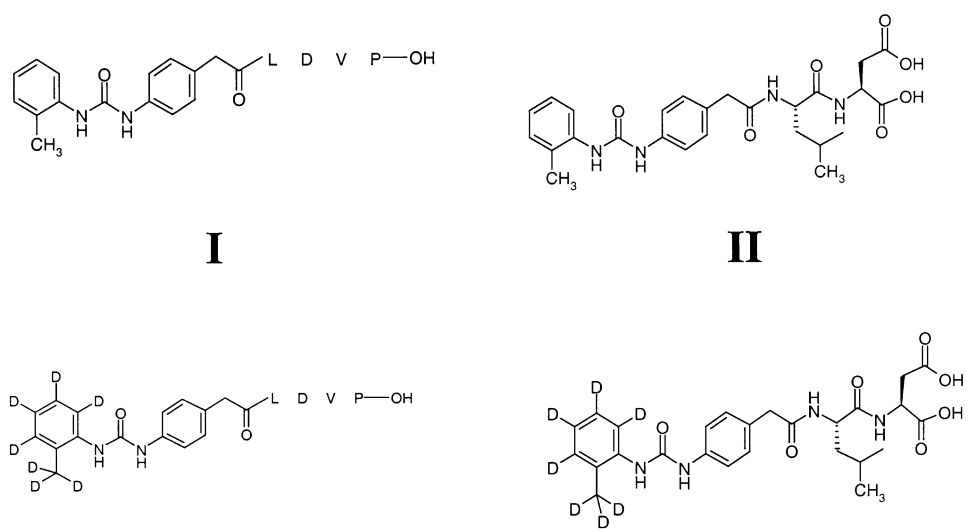


Fig. 1. Chemical structures of **I**, **II** and deuterated internal standards.

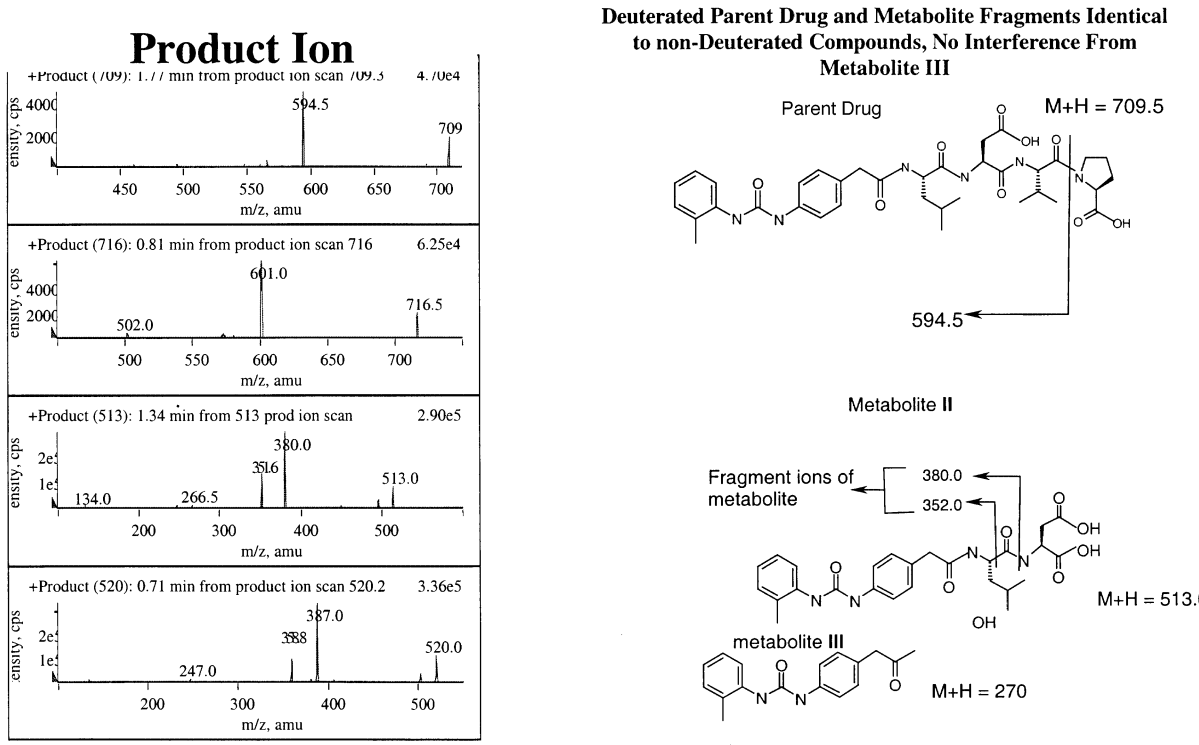
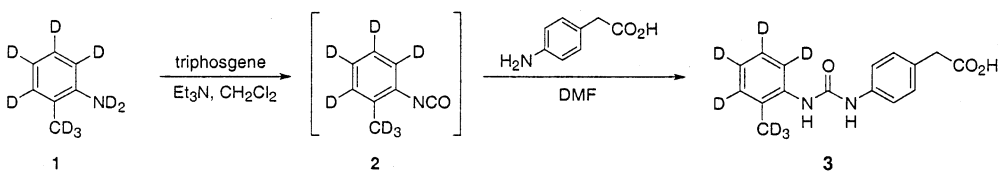


Fig. 2. Product ion scans of **I** and **II** and information concerning metabolite **III.**

2.1.1. Preparation of deuterated internal standards



To a mixture of commercially available d9-*o*-toluidine **1** (8.60 mmol) and triphosgene (3.10 mmol) in anhydrous CH_2Cl_2 (10 ml) at 0 °C was added triethylamine (18.9 mmol). The reaction mixture was stirred at room temperature for 1 h, then diluted with hexanes (20 ml) and filtered. The filtrate was concentrated to give 1.143 g (95%) of d7-isocyanate **2**. Intermediate **2** was stirred with *p*-aminophenylacetic acid (8.20 mmol) in anhydrous DMF (5 ml) at room temperature for 1 h, before quenching with H_2O (10 ml). The resultant white precipitate was removed by filtration, washed with H_2O, and dried in vacuo to yield 2.112 g (88%) of d7-urea **3**.

Urea **3** was coupled under standard EDC/HOBT conditions with the corresponding dibenzyl-protected peptides (obtained from Merck Process Research). The resultant amides were deprotected by hydrogenation over Pd/C in 9:1 THF/H_2O (40 psi H_2) to yield crude d7-**I** and d7-**II**. The compounds were purified by precipitation from THF solution upon addition of toluene, giving d7-**I** (96.3% purity)and d7-**II** (98.6% purity) in 43 and 50% yield, respectively, from **3**.

