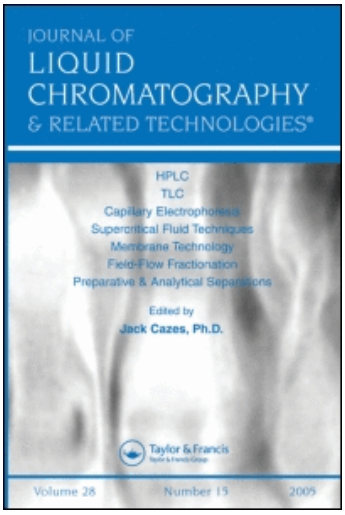

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Determination of a Selective GABA-A $\alpha_{2,3}$ Partial Agonist in Human Plasma by High Performance Liquid Chromatography with Tandem Mass Spectrometric Detection

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Abstract: An automated solid phase extraction, utilizing a 96-well plate format, was used to isolate a selective GABA-A $\alpha_{2,3}$ partial agonist (**I**) and internal standard (**II**) 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 ionization mode. Based upon the peak area ratio (analyte:internal standard), the analyte was quantified over a concentration range of 0.1 to 50 ng/mL. The absolute and relative matrix effects from different sources of human plasma on the ionization efficiency were examined and the absence of these effects was confirmed. Assay validation results including parameters such as intra- and inter-day precision and accuracy are presented. The validated method was subsequently used to support human pharmacokinetic studies.

Keywords: GABA-A, Plasma, Agonist, HPLC, Tandem mass spectrometry

INTRODUCTION

Recent estimates suggest that over 70 million people in the United States, Western Europe, and Japan suffer from anxiety. Benzodiazepines are the

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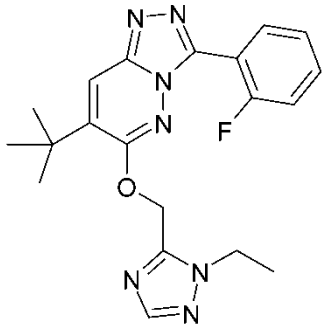
most popular treatment for generalized anxiety disorder (GAD) and posses several advantages over other anxiolytics, including rapid action, excellent efficacy, ease of use, and a wide margin of safety. The usefulness of benzodiazepines, however, is limited by side effects, which include drowsiness, fatigue, motor impairment, hypotension, amnesia, disorientation, headaches, and the potential for addiction. Replacement of benzodiazepines with a selective GABA-A $\alpha_{2,3}$ partial agonist may result in comparable anxiolytic efficacy as nonselective benzodiazepines, but not produce undesirable sedating side effects. The GABA-A receptors constitute a family of ligand gated chloride channels,[1–3] whose activation by the agonist leads to chloride influx, membrane hyperpolarization, and subsequent reduction in the excitability of the neurons. The lack of sedating side effects of GABA-A $\alpha_{2,3}$ agonists is believed to be due to their lack of efficacy at the α_1-subtype, the subtype believed to mediate the sedative effects of benzodiazepines.[4] Ideally, other components of the selective GABA-A $\alpha_{2,3}$ agonist profile (e.g., development of tolerance to anxiolytic effects, dependence liability, withdrawal effects, drug interactions, general safety, and impairment of cognition) would be expected to be comparable to currently used benzodiazepine anxiolytic therapies.

Compound **I** (7-*tert*-butyl-6-(2-ethyl-2H-[1,2,4]triazol-3-ylmethoxy)-3-(2-fluoro-phenyl)-[1,2,4]triazolo[4,3-B]pyridazine; Figure 1) was previously shown to be a selective GABA-A $\alpha_{2,3}$ partial agonist in preclinical species. 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)[5–9] or tandem mass spectrometry (MS/MS)[10,11] 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 the references.[12–19] 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 an automated 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.[20]

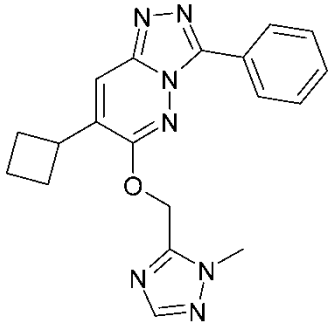
EXPERIMENTAL

Materials

Compound **I** and compound **II** (internal standard; Figure 1) were provided by the Compound Repository of Merck Research Laboratories (West Point, PA,



Compound I



Compound II (Int. Std.)

