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Quantitative determination of a selective alpha-1a receptor antagonist in human plasma by high-performance liquid chromatography with tandem mass spectrometric detection

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Abstract

Solid-phase extraction, utilizing a 96-well plate format, was used to isolate an alpha-1a receptor antagonist and internal standard from human plasma. Following the isolation procedure, the analyte and internal standard were separated and detected using reversed-phase HPLC coupled with atmospheric pressure chemical ionization (APCI) mass spectrometry operated in the positive ion multiple reaction monitoring (MRM) mode. Based upon the peak area ratio (analyte: internal standard) the analyte was quantified over a concentration range of 0.02–2 ng/ml. Assay validation results including parameters such as precision and accuracy are presented. The validated method was subsequently used to support human pharmacokinetic studies.

Keywords: $\alpha_{1\alpha}$-Receptor antagonist

1. Introduction

Benign prostatic hyperplasia (BPH) is quite common in older males, with some data suggesting an incidence rate as high as 50% for men over 60 years of age [1]. Current treatment strategies for BPH may include surgery or administration of a 5-α reductase inhibitor (such as finasteride) or an alpha-1 andrenergic receptor antagonist. One of the known physiological processes associated with BPH is an increased andrenergic tone mediated by alpha-1 andrenergic receptors in the smooth muscle of the bladder, prostate capsule and prostate stroma, all of which contribute to bladder outlet obstruction associated with BPH. Treatment with alpha-1 andrenergic receptor antagonists results in a relaxation of smooth muscle in the bladder neck and prostate, resulting in improved urine flow. However, side effects such as hypotension and/or dizziness have been associated with the administration of currently available alpha-1 andrenergic receptor antagonists due to their non-selective blockade of alpha-1 receptors in both prostate tissue as well as vascular tissue. At the present time, three alpha-1 receptor subtypes (1a, 1b, 1d) have been identified and shown to exist in human and animal tissue [2]. The predominant subtype in the human prostate capsule and prostatic urethra is the alpha-1a subtype [3–5]. The alpha-1a subtype is found in some vascular smooth muscle beds, but is not known to contribute to blood pressure control. Administration of a selective alpha-

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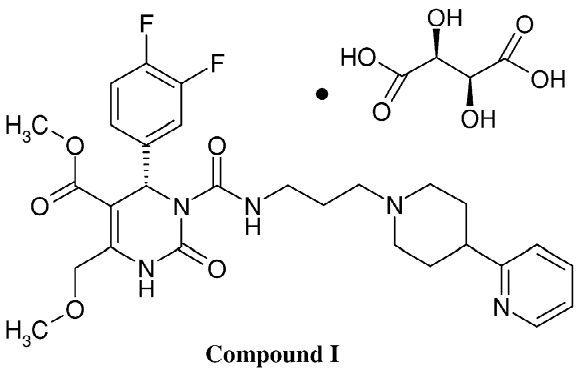
1a receptor antagonist may provide the desired relaxation of the prostate tissue, but not bind with alpha-1 andrenergic receptors in the smooth muscle of the vascular system, thus avoiding the undesirable side effects produced by a non-selective antagonist.

Compound **I** ((+)-1,2,3,6-tetrahydro-1-{*N*-[4-(2-pyridyl)piperidin - 1 - yl]propyl}carboxamido-5-methoxycarbonyl - 4 - methoxymethyl - 6 - (3, 4 - difluorophenyl)-2-oxopyrimidine; supplied as tartaric acid salt; Fig. 1) was previously shown to be a specific alpha-1a receptor antagonist [6]. In order to provide bioanalytical support for human pharmacokinetic evaluation of **I** at initial low dose regimens, an analytical method with a limit of quantification (LOQ) of less than 1 ng/ml was required. The use of HPLC coupled with atmospheric-pressure chemical ionization (APCI) mass spectrometry (MS) [7–11] or tandem mass spectrometry (MS–MS) [12,13] has been used previously for numerous highly selective trace-level quantitative determinations of analytes in a variety of biological matrices. Some recent examples from our laboratories are included in Refs. [14–19]. Due to the sensitivity and selectivity required for the present assay, the use of HPLC–MS–MS employing an APCI interface was evaluated as the analytical method of choice. Concomitant use of a 96-well solid-phase extraction sample preparation step resulted in an assay which was rapid, selective and sensitive. 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.



