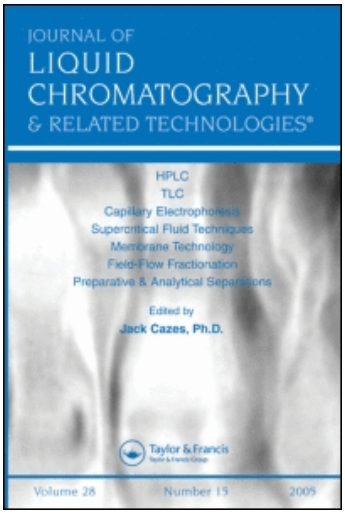

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Simpson, Richard C. , Patti, Adrienne L. and Matuszewski, Bogdan K.(2006) 'Determination of a Selective Neuropeptide Y5 Receptor Antagonist in Human Plasma and Urine by HPLC with Tandem Mass Spectrometric Detection', Journal of Liquid Chromatography & Related Technologies, 29: 2, 273 — 291
To link to this Article: DOI: 10.1080/10826070500416668
URL: http://dx.doi.org/10.1080/10826070500416668

Journal of Liquid Chromatography & Related Technologies®, 29: 273–291, 2006

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070500416668

Determination of a Selective Neuropeptide Y5 Receptor Antagonist in Human Plasma and Urine by HPLC with Tandem Mass Spectrometric Detection

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Abstract: An automated 96-well solid phase extraction method was developed for the isolation of a selective neuropeptide Y5 receptor antagonist and internal standard from human plasma and urine. Following isolation, the analyte was selectively quantified using a reverse phase HPLC system coupled with atmospheric pressure chemical ionization (APCI) mass spectrometry operated in the positive ionization MRM mode. Based upon the peak area ratio (analyte:internal standard) vs standard concentrations, the analyte was quantified over a concentration range of 2–1000 ng/mL. The absolute and relative matrix effects from different sources of sample matrices on the ionization efficiency were examined and the absence of these effects was confirmed. The extraction efficiency was found to be approximately 90% and reproducible in both matrices. Assay validation results, including intra-day precision and accuracy values, are presented. The validated assay has been used to support several pharmacokinetic studies, and some representative data are presented.

Keywords: HPLC-MS/MS, Quantitative, Bioanalytical, Plasma, Urine

INTRODUCTION

Obesity is an increasingly common disease in present day society resulting from a chronic imbalance between energy intake and energy expenditure.

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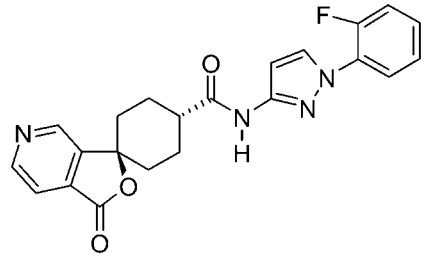
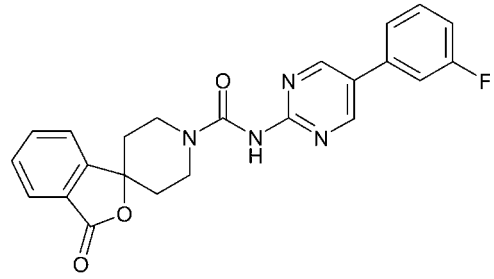
New recommendations have been made for the treatment of this disease based on evidence that relates obesity to both increased mortality as well as increased risk for several other diseases including diabetes, hypertension, etc.[1] Recent discoveries have shown that neuropeptide Y (NPY), a highly conserved 36-amino-acid peptide, has appetite stimulating effects in preclinical species.[2,3] In 1996, researchers at Synaptic reported the isolation of the Y5 receptor, which was proposed to play a dominant role in mediating NPY-induced food intake.[4] Although the relevance of Y5 on human appetite control remains unknown, it is currently postulated that the administration of NPY Y5 antagonists may decrease human appetite while increasing the metabolic rate, resulting in lower incidents of obesity.

Compound **I** (*trans*-N-[1-(2-fluorophenyl)-1H-pyrazol-3-yl]-1′-oxospiro [cyclohexane-1,3′(1′H)-furo[3,4-C]pyridine]-4-carboxamide (Figure 1), is a potent, orally available, and reversible antagonist specific to the NPY Y5 receptor, and represents a potential new therapy for treating human obesity syndromes. A highly selective and sensitive assay was needed for the quantitation of **I** to support human pharmacokinetic studies. A wide linear response range, including a low ng/mL limit of quantification, was required due to the significant spread in dosing regimens used in the initial studies. Previous studies have shown the use of HPLC coupled with atmospheric-pressure chemical ionization (APCI) mass spectrometry (MS)[5–9] or tandem mass spectrometry (MS/MS),[10,11] to be highly successful for the selective



Compound I



Compound II (Int. Std.)

Figure 1. Chemical structures of **I** and **II** (Internal Standard).

quantification of trace concentrations of analytes in a variety of biological matrices. Numerous such assays have been developed within our group, some of which are presented as examples in references.[12–19] Because of the high selectivity/sensitivity required, the use of HPLC-MS/MS employing an APCI interface was evaluated as the analytical method of choice. Partially automated 96-well format solid phase extraction sample preparation has been successfully employed in previously developed methods[20,21] and was, therefore, incorporated into the present method as well. The present paper describes the methodology and performance characteristics of the validated HPLC-MS/MS assay for compound **I**, and the application of the assay to support human pharmacokinetic studies.