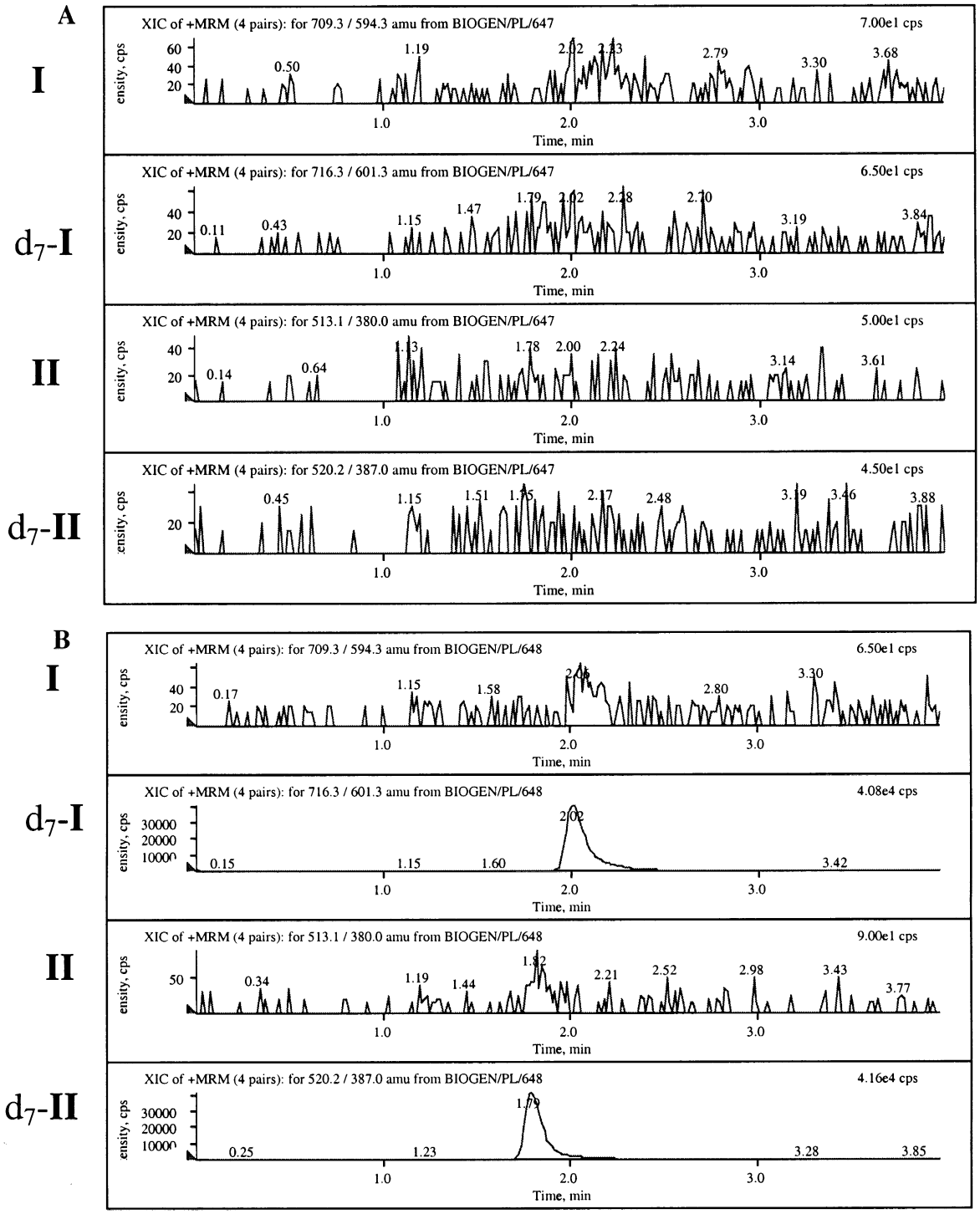


Fig. 3. Extracted ion chromatograms of human control plasma blank. Chromatograms of control plasma containing internal standards d7-**I** and d7-**II**. Chromatograms of 0.5 ng/ml **I** and **II** standards with internal standards (channels for **I**, d7-**I**, **II** and d7-**II**).

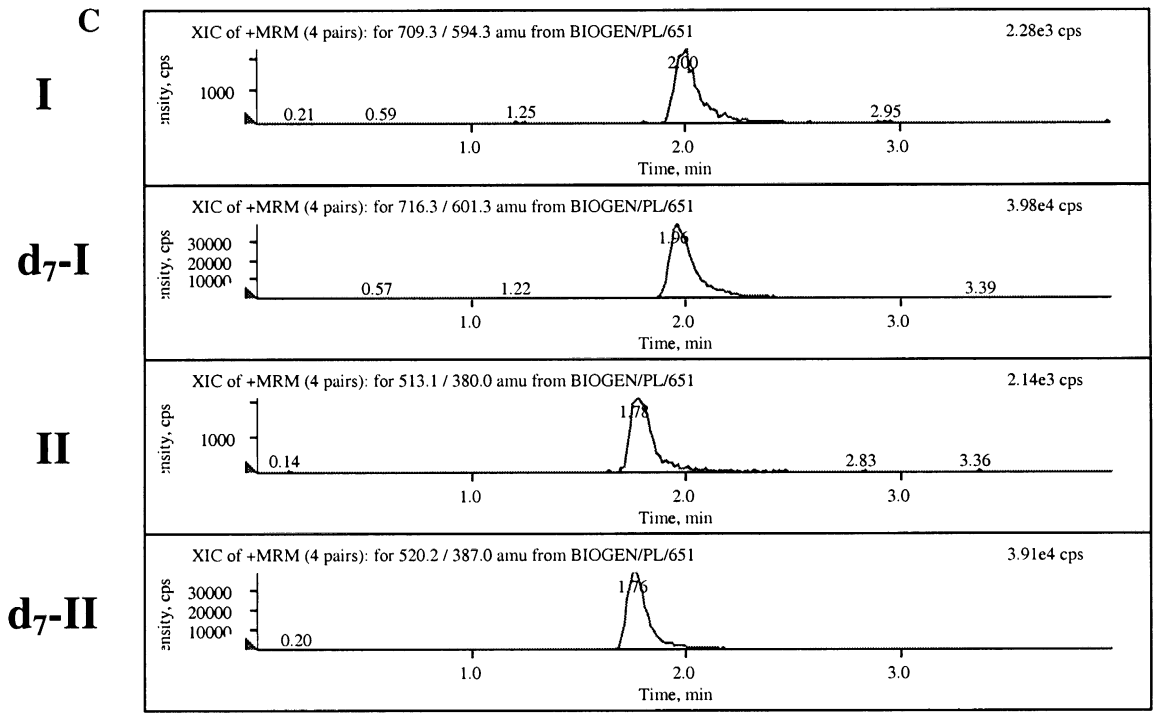


Fig. 3. (*Continued*)