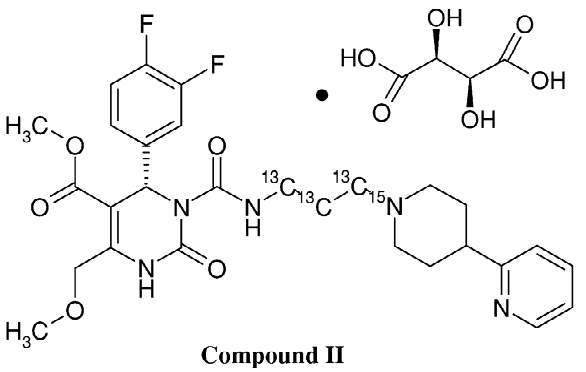


Fig. 1. Chemical structures of **I** and **II** (Internal Standard).

2. Experimental

2.1. Materials

Compound **I** and compound **II** (isotopically labeled internal standard; Fig. 1) were provided by the Medicinal Chemistry Department of Merck Research Laboratories (West Point, PA, USA) and the Labeled Compound Synthesis group (Merck Research Laboratories, Rahway, NJ, USA), respectively. 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). The drug free heparinized human plasma was obtained from Sera Tech Biologicals (Athens, OH, USA). Nitrogen (99.999%) was provided by West Point Cryogenics (West Point, PA, USA). Empore Mixed Phase Cation (MPC) standard density 96-well solid-phase extraction plates were purchased from 3M (St. Paul, MN, USA). An EvapArray 96-well plate sample evaporator was purchased from Jones Chromatography (Lakewood, CO, USA). A Combi-Heat thermal sealer for 96-well collection plates was obtained from Marsh Biomedical Products (Rochester, NY, USA).

2.2. Instrumentation

A Perkin-Elmer Sciex (Thornhill, Ontario, Canada) API 3000 tandem mass spectrometer equipped with a heated nebulizer interface, and a Perkin-Elmer (Norwalk, CT, USA) Series 200

quaternary pump and Series 200 Autosampler were used for all HPLC–MS–MS analyses. Data was processed on a Power MacIntosh G3 computer using MacQuan software (Sciex).

2.3. Chromatographic conditions

HPLC separation was performed on a 30×3 mm I.D., 3 μm BDS Hypersil cyano column (Keystone Scientific, Bellefonte, PA, USA) coupled with a 0.5-μm in-line filter (Supelco, Bellefonte, PA, USA). The mobile phase was a mixture of 10 m*M* ammonium acetate, adjusted to pH 4.0 with acetic acid:acetonitrile (60:40; v/v) and was delivered at a flow rate of 1 ml/min. The retention times for both **I** and **II** were approximately 0.6 min.

2.4. HPLC–MS–MS conditions

The HPLC system was interfaced via a heated nebulizer probe to a PE Sciex triple quadrupole mass spectrometer, which was operated in the positive ion mode. Atmospheric pressure chemical ionization was effected by a corona discharge needle (4 μA). The heated nebulizer probe was maintained at 475 °C. The nebulizing gas (N_2) and auxiliary gas (N_2) were each set to a pressure of 90 p.s.i. The orifice potential was set at +32 V. The dwell time for each ion transition was 175 ms. The mass spectrometer was programmed to admit the protonated molecular ions $[M+H]^+$ at *m/z* 558 (**I**) and *m/z* 562 (**II**) via the first quadrupole mass filter (Q1), with collision-induced fragmentation in Q2 (collision energy of 37 eV; N_2 collision gas at an estimated cell pressure of 4.42×10^{-3} Torr), and monitoring the product ions via Q3 at *m/z* 246 (**I**) and *m/z* 250 (**II**). Peak-area ratios obtained from multiple reaction monitoring (MRM) of the analyte **I** (*m/z* 558→246) to internal standard **II** (*m/z* 562→250) were utilized for the construction of calibration curves, using weighted ($1/x^2$) linear least square regression of the plasma concentrations and the measured peak area ratios.

2.5. Standard solutions

A stock solution of **I** (10 μg free base/ml) was prepared in 1:1 (v/v) water–acetonitrile. This solution was further diluted to give a series of working standards having concentrations of 40, 20, 10, 4, 2, 1 and 0.4 ng free base/ml. The internal standard **II** was also prepared as a stock solution (100 μg free base/ml) in 1:1 (v/v) water–acetonitrile. A working solution of 5 ng free base/ml was prepared by dilution and was used for all analyses.

A series of quality control (QC) samples in control human plasma at nominal concentrations of 0.075, 0.375 and 1.25 ng/ml were also prepared from a separate weighing of **I**. Aliquots (1.25 ml) of these solutions were placed in 3.6 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.