EXPERIMENTAL

Materials

Compound **I** and compound **II** (internal standard; Figure 1) were provided by the Compound Repository of Merck Research Laboratories (West Point, PA, USA). All solvents and reagents were of HPLC or analytical reagent grade and were purchased from Fisher Scientific (Fair Lawn, NJ, USA) or EM Science (Philadelphia, PA, USA). Drug free heparinized human plasma was obtained from Biological Specialties Corp. (Colmar, PA, USA). Bovine Serum Albumin (35%) was obtained from Sigma (Saint Louis, MO, USA). Control urine was obtained from Drug Metabolism personnel (West Point, PA, USA). Nitrogen (99.999%) was provided by West Point Cryogenics (West Point, PA, USA). Ansys SPEC® C8 96-well solid phase extraction plates were purchased from Ansys Technologies (Lake Forest, CA, USA). Plastic sealing mats for the polypropylene 96-well collection plates were obtained from Marsh Biomedical Products (Rochester, NY, USA).

Instrumentation

The HPLC/MS/MS system consisted of an Applied Biosystems/MDS SCIEX (Foster City, CA, USA) API 4000 tandem mass spectrometer equipped with a heated nebulizer interface, a Perkin Elmer (Norwalk, CT, USA) Series 200 quaternary pump, and a Varian (Palo Alto, CA, USA) Pro Star, Model 430 autosampler. Data was processed on a Dell Pentium 4 computer using Analyst software (Sciex).

Chromatographic Conditions

The mobile phase consisted of 55/45 (v/v) 10 mM ammonium acetate buffer (adjusted to pH 4 with acetic acid)/acetonitrile delivered at a flow rate of

1 mL/min. Chromatography was performed on a Waters Symmetry Shield RP_8 50 × 4.6 mm 3.5 μm analytical column (Waters Corporation Milford, MA, US) coupled to a Waters Symmetry Shield RP_8 guard column (20 × 3.9 mm 5 μm). The retention times for **I** and **II** were approximately 2.4 and 2.8 min, respectively.

HPLC/MS/MS Conditions

A PE Sciex API 4000 triple quadrupole mass spectrometer, operated in the positive ionization mode, was interfaced with a heated nebulizer probe to the HPLC system. Gas phase chemical ionization was effected by a corona discharge needle (3 μA). The heated nebulizer probe was maintained at 600°C. The nebulizing gas (N_2) was set to a pressure of 90 psi. The declustering potential was set at +85 V. The dwell time for each ion transition was 400 msec. The mass spectrometer was programmed to admit the protonated molecules $[M + H]^+$ at m/z 407 (**I**) and m/z 419 (**II**) via the first quadrupole mass filter (Q1), with collision-induced fragmentation in Q2 (collision energy of 35 eV; N_2 collision gas at a cell pressure of 4.7×10^{-3} torr), and monitoring the product ions via Q3 at m/z 178 (**I**) and m/z 204 (**II**). Peak-area ratios obtained from multiple reaction monitoring (MRM) of the analyte **I** (m/z 407 → 178) to internal standard **II** (m/z 419 → 204) were utilized for the construction of calibration curves, using weighted ($1/x^2$) linear least square regression of the analyte concentrations and the measured peak area ratios.

Standard Solutions

A standard stock solution of **I** (100 μg/mL) was prepared in 1:1 water:acetonitrile. This solution was further diluted to give a series of working standards having concentrations of 10, 5, 2, 1, 0.5, 0.2, 0.1, 0.05, and 0.02 μg/mL. The internal standard **II** was also prepared as a stock solution (100 μg/mL) in acetonitrile. A working solution of 1 μg/mL was prepared by dilution and used for all analyses.

The human urine (both control and clinical samples) was treated with sufficient volume of the 35% BSA reagent to give a final BSA concentration of 0.5% (v/v) in the urine prior to preparation of the urine calibration standards, as well as before freezing the urine quality control samples or clinical urine samples.

Quality control (QC) samples in control human plasma at nominal concentrations of 7.5, 75, and 750 ng/mL were prepared from a separate weighing of **I**. A series of QC samples was similarly prepared in control human urine containing 0.5% bovine serum albumin. Aliquots (0.7 mL) of these samples were placed in 1.8 mL polypropylene tubes, stored at −20°C, and analyzed daily with clinical samples. The calculated concentrations of

the QC samples were compared on a day-to-day basis to assess the inter-day assay performance. The QC samples were also analyzed for bench-top and freeze thaw stability.

Sample Preparation

A 250 μL aliquot of sample was pipetted into a 13 × 85 mm polypropylene test tube, followed by addition of 25 μL of the appropriate working solution of **I** (or 1 : 1 water : acetonitrile for clinical samples) and 840 μL 0.1M phosphate buffer (pH2). The samples were briefly vortexed, then 50 μL of the working solution of internal standard **II** (yielding a concentration of 200 ng/mL) was added to each tube (excluding the double blank), followed by an additional vortex mix. An 1100 μL aliquot of the resulting solution was transferred to a 1.2 mL deep well collection plate. Using a Tomtec Quadra 320 SPE workstation, a 1 mL aliquot of the resulting solution was transferred to the 96-well Ansys SPEC C8 solid phase extraction plate, which had been conditioned with 1 mL of acetonitrile, followed by 1 mL of water. The sample solution was drawn through the plate using vacuum. The Tomtec was then used to wash the extraction plate wells with 0.5 mL of water, followed by 0.5 mL of water/acetonitrile (80 : 20 v/v). The plate was removed from the workstation and centrifuged for 5 min at 188 × g to remove any residual liquid from the plate. The extraction plate was then placed onto a fresh 1.2 mL deep-well 96-well collection plate, repositioned onto the Tomtec workstation, and 400 μL of water/acetonitrile (1 : 1; v/v) were added to each well. The assembly was centrifuged for 5 min at 161 × g to elute the analyte and internal standard into the collection plate. The collection plate was then sealed and gently vortexed for 1 minute. The sealed collection plate was placed in the HPLC autosampler, which was programmed to make 5 μL injections directly from the individual wells of the collection plate.