2.2. Instrumentation and LC/MS/MS conditions

A Hewlett-Packard Series 1050 quaternary HPLC system (Wilmington, DE, USA) was operated at ambient temperature using a YMC ODS-AM analytical column (Kyoto, Japan) (50 × 2.0 mm, 3 μm) with an isocratic mobile phase composed of methanol-ammonium formate (5 mM, pH 3) (75:25, v/v). The pump flow rate was 0.16 ml/min and autosampler injection volume was 40 μl. The run time was 4.0 min. The effluent was delivered into the electrospray interface (ESI) of a Sciex API 300 triple-quadrapole mass spectrometer (Toronto, Ont., Canada). Nitrogen served as the drying gas (450 °C) and nebulizing gas at flow-rates of 0.9 and 8.0 ml/min, respectively. The LC/MS/MS was programmed for a scan dwell time of 200 ms. The responses of **I** and **II** were measured in the positive ion mode using multiple reaction monitoring (MRM). The mass spectrometer was set to selectively monitor parent fragment ions of m/z 709 → 594 for **I** and m/z 513 → 380 for **II**. For deuterated internal standards the parent to fragment ions were m/z 716 → 601 for d7-**I** and m/z 520 → 387 for d7-**II**. The peak area ratios for calibration curves and quantitation were calculated using MacQuan version 1.1 (Sciex, Toronto, Canada).

2.3. Standards and quality controls

Separate stock solutions of **I** and **II** were prepared at 100 μg/ml by weighing 1.0 mg of material into 10 ml volumetric flask and diluting with methanol–water (4:1, v/v). Using the stock solutions and mobile phase as diluent, working standard solutions were prepared from 0.005 to 10 μg/ml containing both **I** and **II** in 10 ml silanized glass screw-cap centrifuge tubes and stored at −70 °C. A stock solution containing both internal standards was prepared at 0.1 μg/ml with mobile phase. Calibration standards from 0.1 to 100 ng/ml were prepared by mixing 1.47 ml of plasma containing EDTA with 30 μl of working standard solution (or 30 μl of mobile phase for a plasma blank) in polypropylene tubes. Urine stan-

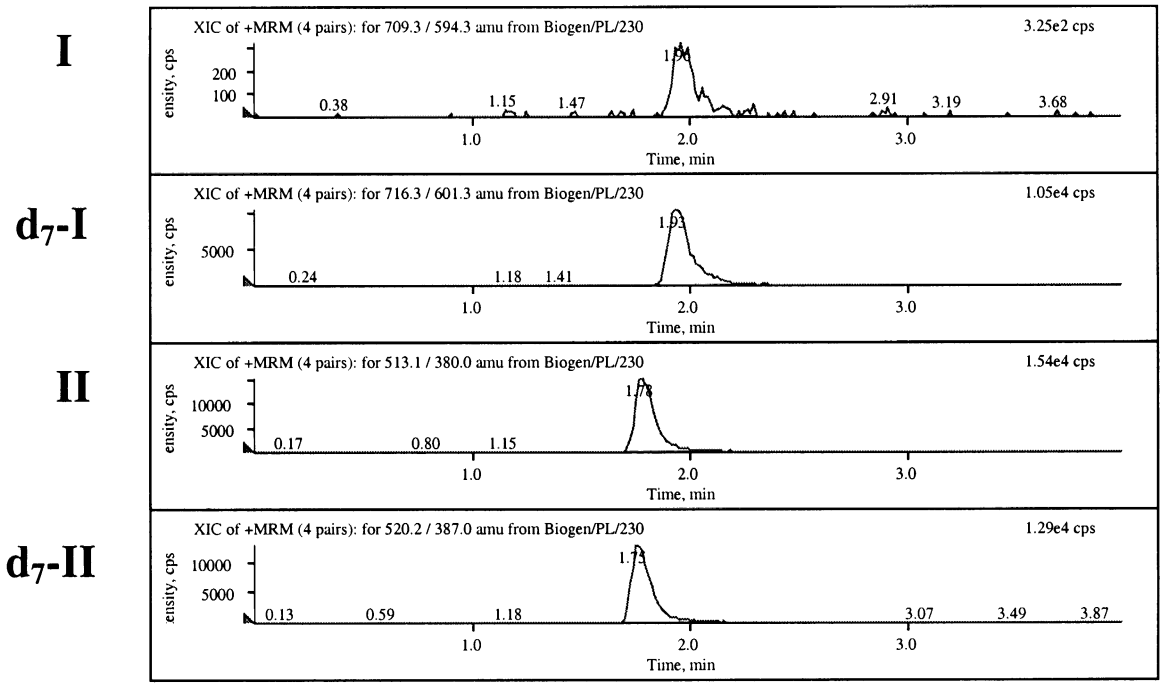


Fig. 4. Extracted ion chromatograms of post-dose plasma sample (12 h) from a subject who received 25 mg of **I** by inhalation (concentration of **I** = 0.24 ng/ml and **II** = 11.1 ng/ml).

dards were prepared similarly from 0.13 to 53 ng/ml.

Plasma quality control (QC) samples containing **I** and **II** were prepared similarly to the calibration standards. A second set of stock solutions at 100 μg/ml were prepared with a second weighing of **I** and **II** for QC samples. Working standards at 0.025, 0.125, 1.0 and 2.5 μg/ml were mixed from the stock solutions. The QC samples at 50, 20, 2.5 and 0.5 ng/ml were prepared individually in polypropylene tubes as described for the calibration standards and then stored at −70 °C. Urine QC samples were prepared as described for plasma QC samples at 0.5, 5.0 and 50 ng/ml and stored at −70 °C.

2.4. Extraction and precipitation procedures for plasma

Stock solutions of the deuterated internal standards (IS) were prepared at 100 μg/ml by weighing 1.0 mg of d7-**I** and d7-**II** and then dissolving and diluting the compounds with methanol–water (4:1 v/v) in a 10 ml volumetric flask. Secondary IS stock solutions were prepared at 10 μg/ml in the same diluent. Working stock solutions containing both internal standards were mixed and diluted to 0.1 μg/ml using mobile phase.