2.6. Sample preparation

A 1-ml aliquot of plasma was pipetted into a 13×85 mm polypropylene test tube, followed by addition of 50 μl of the appropriate working solution of **I** (or 1:1 (v/v) water–acetonitrile for clinical samples) and 50 μl of the working solution of internal standard **II** (equivalent to 0.25 ng/ml of **II**). The samples were briefly vortexed, then a 1-ml aliquot of 0.15 *M* sodium phosphate (pH 2) was added to each tube, followed by an additional vortex mix. A 1-ml aliquot of the resulting solution was transferred to the 96-well MPC mixed mode reversed-phase/cation-exchange solid-phase extraction plate, which had been conditioned (prior to sample addition) with 1 ml of methanol, followed by 1 ml of water and then 1 ml of 0.1 *M* sodium phosphate (pH 2). The sample solution was drawn through the plate using vacuum. The plate was then sequentially washed with 1 ml each of water, 1 *M* acetic acid, and methanol. The plate was then removed from the vacuum manifold and the bottom was rinsed with methanol to prevent cross-contamination of samples. The plate was centrifuged for 5 min at 188 *g* to remove any residual liquid from the plate. The extraction plate was then placed onto a 1.2 ml deep-well 96-well collection plate and 300 μl of CH_2Cl_2–isopropanol–NH_4OH (78:20:2; v/v) was 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 eluates were evaporated to dryness (in the collection plate) under a stream of nitrogen at 40 °C. The residue was reconstituted in 300 μl of 10 m*M* ammonium acetate

(pH 4)/acetonitrile (70:30; v/v). The wells in the collection plate were sealed using a thermal sealing foil. The plate was vortexed for 1 min, sonicated for 15 min, then vortexed for an additional 1 min. The collection plate containing the reconstituted extraction residue was placed in the HPLC autosampler, which was programmed to make injections directly from the individual wells of the collection plate. The injection volume for HPLC–MS–MS analysis was set at 50 μl.

2.7. Precision, accuracy, recovery and selectivity

The assay precision was determined by replicate analyses of different lots ($n = 5$) of control human plasma fortified with compound **I** at the concentrations used to construct calibration curves (0, 0.02, 0.05, 0.1, 0.2, 0.5, 1 and 2 ng/ml). The linearity of the assay was verified for the composite curve as well as for each individual plasma lot. 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 plasma drug concentrations. Linear regression analysis gave a calibration curve that was used to calculate unknown plasma 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 plasma as well as clinical subjects' pre-dose plasma samples. No endogenous interferences were observed in either plasma source. The recovery at each standard concentration was determined by comparing the peak area of compounds **I** and **II** extracted from control plasma to that obtained from extracts of blank control plasma which were spiked with compounds **I** and **II** following the solid-phase extraction procedure.

3. Results and discussion

The positive ion full-scan mass spectra (Q1) of **I** and **II** indicated the presence of the protonated molecular ion $[M+H]^+$ as the predominant ion for each compound, with m/z values of 558 and 562 for **I** and **II**, respectively. The corresponding product ion spectra obtained for **I** and **II**, obtained from the $[M+H]^+$ precursor ions, are shown in Fig. 2. Multiple reaction monitoring of the precursor→product ion transitions at m/z 558→246 for **I** and 562→250 for **II** permitted sensitive and selective detection of the analyte and internal standard.

A 96-well solid-phase extraction plate was chosen as the preferred format for this assay, rather than selecting the more traditional discrete extraction cartridge design or a liquid–liquid extraction method. The 96-well plate permitted the parallel extraction of up to 96 samples, which resulted in approximately an 8-h instrument run time for the