Precision, Accuracy, Recovery, and Selectivity

The assay precision was determined by replicate analyses of different lots ($n = 5$) of control matrix fortified with compound **I** at the concentrations used to construct calibration curves (0, 2, 5, 10, 20, 50, 100, 200, 500, and 1000 ng/mL). The linearity of the assay was verified for the composite curve as well as for each individual sample matrix lot. (Note: each individual curve is indicative of a different lot of matrix). The chromatographic peak area ratios of product ions for drug and internal standard (drug:internal standard) were obtained, weighted by a factor of $1/x^2$ (based on analysis of residuals), and plotted versus the nominal analyte concentrations. Linear regression analysis gave a calibration curve that was used to calculate

unknown sample concentrations. The standard curve was generated daily, along with quality control and unknown samples. The accuracy of the method was determined as [(mean found concentration)/(spiked nominal concentration)] × 100. Assay selectivity was determined by running blank human control samples as well as clinical subjects' pre-dose samples. No endogenous interferences were observed in either source. The recovery at each standard concentration was determined by comparing the peak area of compounds **I** and **II** extracted from control matrix to that obtained from extracts of blank control matrix, which were spiked with compounds **I** and **II** following the solid phase extraction procedure.

RESULTS AND DISCUSSION

The positive ion full-scan mass spectra (Q1) of **I** and **II** indicated the presence of the protonated molecules $[M + H]^+$ as the predominant ion for each compound, with m/z values of 407 and 419 for **I** and **II**, respectively. The corresponding product ion mass spectra for **I** and **II**, obtained from the $[M + H]^+$ precursor ions, are shown in Figure 2. Multiple reaction monitoring of the precursor → product ion transitions at m/z 407 → 178 for **I** and 419 → 204 for **II** allowed sensitive and selective detection of the analyte and internal standard.

Chromatographic separation was tested on a variety of short (30–50 × 4.6 mm) columns. Such small column dimensions tend to yield short run times without sacrificing adequate retention (i.e., capacity factor [k′]). After extensive column and mobile phase screening, the Waters Symmetry Shield RP_8 column was found to provide the best overall chromatographic performance, including acceptable peak shapes for the analyte and internal standard, and sufficient retention (capacity factors, k′) of approximately 3.5 and 2.8, for **I** and **II**, respectively. In addition, the selected column also gave chromatographic separation of a series of possible metabolites from both the analyte and internal standard, thus avoiding any potential interference issues.

A primary factor in designing any clinical assay is to incorporate the ability to process a large number of samples on a daily basis. As a result, the 96-well SPE format was chosen as the sample preparation technique. Solid phase extraction was preferred over liquid–liquid extraction because it can more easily be automated using a variety of readily available robotic systems. Aside from the quantity of samples extracted, robotic systems also allow for the consistent exposure of the samples to the extraction material. Using an automated extraction procedure on the Tomtec ensures that all of the samples are introduced to the solid phase extraction material at essentially the same time, which typically is not possible when using manual sample preparation procedures.

As described in the experimental section, the method was validated in both human plasma and BSA-treated urine over the analyte concentration