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

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). 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). Ansys SPEC Plus MP3 96-well solid phase extraction plates were purchased from MetaChem Technologies (Torrance, CA, 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).

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).

Chromatographic Conditions

HPLC separation was performed on a 30 × 4.6 mm I.D., 3 μm Ultra IBD column (Restek Corporation, Bellefonte, PA, USA) coupled with a 0.5 μm in-line filter (Supelco, Bellefonte, PA, USA). The mobile phase was a mixture of 10 mM ammonium acetate, adjusted to pH 4.0 with acetic acid: acetonitrile (65:35; v/v), and was delivered at a flow rate of 1 mL/min. The retention times for **I** and **II** were approximately 2.2 and 2.0 min, respectively.

HPLC/MS/MS Conditions

A PE Sciex triple quadrupole mass spectrometer, operated in the positive ion mode, was interfaced with a heated nebulizer probe to the HPLC system. Gas phase chemical ionization was effected by a corona discharge needle (4 μA). The heated nebulizer probe was maintained at 500°C. The nebulizing gas (N_2) and auxiliary gas (N_2) were each set to a pressure of 90 psi. The orifice potential was set at +41V. The dwell time for each ion transition was 400 msec. The mass spectrometer was programmed to admit the protonated molecular ions $[M + H]^+$ at m/z 396 (**I**) and m/z 362 (**II**) via the first quadrupole mass filter (Q1), with collision induced fragmentation in Q2 (collision energy of 42 eV; N_2 collision gas at a cell pressure of 4.58×10^{-3} torr, corresponding to a collision gas thickness of 3.02×10^{15} molecules/cm^2), and monitoring the product ions via Q3 at m/z 287 (**I**) and m/z 334 (**II**). Peak area ratios obtained from multiple reaction monitoring (MRM) of the analyte **I** (m/z 396 → 287) to internal standard **II** (m/z 362 → 334) 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.

Standard Solutions

A stock solution of **I** (10 μg/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 1, 0.4, 0.2, 0.1, 0.04, 0.02, 0.01, 0.004, and 0.002 μg/mL. The internal standard **II** was also prepared as a stock solution (10 μg/mL) in 1:1 (v/v) water/acetonitrile. A working solution of 0.05 μg/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 0.35, 3.5, and 35 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.

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 2.5 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. Using a Packard MultiProbe liquid handling system, 2 mL of each resulting sample solution were transferred to a 2.2 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 MP3 mixed mode reversed phase/cation exchange solid phase extraction plate, which had been conditioned with 0.8 mL of methanol, followed by 0.8 mL of water and then 0.8 mL of 0.1 M sodium phosphate (pH 2). The sample solution was drawn through the plate using vacuum. Again, using the Tomtec Quadra, a second 1 mL aliquot of the sample solution was transferred to the 96-well MP3 mixed mode reversed phase/cation exchange solid phase extraction plate and drawn through with vacuum. The Tomtec was then used to sequentially wash the extraction plate wells with 1 mL of water, followed by 0.8 mL of 1 M acetic acid, and 0.8 mL of 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 0.65 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 100 μL of 10 mM ammonium acetate (pH 4)/acetonitrile (85 : 15; 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 10 μL.

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, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, and 50 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' predose 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.

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 396 and 362 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 396 → 287 for **I** and 362 → 334 for **II** permitted sensitive and selective detection of the analyte and internal standard.