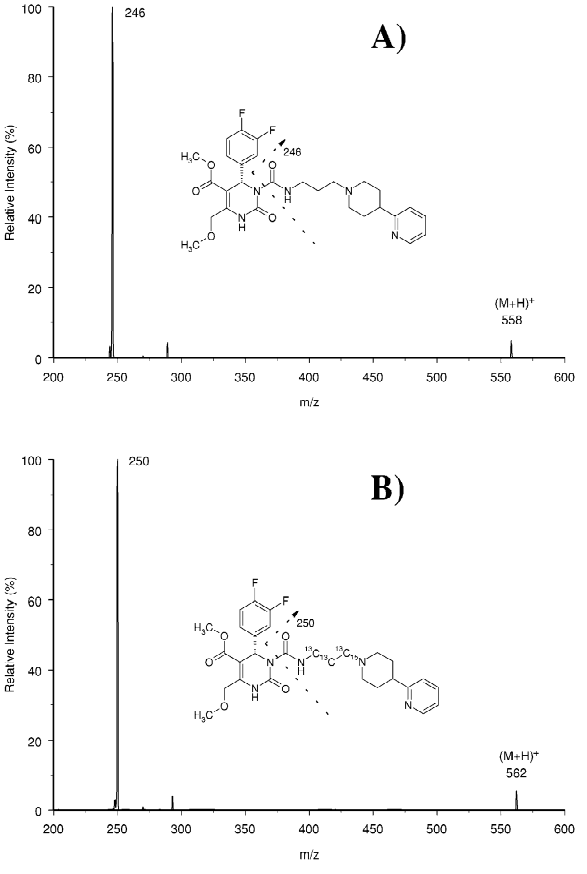


Fig. 2. Product ion mass spectra for **I** (A) and **II** (B) under the MS–MS conditions used in a selected reaction monitoring (SRM) mode.

method described in this paper. The mixed phase cation-exchange/reversed-phase membrane used here provided a very clean sample extract, yielding consistently good analyte recovery across the concentration range examined. The use of a 96-well sample evaporator to dry the sample extracts directly in the deep-well collection plate provided a convenient method of eliminating tedious and time consuming sample transfers to alternative evaporation vessels. The ability to reconstitute the sample extracts directly in the collection plate, seal the entire plate and place it in a 96-well compatible autosampler for sample injection directly from the plate resulted in a substantial reduction of manual sample manipulation.

In addition to the high sample processing capability, the 96-well format is also readily adaptable to automated sample preparation if required in future modifications of the assay. One drawback which we have encountered in the present method is the limited well volume, typically about 1 ml. As a result, extractions are limited to a 1-ml sample volume which may be a limiting factor in obtaining improved assay sensitivity. Serial loading of the wells may be used to compensate for this, but our experience indicates that this approach is more likely to result in an increased incidence of a plugged disk membrane and loss of samples. As an alternative, a decrease in the volume of the reconstitution solvent