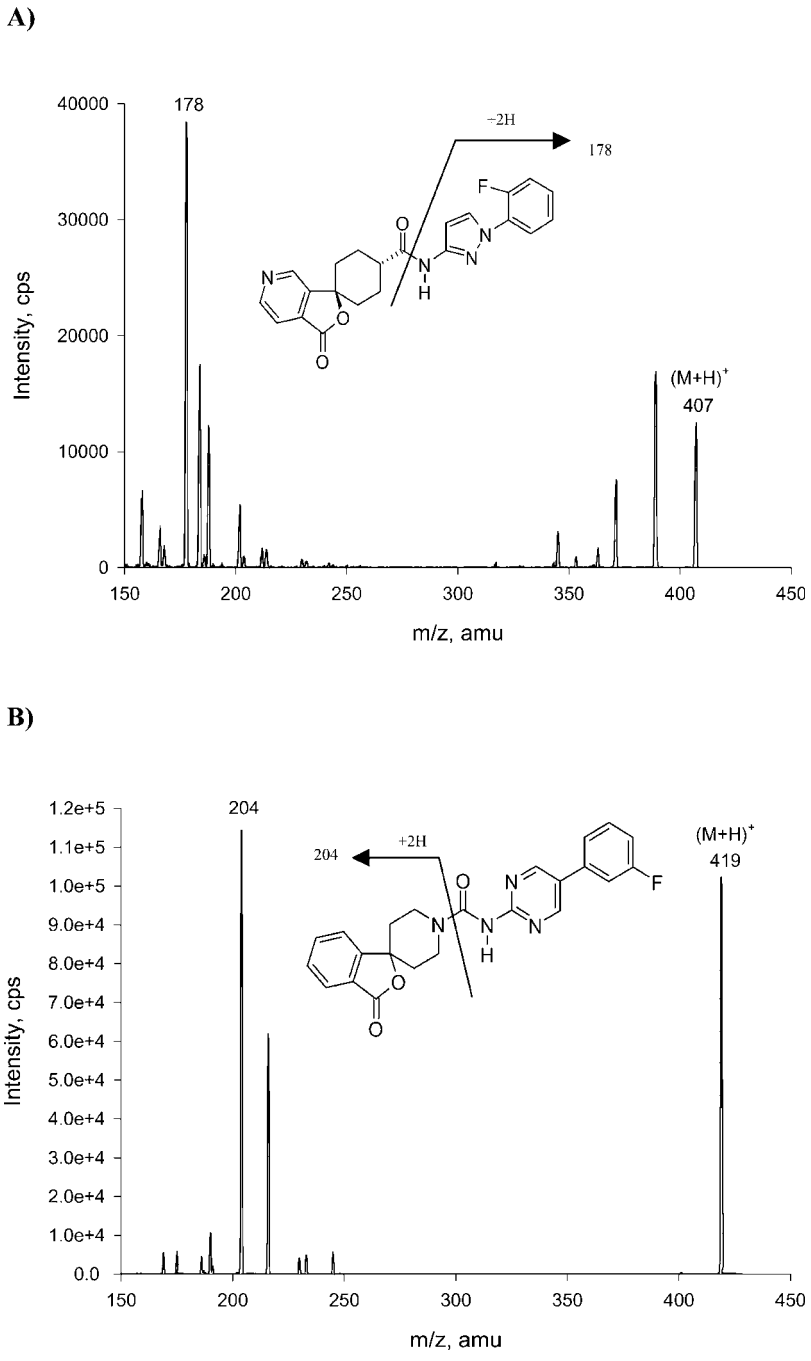


Figure 2. Product ion mass spectra for **I** (A) and **II** (B) under the MS/MS conditions used in the multiple reaction monitoring (MRM) mode.

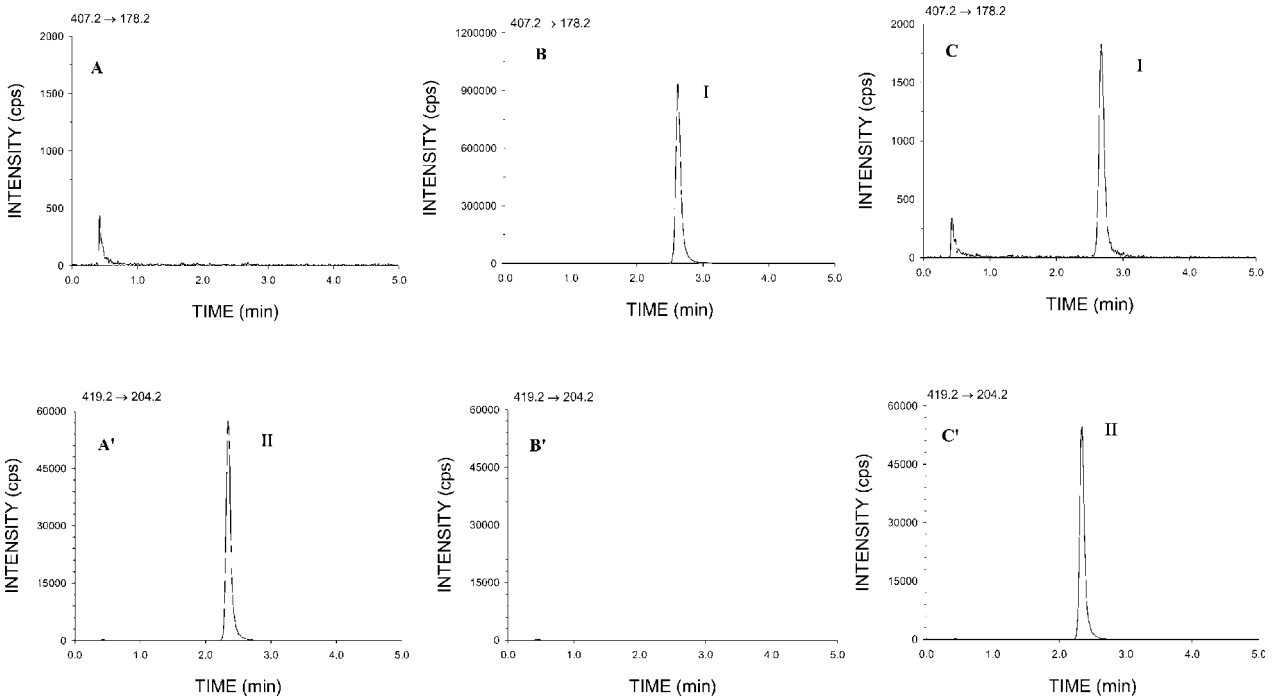


Figure 3. Representative extracted ion chromatograms demonstrating assay selectivity and sensitivity. Chromatograms of control human plasma extracts were obtained by multiple reaction monitoring at m/z 407 → 178 for **I** and m/z 419 → 204 for **II** (Internal Standard). (**A**, **A′**) control plasma spiked with 200 ng/mL of **II**. No response from internal standard (**II**) observed in analyte channel (**A**). (**B**, **B′**) control plasma spiked with 1000 ng/mL of **I** only. No response from the highest concentration of analyte (**I**) observed in internal standard channel (**B′**). (**C**, **C′**) control plasma spiked with 2 ng/mL of **I** (assay LOQ) and 200 ng/mL of **II**.