QC samples and clinical samples were thawed to ambient temperature, mixed by vortex for 10–15 s and centrifuged at 2000 × *g* to sediment fibrin. Aliquots of 1.0 ml of samples and standards were mixed with 100 μl of working internal standard (or 100 μl of mobile phase for blanks) in 16 × 125 mm glass screw-cap culture tubes. The tubes were capped and mixed by vortex for 15 s. The samples were then mixed with 1.0 ml of 10% formic acid and extracted with 6 ml of pentane–ethylacetate (90:10 v/v). Extraction involved mixing the organic and aqueous sample phases by vortex for 5 min and centrifuging at 2000 × *g* for 10 min at 10 °C. The lower aqueous layer of the tubes was then frozen in acetone/dry ice bath and the organic phase was decanted into clean silanized 10 ml conical glass tubes with screw caps. The tubes were evaporated to dryness under

Table 1
Intraday accuracy, precision and extraction recovery and curve statistics of **I** and **II** in EDTA plasma ($n = 5$)

Concentration (ng/ml) of **I**											Concentration (ng/ml) of **II**									
Nominal	0.1	0.25	0.5	1.0	2.5	5.0	10	25	50	100	0.1	0.25	0.5	1.0	2.5	5.0	10	25	50	100
Mean	0.10	0.25	0.49	1.02	2.45	4.96	9.98	25.3	50.9	101	0.10	0.25	0.51	0.98	2.50	4.95	9.97	25.1	50.9	101
S.D.	0.01	0.02	0.02	0.03	0.01	0.11	0.11	0.48	0.43	0.96	0.01	0.02	0.01	0.03	0.06	0.10	0.16	0.35	0.43	0.96
CV(%)	8.75	5.12	4.6	2.51	1.84	1.91	3.65	1.55	1.11	1.06	8.08	7.69	3.93	4.14	3.02	4.08	4.76	0.71	1.11	1.06
Accuracy (%)	101	98.6	97.9	102	97.9	99.2	99.8	101	102	101	100.2	99.3	101.1	98.0	100.2	99.0	99.7	100.5	102	101
Extraction recovery (%)	62.8	66.0	62.3	66.7	62.8	64.4	64.2	64.2	61.9	61.6	61.6	62.6	66.2	66.5	67.5	67.6	68.2	66.0	61.9	61.6

For five separate standard curves analyzed within one day the slope, intercept and correlation coefficient for **I** ranged from 0.001 to 0.001, 0.107 to 0.109, and 0.999 to 1.000. For five separate standard curves analyzed within one day the slope, intercept and correlation coefficient for **II** ranged from −0.001 to 0.001, 0.115 to 0.117, and 0.999 to 1.000.

Table 2
Intraday accuracy, precision and extraction recovery and curve statistics of **I** and **II** in urine containing EDTA ($n = 5$)

Concentration (ng/ml) of **I**							Concentration (ng/ml) of **II**											
Nominal	0.1	0.25	0.5	1.0	2.5	5.0	10	25	50	0.10	0.25	0.5	1.0	2.5	5	10	25	50
Mean	0.10	0.25	0.51	1.0	2.6	5.0	10.3	24.6	49.6	0.10	0.24	0.47	1.05	2.65	4.88	10.3	24.5	49.5
S.D.	0.01	0.01	0.02	0.07	0.03	0.32	0.1	0.95	0.63	0.02	0.02	0.02	0.05	0.16	0.40	0.40	0.90	0.97
CV (%)	13.0	4.4	3.5	7.4	1.1	1.9	3.1	3.9	1.3	20	8.3	4.3	4.8	6.0	8.2	3.9	3.7	2.0
Accuracy (%)	101	102	102	102	105	103	99.3	98.6	99.2	103	95.3	93.6	106	106	103	97.5	98.2	99.0
Extraction recovery (%)	55.2	64.3	61.6	65.0	59.6	59.4	63.1	60.0	63.7	56.6	59.0	57.9	55.8	56.7	55.9	57.6	53.7	58.0

For five separate standard curves analyzed within one day the slope, intercept and correlation coefficient for **I** ranged from 0.001 to 0.001, 0.018 to 0.018, and 0.990 to 0.999. For five separate standard curves analyzed within one day the slope, intercept and correlation coefficient for **II** ranged from −0.001 to 0.001, 0.014 to 0.018, and 0.990 to 0.999.

a stream of air in a 50 °C water bath. The remaining residue was reconstituted by adding and mixing 150 μl of methanol and then adding and mixing 150 μl of water. The reconstituted residues were placed in a −20 °C refrigerator overnight to allow for precipitation of proteins and lipids. The next day samples were allowed to warm to room temperature and they were gently shaken and centrifuged at 2500 × *g* for 10 min at 5 °C. The supernatant was transferred to silanized autosampler tubes and analyzed by LC/MS/MS.

2.5. Extraction procedure for urine

The extraction procedure for a 1.0 ml sample of urine containing EDTA was identical to plasma except for the reconstitution and precipitation step. After extraction and evaporation of pentane–ethylacetate, the residue was reconstituted in a mixture of 300 μl methanol–water (4:1 v/v) and transferred directly to silanized autosampler tubes for LC/MS/MS analysis.

2.6. Extraction recovery

The liquid–liquid extraction efficiency was calculated by comparing the peak areas of extracted plasma standards with areas of reference standards added to blank plasma extract. The reference standards were prepared by extracting human control plasma and reconstituting the evaporated extracts with stock solutions of drug, metabolite and internals standards. The concentration of the labeled internal standards was 10 ng/ml.