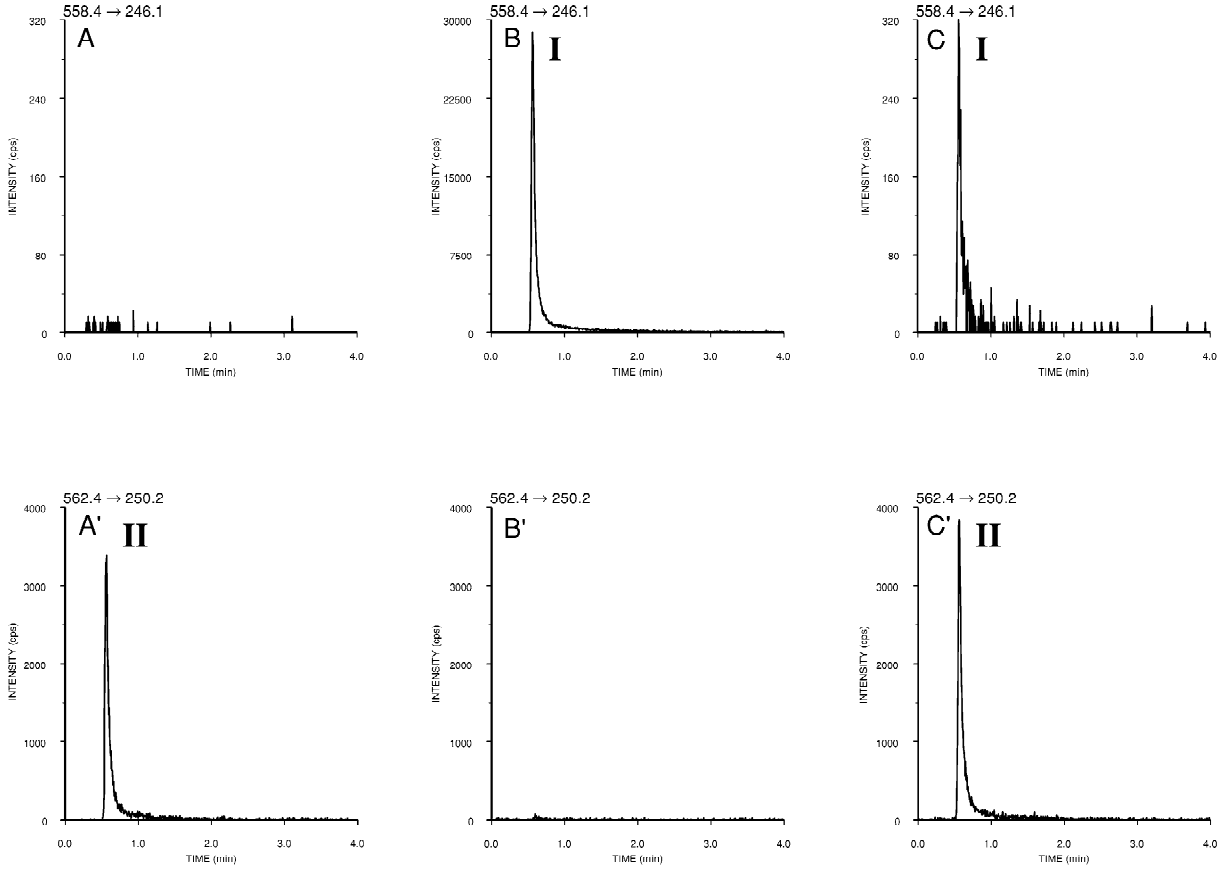


Fig. 3. Representative extracted ion chromatograms demonstrating assay selectivity and sensitivity. Chromatograms of control human plasma extracts were obtained by multiple reaction monitoring at m/z 558→246 for **I** and m/z 562→250 for **II** (Internal Standard. (**A, A′**) Control plasma spiked with 0.25 ng/ml of **II**. No response from internal standard (**II**) observed in analyte channel (A). (**B, B′**) control plasma spiked with 2 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 0.02 ng/ml of **I** (assay LOQ) and 0.25 ng/ml of **II**.

may lead to an improved assay sensitivity, if required, but adequate precision and accuracy performance of such a procedure would need to be separately confirmed.

Chromatographic separation was obtained on a short (30 mm) cyano column used in the reversed-phase mode, with **I** and **II** coeluting and each having a capacity factor (k') of approximately 2. Although a cyano-bonded phase is not typically the first choice for use in a reversed-phase separation, many columns and bonded phases were evaluated and the cyano column provided the best results in terms of peak shapes, capacity factors and chromatographic separation. We have observed no column stability problems, with typical column lifetimes being well over 1000 injections.

In order to meet the sensitivity requirements for the current assay, a large injection volume (50 μl) was used. Such an injection volume is atypical for a 30×3 mm column. However the judicious use of a weak reconstitution solvent (relative to the mobile phase elution strength) resulted in on-column focussing of **I** and **II** during the injection process, and provided acceptable peak shapes.

An issue in many trace-level quantitative methods is the absence of analyte carryover in the run immediately following that of a high concentration sample. No such carryover was observed in this assay. However, a blank injection immediately following injection of a high concentration standard (2 ng/ml) showed a large amount of background signal in the chromatographic baseline. This background noise was attributed to analyte ''bleed'' from the column, and slowly disappeared as the time between injections was increased. In order to consistently obtain accurate quantification of low concentration samples, this ''bleed'' had to be removed between injections. Use of a 3-mm I.D. column rather than a 4.6-mm I.D. column, at identical mobile phase flow-rates and run times, effectively increases the wash volume between injections by a factor of approximately 2.3. By utilizing a 3-mm I.D. column with a mobile phase flow rate of 1 ml/min and a run time of 4 min, the observed analyte ''bleed'' was eliminated, permitting reliable quantification of low concentration samples while maintaining an acceptable run time.

As described in the Experimental Section, the method was validated in human plasma over the analyte concentration range of 0.02–2 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 than 15% with an assay accuracy of 100±15%. Using the experimental conditions described in this paper, the assay LOQ (0.02

Table 1
Precision and accuracy of replicate analyses ($n = 5$) of **I** in human plasma[a], (Analyst 1/*Analyst 2*)

Nominal concentration (ng/ml)	Mean[b] concentration (ng/ml)	Precision[c] C.V.%	Accuracy[d] (%)
0.02	0.021/*0.020*	4.3/*11.4*	104.2/*101.9*
0.05	0.047/*0.049*	7.4/*8.3*	94.7/*98.5*
0.1	0.089/*0.095*	9.8/*8.4*	88.7/*94.5*
0.2	0.201/*0.194*	4.3/*6.2*	100.6/*97.1*
0.5	0.511/*0.508*	6.2/*2.3*	102.2/*101.7*
1	1.012/*1.022*	5.2/*5.4*	101.2/*102.2*
2	2.170/*2.084*	4.3/*2.9*	108.5/*104.2*

[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].