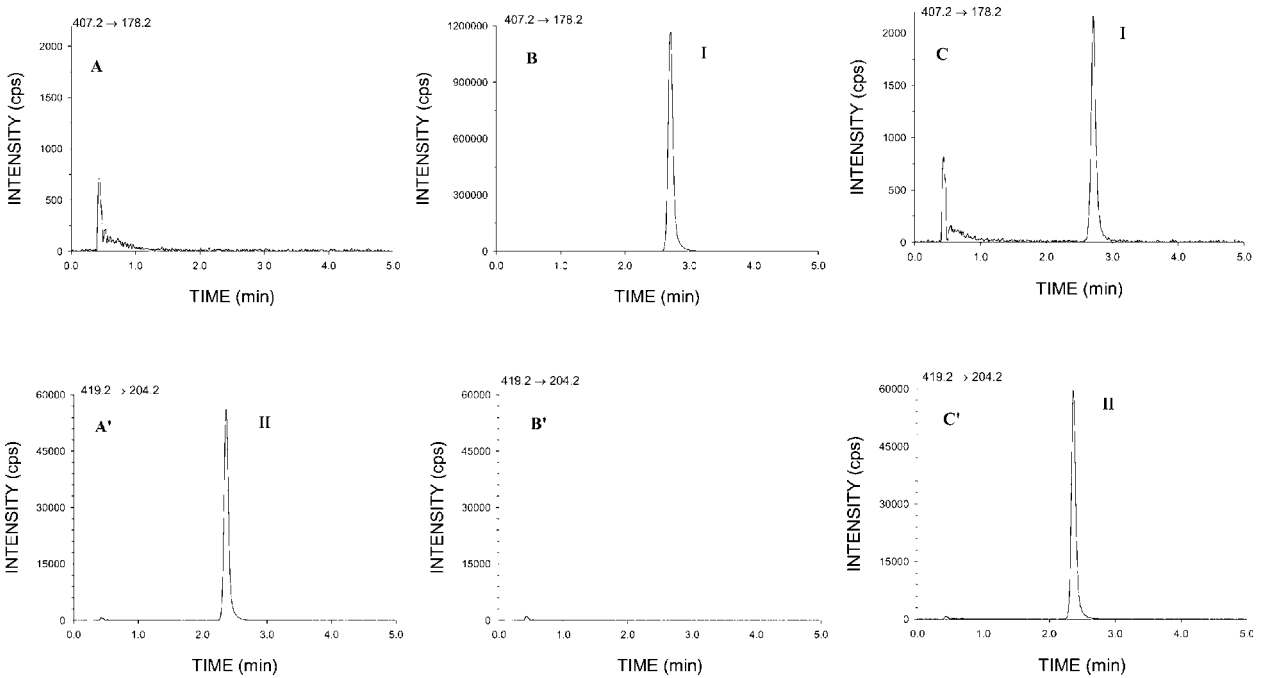


Figure 4. Representative extracted ion chromatograms demonstrating assay selectivity and sensitivity. Chromatograms of control human BSA-treated urine extracts were obtained by multiple reaction monitoring at m/z 407 → 178 for **I** and m/z 419 → 204 for **II** (Internal Standard). (**A**, **A**′) control BSA-treated urine spiked with 200 ng/mL of **II**. No response from internal standard (**II**) observed in analyte channel (**A**). (**B**, **B**′) control BSA-treated urine spiked with 1000 ng/mL of **I** only. No response from the highest concentration of analyte (**I**) observed in internal standard channel (**B**′). (**C**, **C**′) control BSA-treated urine spiked with 2 ng/mL of **I** (assay LOQ) and 200 ng/mL of **II**.

range of 2 to 1000 ng/mL. The limit of quantification (LOQ) was defined as the lowest concentration on the standard curve for which the precision, expressed as the coefficient of variation (C.V.%) was less then 15% and the associated accuracy was $100 \pm 15\%$. Using the experimental conditions described in this paper, the assay LOQ (2 ng/mL) corresponded to approximately 5 pg of **I** injected on-column. Representative chromatograms for spiked human control plasma and BSA-treated urine are found in Figures 3 and 4, respectively. Assay validation statistics, including accuracy and precision, are presented in Tables 1 and 2 for plasma and urine. The data show that the assays are both accurate and reproducible over the entire concentration range. The intraday precision of the assays, as measured by the coefficient of variation (C.V.%) was ≤ 6.1 for all points on the calibration curves. The mean assay accuracy was found to be within 4.1% of nominal concentrations for all standards.

Analyte recovery studies were also conducted. The analyte recovery was determined by comparing the ratio of analyte peak areas obtained from control samples spiked prior to extraction to the peak areas obtained from blank samples from the same matrix lots, which were extracted and subsequently spiked after the solid phase extraction. This approach cancels any effects the extract matrix may have on ionization efficiencies in different biofluid lots, and reflects only the efficiency of the solid phase extraction process.

Table 1. Precision and accuracy of replicate analyses ($n = 5$) of **I** in human plasma[a]

Concentration (ng/mL)		Precision[c]	Accuracy[d]
Nominal	Mean[b]	C.V.%	(%)
2.0	2.0	6.0	100.0
5.0	5.0	5.6	100.0
10.0	10.1	5.5	101.0
20.0	19.2	4.1	96.2
50.0	49.5	6.1	99.0
100.0	99.3	3.8	99.3
200.0	197.4	4.0	98.7
500.0	509.5	3.7	101.9
1000.0	1035.1	4.1	103.5

[a]Data determined using control plasma from five different sources.

[b]Mean concentrations calculated from the weighted linear least-squares regression curve constructed using all five replicate values at each concentration.

[c]Expressed as coefficient of variation (C.V.%).

[d]Expressed as [(mean calculated concentration)/(nominal concentration) $\times$ 100].