2.7. Method validation

Intraday accuracy and precision was determined by analyzing replicate calibration curves ($n = 5$). The peak areas generated by MRM for drug,

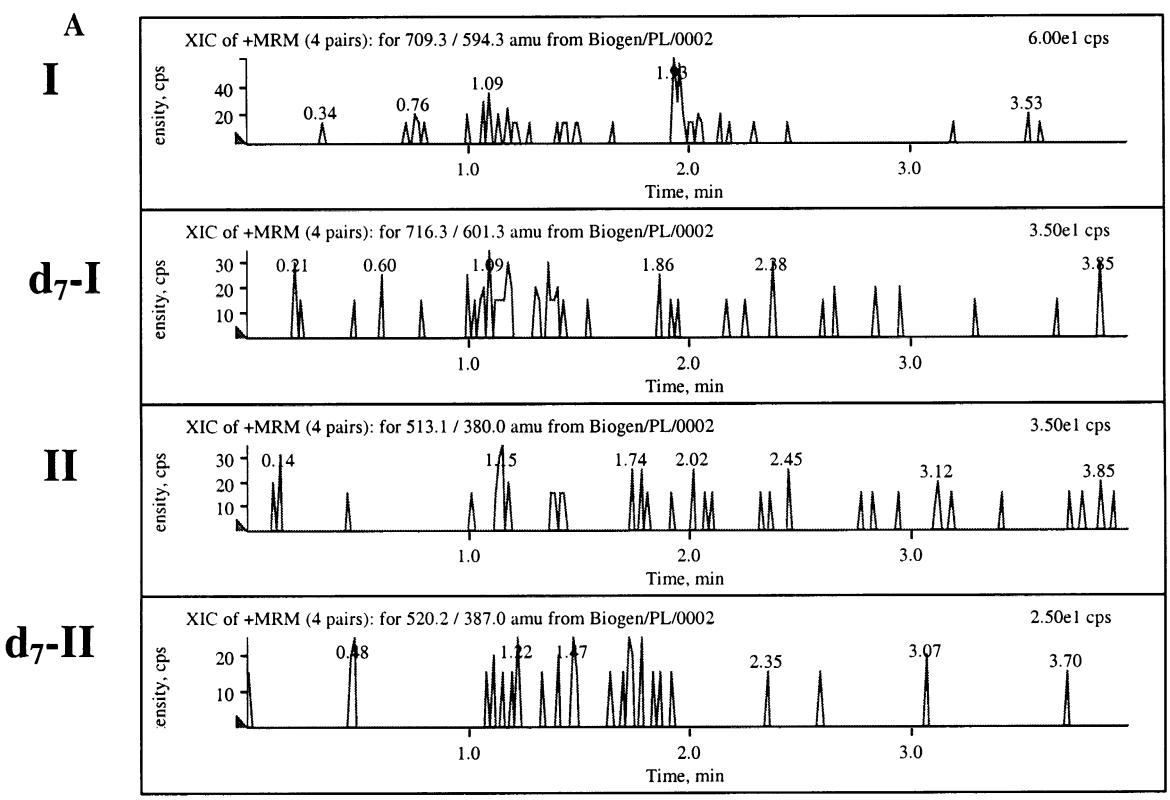


Fig. 5. Extracted ion chromatograms of human control urine blank. Chromatograms of control urine containing internal standards d7-**I** and d7-**II**. Chromatograms of **I** and **II** standards at 0.1 ng/ml with internal standards (channels for **I**, d7-**I**, **II** and d7-**II**).

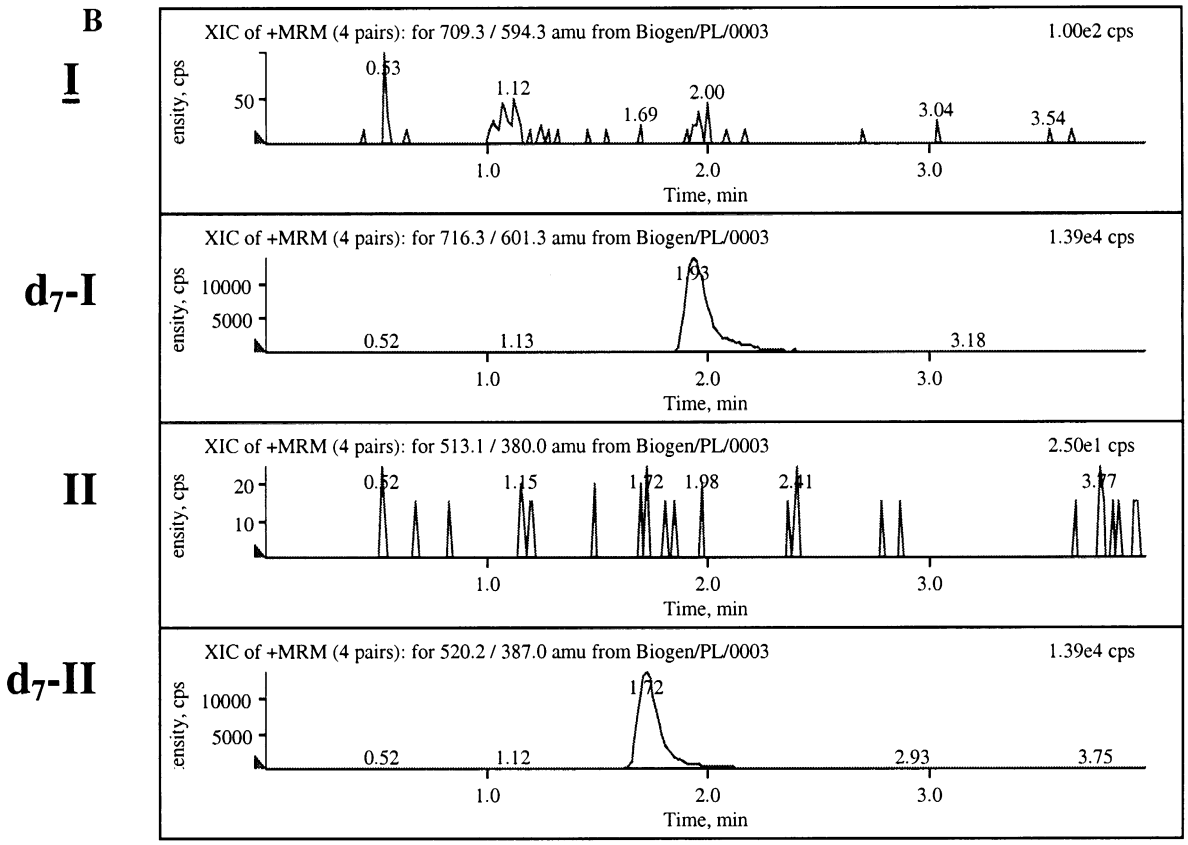


Fig. 5. (*Continued*)

metabolite and respective internal standards were used to determine peak-area ratios at each standard concentration of drug and metabolite. The calibration curves were constructed by weighted $(1/x^2)$ least-squares linear regression analysis of peak-area ratios versus nominal concentrations.

Freeze–thaw stability was evaluated at two concentrations. Samples were quantitated initially before freezing, and then frozen for a day at −70 °C, thawed and quantitated again. This cycle was repeated two more times.

QC samples were used to determine interday assay variability. A set of QC samples at low, medium and high concentrations ($n = 2$) were analyzed with a standard curve prepared daily with clinical samples. Acceptance of sample concentration data was based on QC results in the sample analysis run.