Table 2
Recovery of **I** and **II** from human plasma in replicate analyses ($n = 5$)

Nominal concentration of **I** (ng/ml)	Mean[a,b] recovery of **I** (%)	Mean[a,b,d] recovery of **II** (%)
0.02	86.4	87.8
0.05	85.9	88.0
0.1	82.6	88.7
0.2	89.0	86.6
0.5	87.9	85.4
1	88.4	86.0
2	88.5	83.2
Mean % Recovery	87.0 (Precision[c] = 2.6%)	86.5 (Precision[c] = 2.2%)

[a] Recovery calculated as [(Peak area of sample spiked pre-extraction)/(Peak area of sample spiked post-extraction)] × 100.
[b] Mean of replicates of 5 different lots of plasma.
[c] Expressed as coefficient of variation (C.V.%).
[d] Concentration fixed at 0.25 ng/ml.

Table 3
Assessment of the absolute matrix effects on ionization of **I** and **II** in five different lots of human plasma[a]

Nominal concentration of **I** in plasma (ng/ml)	**I** Mean absolute matrix Effect[b] [%C.V.] ($n = 5$)	**II (0.25 ng/ml)** Mean Absolute Matrix effect[b] [%C.V.] ($n = 5$)
0.020	1.3 [7.3]	1.3 [4.9]
0.050	1.3 [7.5]	1.3 [4.7]
0.100	1.2 [6.2]	1.3 [1.4]
0.200	1.2 [4.4]	1.3 [5.8]
0.500	1.2 [4.9]	1.3 [6.1]
1.000	1.2 [3.3]	1.3 [2.5]
2.000	1.2 [5.5]	1.3 [4.7]
Mean absolute matrix effect[b]	1.2	1.3

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

ng/ml) corresponded to approximately 1.4 pg of **I** injected on-column. Representative chromatograms for spiked human control plasma are shown in Fig. 3. Table 1 presents the validation statistics, namely precision and accuracy, obtained by two different analysts. The data indicate the assay performs well across the entire concentration range. The intra-day precision was below 10% in all cases except for the

Table 4
Analysis of plasma quality control samples spiked with **I**

	Low QC concentration (ng/ml)		Mid QC concentration (ng/ml)		High QC concentration (ng/ml)	
Nominal concentration	0.075		0.375		1.250	
Initial mean ($n = 5$)						
Assayed concentration[a]	0.072		0.375		1.204	
S.D.[b]	0.006		0.022		0.036	
C.V.[c] (%)	8.6		5.8		3.0	
Daily results[d]	Analyst 1	Analyst 2	Analyst 1	Analyst 2	Analyst 1	Analyst 2
Run 1		0.074		0.357		1.147
Run 2	0.073		0.359		1.096	
Run 3		0.072		0.358		1.112
Run 4		0.063		0.365		1.158
Run 5	0.069		0.330		1.136	
Run 6		0.075		0.344		1.117
Run 7	0.069		0.386		1.180	
Mean	0.070	0.071	0.358	0.356	1.137	1.133
SD	0.002	0.006	0.028	0.008	0.042	0.022
C.V. (%)	3.1	7.8	7.8	2.4	3.7	2.0

[a] Mean of $n = 5$.
[b] Standard Deviation.
[c] Coefficient of Variation, $n = 5$.
[d] Mean of two determinations.

limit of quantification, at which the precision was still within acceptable limits. The accuracy was within 12% of nominal values across the entire concentration range. The data also indicate that the assay was robust enough to produce comparable results amongst different analysts.

Analyte recovery studies were also conducted. The analyte recovery was determined by comparing the ratio of analyte peak areas obtained from plasma samples spiked prior to extraction to the peak areas obtained from blank samples from the same plasma lots which were extracted and subsequently spiked after the solid-phase extraction. This approach cancels any effects the matrix may have on ionization efficiencies in different plasma lots, and reflects only the efficiency of the solid-phase extraction process. The results are summarized in Table 2. The mean recovery for **I** across the concentration range of the assay was 86.7% with a coefficient of variation of only 2.6%, indicating excellent reproducibility of extraction.