Table 2. Precision and accuracy of replicate analyses ($n = 5$) of **I** in human BSA-treated urine[a]

Concentration (ng/mL)		Precision[c]	Accuracy[d]
Nominal	Mean[b]	(C.V.%)	(%)
2.0	2.0	3.8	101.0
5.0	5.0	1.3	100.0
10.0	9.9	3.5	99.4
20.0	19.2	1.7	95.9
50.0	49.9	1.9	99.8
100.0	100.9	2.4	100.9
200.0	192.8	2.7	96.4
500.0	578.0	2.2	103.6
1000.0	1037.4	3.0	103.7

[a]Data determined using control plasma from five different sources.

[b]Mean concentrations calculated from the weighted linear least-squares regression curve constructed using all five replicate values at each concentration.

[c]Expressed as coefficient of variation (C.V.%).

[d]Expressed as [(mean calculated concentration)/(nominal concentration) × 100].

The results are summarized in Tables 3 and 4. The mean recovery of **I** in plasma was 93% and 95% for **II** (at its working concentration of 200 ng/mL). The mean recovery of **I** in BSA-treated urine was 87% and 86% for **II** (at its working concentration of 200 ng/mL). Initial studies found that urine

Table 3. Recovery of **I** and **II** from human plasma

Nominal concentration of **I** (ng/mL)	Mean[a,b] recovery of **I** (%)	Mean[a,b,d] recovery of **II** (%)
5	94.4	90.6
50	91.4	96.1
500	91.5	97.1
Mean recovery (%)	92.5	94.6
Precision[c] (%)	1.8	3.7

[a]Recovery calculated as [(Peak area of sample spiked pre-extraction)/(Peak area of sample spiked post-extraction)] × 100, $n = 5$ different lots of plasma.

[b]Mean of replicates of 5 different lots of plasma.

[c]Expressed as coefficient of variation (C.V.%).

[d]Concentration of **II** was 200 ng/mL.

Table 4. Recovery of **I** and **II** from human BSA-treated urine

Nominal concentration of **I** (ng/mL)	Mean[a,b] recovery of **I** (%)	Mean[a,b,d] recovery of **II** (%)
5	88.1	86.6
50	88.2	86.8
500	84.4	83.8
Mean recovery (%)	87.0	85.7
Precision[c] (%)	2.5	2.0

[a]Recovery calculated as [(Peak area of sample spiked pre-extraction)/(Peak area of sample spiked post-extraction)] × 100, $n = 5$ different lots of urine.

[b]Mean of replicates of 5 different lots of BSA-treated urine.

[c]Expressed as coefficient of variation (C.V.%).

[d]Concentration of **II** was 200 ng/mL.

samples, which were spiked with **I** but not pre-treated with BSA, yielded poor recovery of **I** after undergoing a freeze/thaw cycle. Use of urine pre-treated with BSA significantly improved the recovery of **I** from the urine samples. Recovery was found not to vary significantly over the concentration range examined, indicating that **I** and **II** were extracted consistently over the quantitation range of the assay.

An assessment was made of potential matrix effects on the ionization efficiency, and subsequent peak areas, for **I** and **II**. The presence of any

Table 5. Assessment of matrix effects on ionization of **I** and **II** in 5 different lots of human plasma[a]

Nominal concentration of **I** in plasma (ng/mL)	Mean absolute matrix effect[b] [%C.V.] ($n = 5$)	
	I	**II** (200 ng/mL)
5	1.0 [4.6]	1.0 [8.6]
50	1.1 [4.6]	1.0 [11.0]
500	1.0 [3.4]	1.0 [5.5]
Mean absolute matrix effect[b]	1.1	1.0

[a]Spiked after extraction of control plasma.

[b]Absolute matrix effect expressed as the ratio of the mean peak areas of an analyte spiked into control plasma post-extraction to the mean peak areas of the same analyte neat reference standards. A value >1 indicates ionization enhancement, and a value <1 indicates ionization suppression.

Table 6. Assessment of matrix effects on ionization of **I** and **II** in 5 different lots of BSA-treated urine[a]

Nominal concentration of **I** in plasma (ng/mL)	Mean absolute matrix effect[b] [%C.V.] ($n = 5$)	
	I	**II** (200 ng/mL)
5	1.0 [3.6]	1.0 [2.4]
50	1.0 [1.9]	1.0 [2.3]
500	1.0 [3.5]	1.0 [3.5]
Mean absolute matrix effect[b]	1.0	1.0

[a]Spiked after extraction of control BSA-treated urine.

[b]Absolute matrix effect expressed as the ratio of the mean peak areas of an analyte spiked into control plasma post-extraction to the mean peak areas of the same analyte neat reference standards. A value >1 indicates ionization enhancement, and a value <1 indicates ionization suppression.

matrix effect may have an adverse impact on reliable analyte quantification, especially when a chemical analog rather than a stable isotope labeled compound is used as the internal standard.[12,14,18,22] Since the present assay utilized an analog as the internal standard, both absolute and relative matrix effects on the ionization efficiency for both **I** and **II** were investigated.[22]

Table 7. Slopes of standard lines for the determination of **I** in 5 different lots of human plasma and BSA-treated urine

Plasma/urine lot number	Slope	
	Plasma	Urine
1	0.0195	0.0189
2	0.0188	0.0183
3	0.0191	0.0168
4	0.0187	0.0174
5	0.0207	0.0178
Mean	0.0194	0.0178
Std dev.[a]	0.00081	0.0008
Precision[b] (%)	4.2	4.5

[a]Standard deviation.