2.8. Study design

Healthy adult male subjects received rising multiple doses of drug by inhalation for 8 days. Blood was collected using EDTA as the anticoagulant and then centrifuged for plasma. Samples were collected before dosage, during dosage (5 min after start) and postdose from 0 to 24 h. Samples were stored at −70 °C. Urine samples were collected before dosage and after dosage with collection intervals over 24 h. The samples were mixed with 1.8 mg K_3EDTA and frozen at −70 °C.

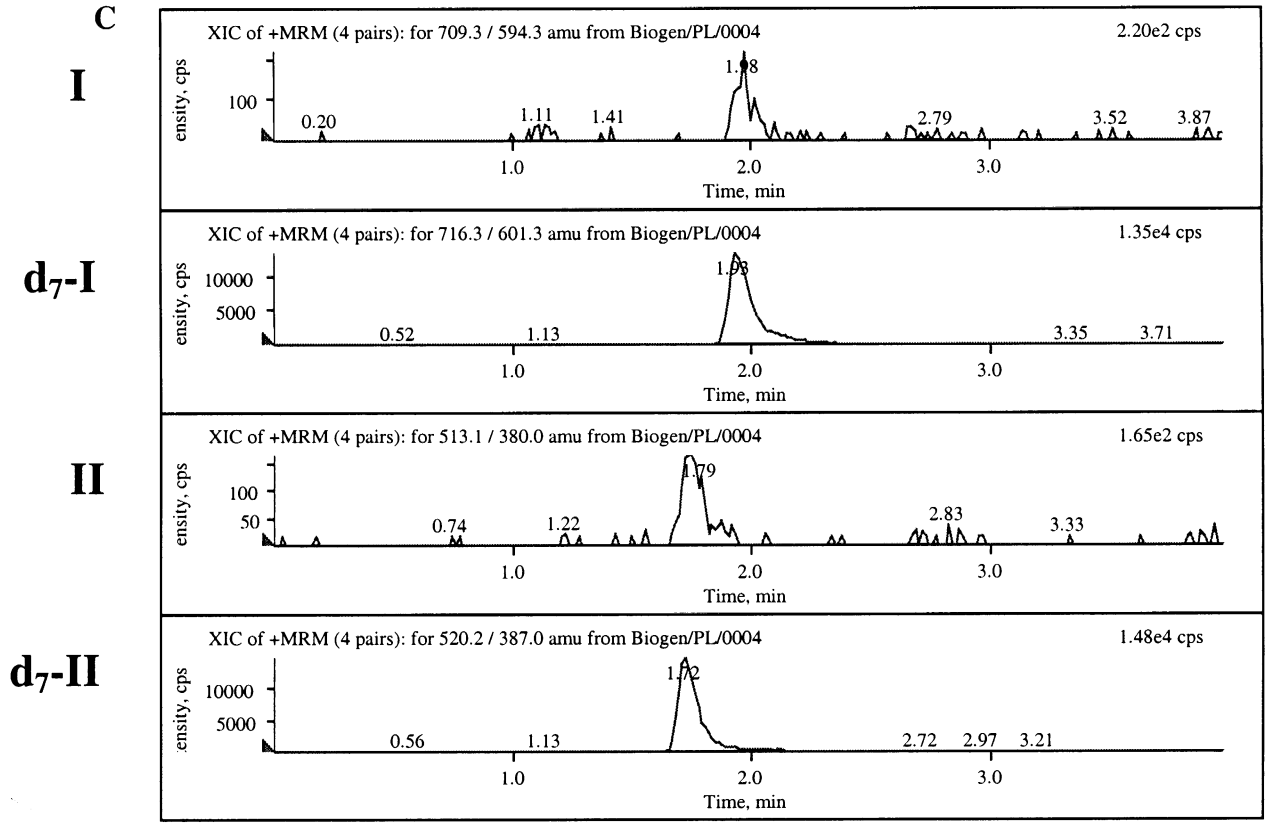


Fig. 5. (*Continued*)

Table 3
Interday precision and accuracy of **I** and **II**

Parameter	QC plasma concentration (ng/ml) of **I**				QC plasma concentration (ng/ml) of **II**			
For plasma QC samples[a]								
Nominal	0.50	2.5	20	50	0.50	2.5	20	50
Mean	0.50	2.44	20.0	50	0.511	2.43	20.6	49.8
SD	0.02	0.05	0.29	0.67	0.01	0.04	0.33	0.63
% CV	3.5	2.1	1.5	1.3	2.4	1.6	1.6	1.3
For urine QC samples[b]								
	QC urine concentration (ng/ml) of **I**				QC urine concentration (ng/ml) of **II**			
Nominal	0.50	4.5	25		0.50	4.5	25	
Mean	0.50	4.20	23.4		0.48	4.20	23.4	
SD	0.04	0.13	0.68		0.06	0.13	0.68	
% CV	7.7	3.2	2.9		12.7	3.2	2.9	

[a] $n = 9$ for 1.5 months at −70 °C.
[b] $n = 7$ for 2 months at −70 °C.

Table 4
Freeze–thaw stability for **I** and **II**

Freeze–thaw cycle[a]	Nominal concentration for **I** (ng/ml)				Nominal concentration for **II** (ng/ml)			
In plasma quality control samples stored at −70 °C								
	0.50	2.5	20	50	0.50	2.5	20	50
Cycle 1	0.49	2.41	20.1	50.6	0.5	2.39	19.7	49.2
Cycle 2	0.49	2.45	19.9	49.9	0.53	2.47	19.9	49.7
Cycle 3	0.51	2.46	19.7	50.0	0.51	2.43	20.1	50.2
In urine quality control samples stored at −70 °C								
Freeze–thaw cycle[b]	Nominal concentration for **I** (ng/ml)				Nominal concentration for **II** (ng/ml)			
	0.50	5.0	25		0.50	5.0	25	
Cycle 1	0.48 (0.02)[c]	4.42 (0.12)	24.3 (0.26)		0.45 (0.03)	4.75 (0.16)	25.2 (0.55)	
Cycle 2	0.41 (0.04)	4.09 (0.04)	22.5 (0.1)		0.51 (0.01)	4.44 (0.32)	22.9 (0.60)	
Cycle 3	0.47 (0.15)	3.85 (0.16)	21.9 (0.54)		0.45 (0.15)	3.96 (0.20)	20.7 (0.84)	

[a] Single determination.
[b] $n = 3$.
[c] () S.D.