A primary factor in designing any clinical assay is to incorporate the ability to process a large number of samples on a daily basis. In response to this concern, 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 rapid, parallel extraction of up to 96 samples, which resulted in approximately a 10 hr instrument run time for the 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

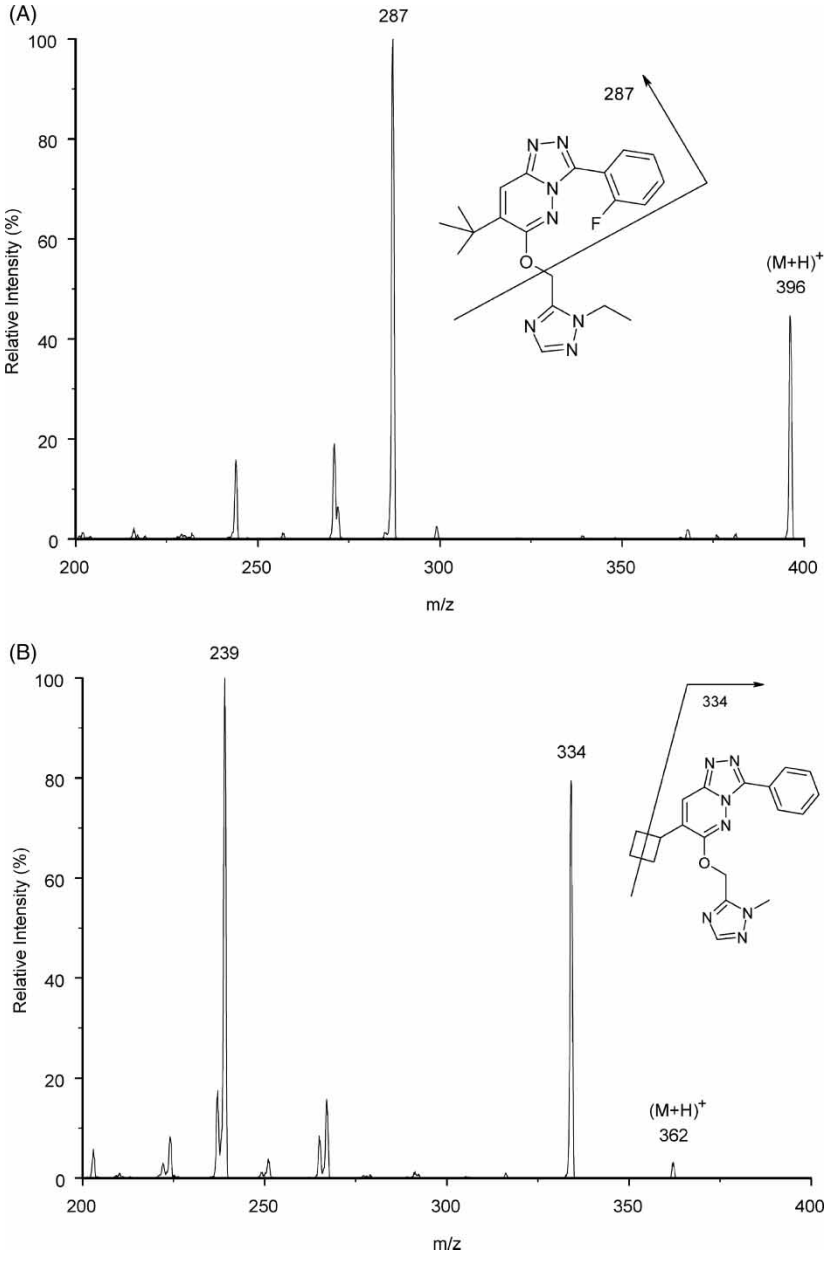


Figure 2. Product ion mass spectra for **I** (A) and **II** (B) under the MS/MS conditions used in the multiple reaction monitoring (MRM) mode.