[b]Coefficient of variation; $n = 5$.

Table 8. Analysis of plasma quality control samples spiked with **I**

	Concentration (ng/mL)		
	Low QC	Mid QC	High QC
Nominal concentration	7.5	75.0	750.0
Initial experimentally measured concentrations	7.7	73.3	787.0
	7.9	73.0	779.4
	7.5	72.3	768.7
	7.5	72.6	791.3
	7.1	75.0	788.6
Initial mean ($n = 5$) assayed concentration[a]	7.5	73.2	783.0
Accuracy[b]	100.5	97.7	104.4
C.V.[c] (%)	4.0	1.4	1.2
Daily results[d]			
Run 1	7.0	69.9	722.3
Run 2	7.2	72.3	720.9
Run 3	7.3	70.6	719.9
Run 4	7.2	72.3	741.1
Run 5	7.3	73.0	727.8
Run 6	7.0	70.0	706.6
Run 7	7.2	69.2	724.9
Run 8	6.8	69.6	743.9
Run 9	7.0	70.4	728.7
Run 10	7.5	70.8	761.4
Run 11	7.5	70.8	761.4
Run 12	6.8	67.7	672.3
Mean concentration	7.1	70.5	722.8
Interday accuracy (%)[b]	94.6	93.9	96.4
S.D.	0.2	1.5	22.4
C.V. (%)	3.0	2.1	3.1

[a]Mean of $n = 5$.

[b]Expressed as [(mean calculated concentration)/(nominal concentration) $\times$ 100].

[c]Coefficient of variation, $n = 5$.

[d]Mean of two determinations.

Verification of a lack of any significant absolute matrix effect between different biofluid lots was confirmed experimentally. Both **I** and **II** were spiked into several different lots of human control fluids after plasma extraction, and the resulting peak areas were compared between each biofluid lot as well as against neat reference standards. Addition of **I** and **II** to the biofluid after the extraction process reflected changes in analyte peak areas due solely to matrix effects on ionization efficiency, and eliminated any variability due to

Table 9. Analysis of BSA-treated urine quality control samples spiked with **I**

	Concentration (ng/mL)		
	Low QC	Mid QC	High QC
Nominal concentration	7.5	75.0	750.0
Initial experimentally measured concentrations	7.0	69.0	725.4
	6.9	70.4	752.7
	7.2	71.8	727.0
	7.0	72.6	730.5
	7.0	68.1	739.7
Initial mean ($n = 5$)			
Assayed concentration[a]	7.0	70.4	735.1
Accuracy[b]	93.6	93.8	98.0
C.V.[c] (%)	1.3	2.7	1.5
Daily results[d]			
Run 1	6.9	66.3	683.4
Run 2	7.2	67.9	708.9
Run 3	7.1	66.5	671.2
Mean concentration	7.0	66.9	687.8
Interday accuracy (%)[b]	93.8	89.2	91.7
S.D.	0.1	0.9	19.2
C.V. (%)	1.8	1.3	2.8

[a]Mean of $n = 5$.

[b]Expressed as [(mean calculated concentration)/(nominal concentration) × 100].

[c]Coefficient of variation, $n = 5$.

[d]Mean of two determinations.

the extraction process itself. The results are summarized in Tables 5 and 6. The inter-lot variability of the peak areas at each concentration level was small (low %C.V.), indicating very little relative (i.e, inter-lot) matrix effect for either compound. Comparison of the mean peak areas for each compound in sample extracts (post-extraction spike of the compounds) vs the mean peak areas observed in neat reference standards shows no significant absolute matrix effect, indicating essentially no signal suppression or enhancement in sample extracts vs. neat reference standards. In addition, slopes of the standard lines constructed in 5 different lots of human plasma and/or urine were practically the same. The precision of the slopes were 4.2% and 4.5% in plasma and urine, respectively (Table 7), confirming the absence of any significant relative matrix effect. As a result of these studies,

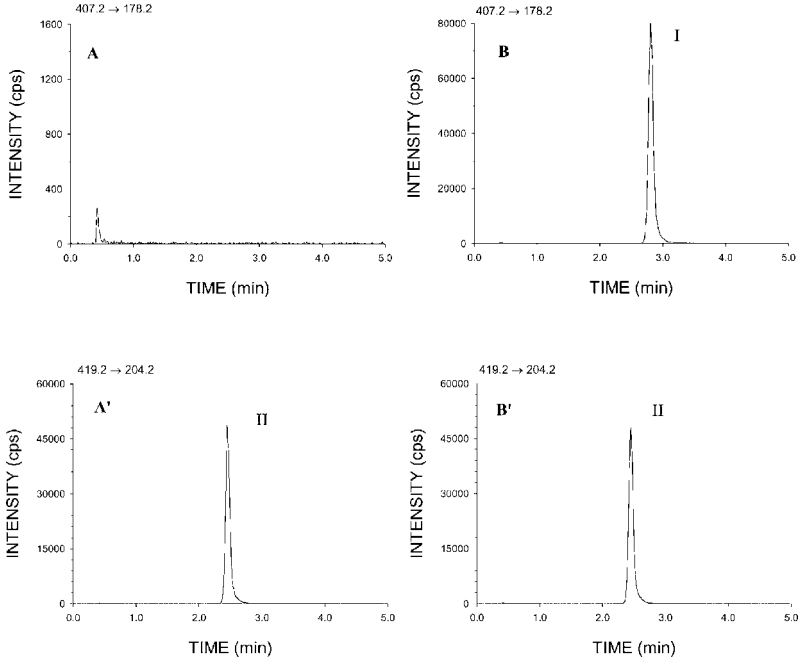


Figure 5. Representative extracted ion chromatograms of pre- and post-dose clinical samples. Ion chromatograms of clinical plasma extracts were obtained by multiple reaction monitoring at m/z 407 → 178 for **I** and m/z 419 → 204 for **II** (Internal Standard). (**A**, **A**′) Pre-dose clinical plasma sample spiked with 200 ng/mL of **II**. (**B**, **B**′) Post-dose clinical plasma sample spiked with 200 ng/mL of **II**. Calculated analyte (**I**) concentration was 126.0 ng/mL.

sample matrix effects were shown to have no impact on the accurate quantification of **I**.