An assessment of potential matrix effects on the ionization efficiency, and subsequent peak areas, for **I** and **II** was also investigated. The presence of any 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 [14,16,20]. Since the present assay utilized a stable-isotope labeled internal standard, any absolute matrix effects on the

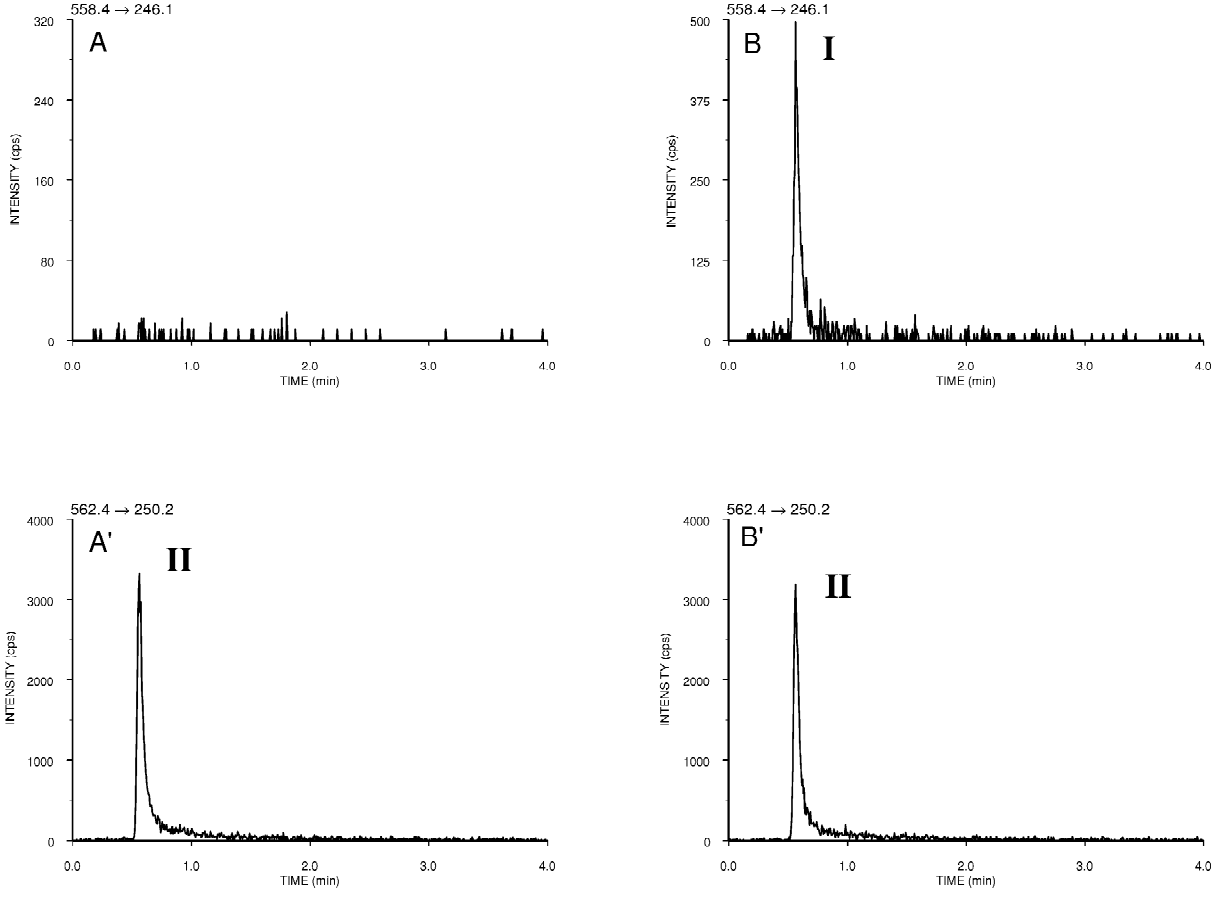


Fig. 4. Representative extracted ion chromatograms of pre- and post-dose clinical samples. Ion chromatograms of clinical plasma extracts were obtained by multiple reaction monitoring at m/z 558→246 for **I** and m/z 562→250 for **II** (Internal Standard). (**A, A′**) Pre-dose clinical plasma sample spiked with 0.25 ng/ml of **II**. (**B, B′**) Post-dose clinical plasma sample spiked with 0.25 ng/ml of **II**. Calculated analyte (**I**) concentration was 0.034 ng/ml.