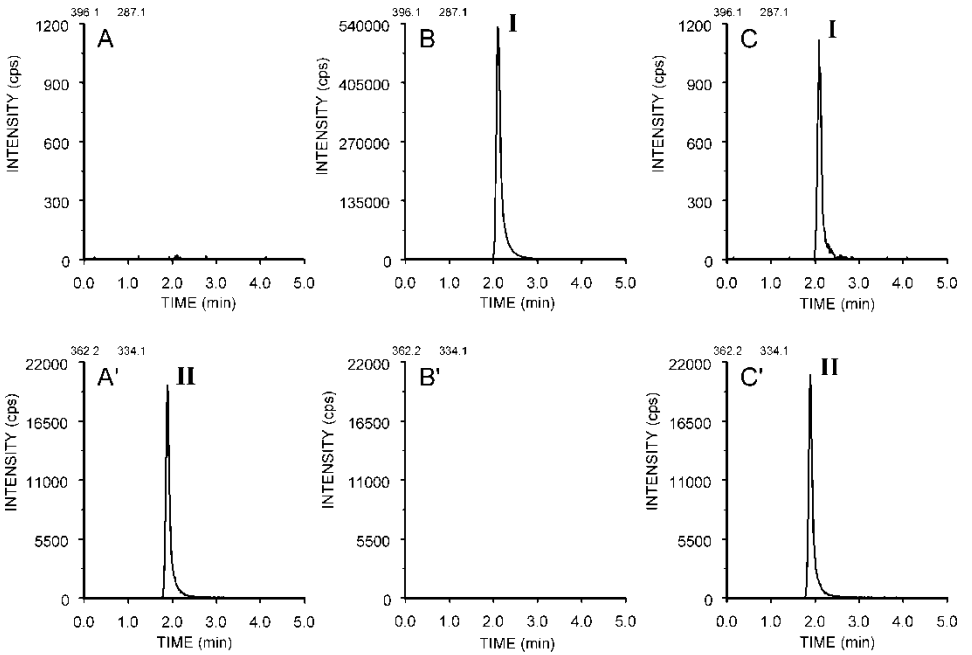


Figure 3. Representative extracted ion chromatograms demonstrating assay selectivity and sensitivity. Chromatograms of control human plasma extracts were obtained by multiple reaction monitoring at m/z 396 → 287 for **I** and m/z 362 → 334 for **II** (Internal Standard. (A, A′) control plasma spiked with 2.5 ng/mL of **II**. No response from internal standard (**II**) observed in analyte channel (A). (B, B′) control plasma spiked with 50 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 0.1 ng/mL of **I** (assay LOQ) and 2.5 ng/mL of **II**.

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. Use of the Packard unit for automated sample transfer from individual tubes into the 96-well format required by the Tomtec system is a non-critical volumetric measurement step, and relieves the analyst from the tedium of repetitive sample transfers. The Tomtec Quadra greatly simplifies the SPE sample extraction procedure, especially in light of the serial sample extraction procedure required due to the limitation of the extraction well volume (1.2 mL), and the tendency of some samples to pass through the Ansys extraction membrane due to simple gravity flow. The result of the automated simultaneous sample application to the extraction plate is a much more consistent sample treatment, relative to the use of manual sample loading and extraction.

Chromatographic separation was obtained on a very short (30 mm) column packed with one of the new "intrinsically base deactivated" bonded phases. These phases contain a typical non-polar chain, but also contain a more polar group, such as a carbamate or amide functionality, embedded in the chain.

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

Nominal concentration (ng/mL)	Mean[b] concentration (ng/mL)	Precision[c] C.V. (%)	Accuracy[d] (%)
0.10	0.10	4.2	100.2
0.20	0.20	3.8	99.4
0.50	0.51	3.4	102.0
1.00	0.98	2.7	98.1
2.00	1.97	4.2	98.3
5.00	4.96	4.5	99.1
10.00	9.79	1.3	97.9
20.00	20.29	5.0	101.4
50.00	51.73	2.3	103.5