Tables 8 and 9 present inter-day precision results based upon the analyses of QC samples over a 5-week period. The data show the interday precision at all QC concentrations was $<3.2\%$.

The validated method has been used to support four different pharmacokinetic studies, involving oral administration of **I** to humans, requiring the quantification of over 3000 samples. Representative chromatograms of pre- and post-dose clinical samples, in human plasma and BSA-treated urine, are shown in Figures 5 and 6, respectively. As shown, there are no interferences within the chromatograms from either circulating metabolites and/or endogenous interferences, confirming assay selectivity. An example of a plasma drug concentration vs time profile obtained using the described method is shown in Figure 7.

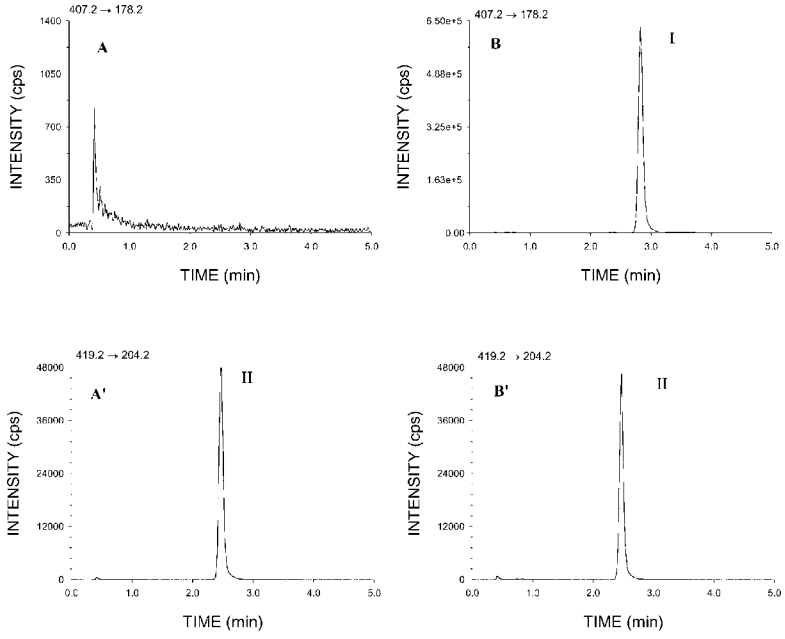


Figure 6. Representative extracted ion chromatograms of pre- and post-dose clinical samples. Ion chromatograms of clinical BSA-treated urine extracts were obtained by multiple reaction monitoring at m/z 407 → 178 for **I** and m/z 419 → 204 for **II** (Internal Standard). (**A**, **A′**) Pre-dose clinical BSA-treated urine sample spiked with 200 ng/mL of **II**. (**B**, **B′**) Post-dose clinical BSA-treated urine sample spiked with 200 ng/mL of **II**. Calculated analyte (**I**) concentration was 936.1 ng/mL.

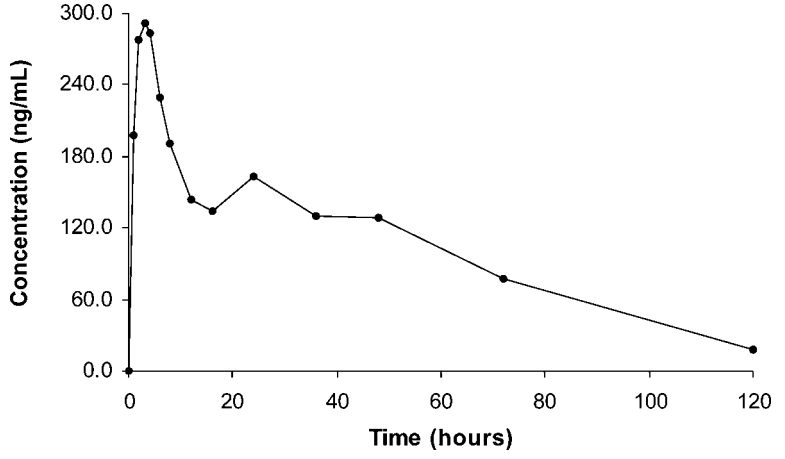


Figure 7. A representative plasma concentration vs. time profile following oral administration of **I** to a human subject.

CONCLUSIONS

HPLC-MS/MS methods for the determination of **I** in human plasma and BSA-treated urine were developed and validated. The assays were shown to be sensitive, selective, and robust over an extended time period. The absence of any significant absolute or relative matrix effects and interferences from metabolites were demonstrated. The highly efficient sample preparation and extraction procedure permitted a high-throughput analysis of clinical samples required to support clinical studies. The method has successfully been used to provide bioanalytical support for human pharmacokinetic evaluation of the analyte in both human plasma and urine samples. It has been used to assay over 3000 samples from four different clinical studies.

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Received June 12, 2005
Accepted July 8, 2005
Manuscript 6665