3. Results

3.1. LC/MS/MS optimization

The Q1 mass spectra of **I** and **II** showed protonated molecular ions (MH^+) at m/z 709 and 513, respectively. The MRM product ion spectra for **I** and **II** showed high-abundance fragment ions at m/z 594 and 380 (Fig. 2) with fragmentation occurring at the peptide bonds. The product ions at m/z 594 and 380 were used to monitor **I** and **II**, respectively. The Q1 and product ion mass spectra for the internal standards d7-**I** and d7-**II** were similar to **I** and **II** but with seven added deuterium's. The product ions monitored were m/z 601 and 387, respectively.

3.2. Separation and specificity

Typical chromatograms of control human blank plasma, blank plasma with internal standards and plasma with **I**, **II**, d7-**I** and d7-**II** are shown in Fig. 3a–c. A representative plasma chromatogram from a subject dosed with **I** is shown in Fig. 4. Retention times of about 2.0 min or less were achieved for both **I** and **II** on a YMC C18 ODS-AM, 3 micron, 50×2.0 mm column. Separation of **I** and **II** was attained with $R_s = 1.23$. The specificity of the assay for **I**, **II** and deuterated internal standards in plasma was demonstrated by absence of interferences in the extracted chromatograms of a blank plasma sample from five separate sources of plasma; similarly, chromatrograms of a sample containing only the deuterated analogs demonstrated the negligible presence of **I** and **II** in their respective chromatograms. The metabolite **III** (*o*-methyl PUPA; MH^+ m/z 270 and shown in Fig. 2), was not detected under the conditions used for MRM or product ion monitoring. Representative chro-

Table 5
Assessment of ESI matrix effect by comparing the following: (a) peak areas of plasma standards spiked pre-extraction with **I** and **II** to (b) peak areas of reconstituted blank plasma extracts spiked post-extraction with neat **I** and **II**

Concentration (ng/ml)	Peak area ratio (extract/neat)	
	I	**II**
0.25	0.94	0.94
0.5	1.26	1.16
1	1.22	1.00
2.5	1.04	0.92
5	1.11	0.94
10	0.99	0.83
25	1.16	0.94
50	1.00	0.82
100	1.05	0.88
Mean	1.09	0.94

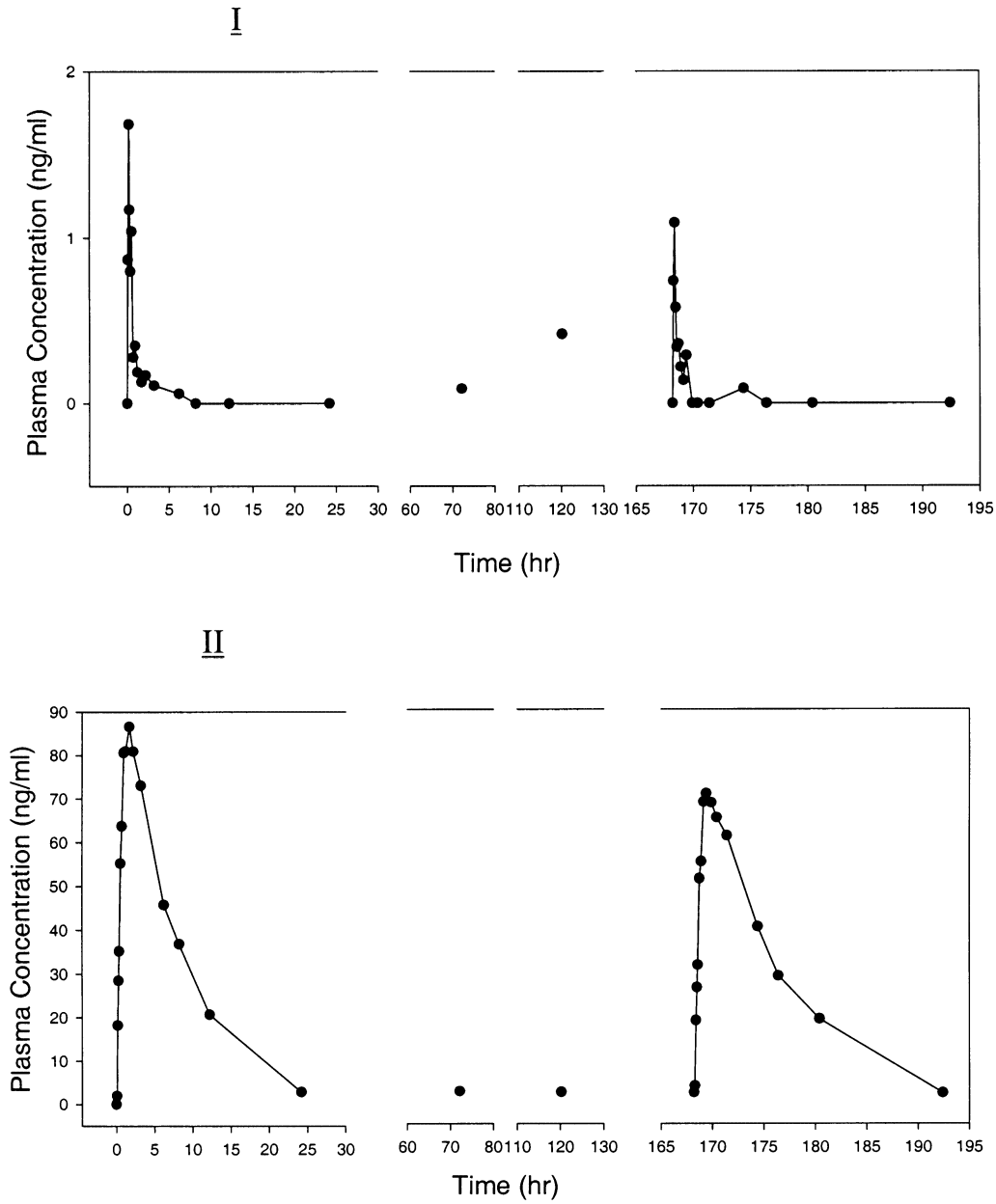


Fig. 6. Individual plasma profiles of **I** and metabolite **II** from a healthy male subject receiving **I** at 25 mg q.d. by inhalation for 8 days.

matograms of human urine samples are given in Figs. 3–5.