ionization efficiency should be similar for both **I** and **II**, resulting in no net relative effect and thus permit accurate quantification of **I** using **II** as the internal standard. In order to more fully characterize the assay performance, verification of a lack of any significant absolute matrix effect between different plasma lots may also be valuable and was confirmed experimentally. Both **I** and **II** were spiked into several different lots of human control plasma after plasma extraction and the resulting peak areas were compared between each plasma lot as well as against neat reference standards. Addition of **I** and **II** to the plasma after the extraction process reflected changes in analyte peak areas due solely to matrix effects on ionization efficiency, and eliminated any variability due to the extraction process itself. The results are summarized in Table 3. The inter-lot variability of the peak areas at each concentration level are small (low %C.V.), indicating very little relative (ie inter-lot) matrix effect for either compound. On the other hand, comparison of the mean peak areas for each compound in plasma extracts (post-extraction spike of the compounds) vs. the mean peak areas observed in neat reference standards shows an increase of about 20–30%, indicating some signal enhancement in plasma extracts vs. neat reference standards and the presence of an absolute matrix effect. However, the degree of the absolute matrix effect was essentially the same for both **I** and **II** and between different plasma lots, and thus did not have any effect on the determination of the **I**/**II** peak area ratio used in the assay.

Table 4 presents inter-day precision results, for two different analysts, based upon the analyses of QC samples over a 2-week period. The data show the inter-day precision was $<8\%$ for each analyst.

The validated method was successfully applied to the analysis of over 800 plasma samples to support pharmacokinetic evaluation of **I** following oral administration to humans. Representative chromatograms of pre- and post-dose clinical samples are shown in Fig. 4. No interferences from metabolites in post-dose samples were observed, confirming assay selectivity. An example of a plasma drug concentration vs. time profile obtained with data generated by the described method is shown in Fig.

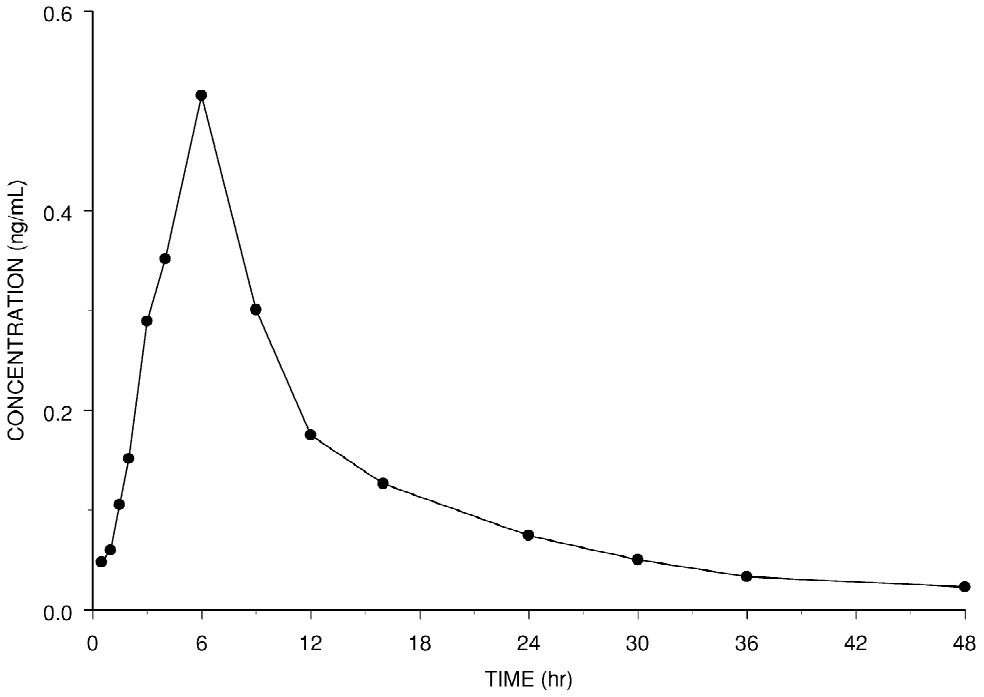


Fig. 5. A representative plasma concentration vs. time profile following oral administration of **I** to a human subject.

5. The figure demonstrates adequate sensitivity and range of quantification for the method to characterize the plasma concentration profile of **I** in post-dose clinical samples at the administered oral doses.

4. Conclusions

An HPLC–MS–MS method for the determination of **I** in human plasma was developed and validated. The assay was shown to be sensitive, selective, and robust when used by multiple analysts over an extended time period. The absence of a relative matrix effect and interferences from metabolites were demonstrated. The highly efficient sample preparation 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.

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