3.3. *Sensitivity, linearity, precision and accuracy*

Representative calibration curves for **I** and **II** in plasma have a correlation coefficient > 0.999 and fit a linear regression curve of $y = 0.002 + 0.108x$ and $y = 0.001 + 0.116x$, respectively. The assay procedures were developed to obtain a LLOQ at 0.1 ng/ml for **I** and **II** by extracting 1.0 ml of sample. Table 1 presents the accuracy and preci-

sion of five standards curves of **I** and **II** in plasma. Accuracy and precision for **I** ranged from 98 to 102% of nominal and from 0.84 to 13.5 percent coefficient of variation (%CV) at the LLOQ, respectively; similarly for metabolite, 99–101% and 1.4–9.0%, respectively. Data for urine containing EDTA is summarized in Table 2.

3.4. Extraction recovery

Extraction recovery is calculated by comparing the peak area ratios of **I** in plasma samples with the peak area ratios of **I** added to blank plasma extract. The recoveries for **I** and **II** from 0.1 to 100 ng/ml are listed in Table 1 and show an overall mean percent recovery of 63.7% for **I** and 64.9% for **II**. Similarly for urine, mean recovery was 61.4% for **I** and 56.7% for **II**.

3.5. Matrix effect

In MRM the peak areas over the concentration range of 0.25–100 ng/ml were compared with assess matrix effect as follows: (a) neat standards injected into the LC/MS/MS were compared with (b) extracted blank plasmas reconstituted with the same neat standard. This comparison (Table 5) shows no significant matrix effect using electrospray ionization (ESI) for plasma. Similar comparisons were made for internal standard in plasma and for **I** and IS in urine showing no significant matrix effect (data for urine not shown). Further support for these general findings are intraday precision and accuracy data for plasma and urine (Tables 1 and 2). 'Tight' precision and accuracy data from five different sources of plasma and urine also support no significant matrix effect.

3.6. Stability

Interday stability of QC samples in Table 3 show no degradation of **I** and **II** at -70 °C over 1.5 months in EDTA plasma and with no change in urine samples over 2 months at -70 °C. Freeze–thaw stability for plasma and urine QC samples stored at -70 °C are given in Table 4. As with QC stability, frozen and thawed plasma samples did not show any loss of **I** and **II** in EDTA plasma over three successive freeze–thaw cycles; freeze–thaw stability of **I** and **II** in urine decreased between 10 and 20% with the third freeze–thaw cycle.

3.7. Application

Plasma profiles for **I** and metabolite from a healthy male subject administered **I** by inhalation at 25 mg daily for 8 days is shown in Fig. 6. The plasma concentrations of **I** reach a maximum at the end of inhalation (12 min) and fall below the LLOQ between 0.5 and 6.0 h. For Day 8, drug profiles were similar to day 1. For the metabolite **II**, plasma concentrations reached a mean maximum concentration on day 1 at 101 ± 59 ng/ml between 20 and 90 min and similar profiles were observed on day 8.

For urine samples from a subject administered 25 mg daily for 8 days, drug and metabolite were quantitated at concentration ranges from the LLOQ to 0.005 and 3.9 μg/ml, respectively (urine samples were diluted with control urine in some cases).

4. Discussion

The liquid–liquid extraction, LC/MS/MS method described for **I** and **II** from plasma and urine may have application to small acidic and neutral peptides containing hydrophobic side chains. This method may also be applied to small basic peptides by using basic extraction conditions.

Key points for high recovery of peptides **I** and **II** with minimal interference from matrix are the following: (1) constant methanol/aqueous ratio in plasma standards, quality controls and samples for reproducible recovery, (2) pentane–ethyl acetate extraction solvent (90:10 v/v) for optimal recovery, (3) silanized tubes to prevent sticky peptides from adsorbing to charged and hydrophobic surfaces, especially during the dry down step, (4) cold precipitation of proteins and other insoluble macromolecules in the extract for a clear supernatant for injection onto the LC/MS/MS. These points are discussed below.

Having constant and low methanol–plasma ratio in plasma standards and quality controls allowed reproducible recovery of **I** and **II** in plasma. Variations in organic content from 5 to 20% greatly effected extraction efficiency and reproducibility whereas with 5% organic reproducibility was achieved. As a result concentrated QC samples and internal standard stock solutions were prepared in mobile phase and diluted with control plasma for standards and QC samples so that the final concentration of methanol in the plasma was $< 4\%$.

The initial use of MTBE or MTBE-IPA (90:10 v/v) for sample extraction resulted in a pronounced emulsion between the aqueous and organic layers with $< 50\%$ recovery of both analytes. Extraction with pentane–ethyl acetate (90:10 v/v) allowed clean separation of the organic and aqueous layers and about 60–65% recovery of peptides from plasma and urine.

When the pentane-ethyl acetate mixture was taken to dryness **I** and **II** adsorbed strongly to untreated glass, polypropylene, surface deactivated glass, and to a certain extent to aqueous silanized surface-deactivated glass. Organic solution silanization in toluene was the most effective remedy to prevent sticking to glass.

Reconstitution of the plasma extract into a variety of solvents compatible with LC/MS/MS resulted in a cloudy solution containing insoluble components (most likely proteins, lipoproteins, glycoproteins and cholesterol) that occluded the 2 mm analytical HPLC column used for the assay. The initial solution was a reconstitution of the organic extract into DMSO–water (1:1 v/v) and the use of a Prospekt on-line solid phase extraction system before chromatography to further 'clean-up' and filter the plasma extract. For this approach, the extract containing DMSO–water was loaded onto a C18 cartridge, washed with water and then eluted in mobile phase containing methanol–ammonium acetate (5 mM, pH 3.0) (65:45 v/v). However, the large dead volume in the Prospekt sample loop and cartridge resulted in excessive peak broadening with the 50×2 mm analytical column and this diminished assay sensitivity. This broadening did not allow the required sensitivity of 0.1 ng/ml to be achieved using the Prospekt (LLOQ with Prospekt was 0.5 ng/ml).

As the end result, the Prospekt was eliminated and the dry extract was directly reconstituted into methanol–water (1:1 v/v) and frozen overnight at -20 °C allowing two layers to form. The following day the cloudy layers of extract were thawed and centrifuged, and the clear supernatant injected onto the LC/MS/MS. Freezing the layers overnight was key to obtaining a clear extract as cold methanol–water promoted complete precipitation of protein and other insoluble macromolecules at the bottom of the centrifuge tube and allowed high recovery of both acidic and neutral peptides in the supernatant.

5. Conclusions

This method for quantitation of acidic and neutral peptides in the pg/ml range from plasma and urine may have wide utility and uses a unique approach of extraction of peptides from a biological matrix into a polar organic solvent and then subsequent precipitation of the reconstituted extract for further purification. The method is free from matrix effect, is rugged, reproducible, and accurate.

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