[a]Data obtained 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 result is a reduction of undesirable secondary interactions of basic analytes with residual surface silanol groups. This translates into improved peak shape for basic compounds, and often times give the ability to separate a "non-polar" drug and its "polar" metabolites with the same chromatographic conditions. After extensive column and mobile phase screening, the IBD column gave the best overall results, including acceptable peak shapes for the analyte and internal standard, sufficient retention (capacity factors (k′)) of approximately 3.4 and 3.0 for **I** and **II**, respectively. In addition, the IBD column also gave chromatographic separation of a series of potential metabolites from both the analyte and internal standard, thus avoiding any potential interference issues. 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, the combined approach of extraction of 1 mL of plasma, eluate evaporation, and reconstitution in a small volume (100 μL) was required. In addition to these sample preconcentration techniques, use of a reconstitution solvent significantly weaker than the mobile phase resulted in on-column focusing of **I** and **II** during the injection process and provided peak shapes, which were better than those observed when using a stronger reconstitution solvent.

As described in the Experimental section, the method was validated in human plasma over the analyte concentration range of 0.1 to 50 ng/mL. The limit of quantification (LOQ) was defined as the lowest concentration

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** (%)
0.10	63.4	65.0
0.20	61.9	60.8
0.50	61.8	59.9
1.00	66.4	64.6
2.00	69.0	66.4
5.00	68.8	64.7
10.00	67.0	63.2
20.00	69.9	67.2
50.00	65.2	61.3
Mean % recovery	65.9 (Precision[c] = 4.6%)	63.7 (Precision[c] = 4.0%)

[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 of **II** was 2.5 ng/mL.

Table 3. Assessment of matrix effects on ionization of **I** and **II** in 5 different lots of human plasma[a]

	I	**II** (2.5 ng/mL)
Nominal concentration of **I** in plasma (ng/mL)	Mean absolute matrix effect[b] (%CV, $n = 5$)	Mean absolute matrix effect[b] (%CV, $n = 5$)
0.10	1.1 [5.3]	1.1 [5.3]
0.20	1.2 [3.5]	1.1 [4.4]
0.50	1.1 [5.4]	1.1 [6.5]
1.00	1.1 [6.4]	1.1 [7.4]
2.00	1.1 [3.7]	1.1 [4.9]
5.00	1.2 [4.9]	1.1 [5.8]
10.00	1.2 [3.5]	1.1 [5.3]
20.00	1.1 [3.4]	1.1 [5.2]
50.00	1.1 [1.9]	1.1 [4.3]
Mean absolute matrix effect[b]	1.1	1.1

[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.

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 $100 \pm 15\%$. Using the experimental conditions described in this paper, the assay LOQ (0.1 ng/mL) corresponded to approximately 7 pg of **I** injected on-column. Representative chromatograms for spiked human control plasma are shown in Figure 3. Table 1 presents the validation statistics, namely precision and accuracy. The data indicate the assay performs well across the entire concentration range. The intra-day precision was within 5% and the accuracy was within 4% of nominal values across the entire concentration range.

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 extract 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 65.9% with a coefficient of variation

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:	0.35	3.50	35.00
Initial experimentally	0.32	3.49	35.69
measured concentrations	0.34	3.52	34.57
	0.35	3.77	35.93
	0.37	3.57	35.60
	0.35	3.48	35.32
Initial mean ($n = 5$)			
Assayed concentration[a]	0.35	3.57	35.42
Accuracy[b]	99.3	102.0	101.2
C.V.[c](%)	5.0	3.4	1.5
Daily results[d]			
Run 1	0.35	3.42	36.25
Run 2	0.35	3.51	35.87
Run 3	0.36	3.77	37.45
Run 4	0.38	3.70	39.10
Run 5	0.36	3.57	35.41
Run 6	0.37	3.72	36.75
Run 7	0.36	3.59	34.74
Run 8	0.37	3.77	36.48
Run 9	0.37	3.62	35.63
Run 10	0.36	3.63	36.56
Run 11	0.35	3.66	36.19
Mean concentration	0.36	3.63	36.40
Interday accuracy (%)	103.1	101.7	102.8
S.D.	0.01	0.11	1.15
C.V.(%)	2.5	3.0	3.2

[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.

of only 4.6%, indicating excellent reproducibility of extraction from different plasma lots.

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.[12,14,21] 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.

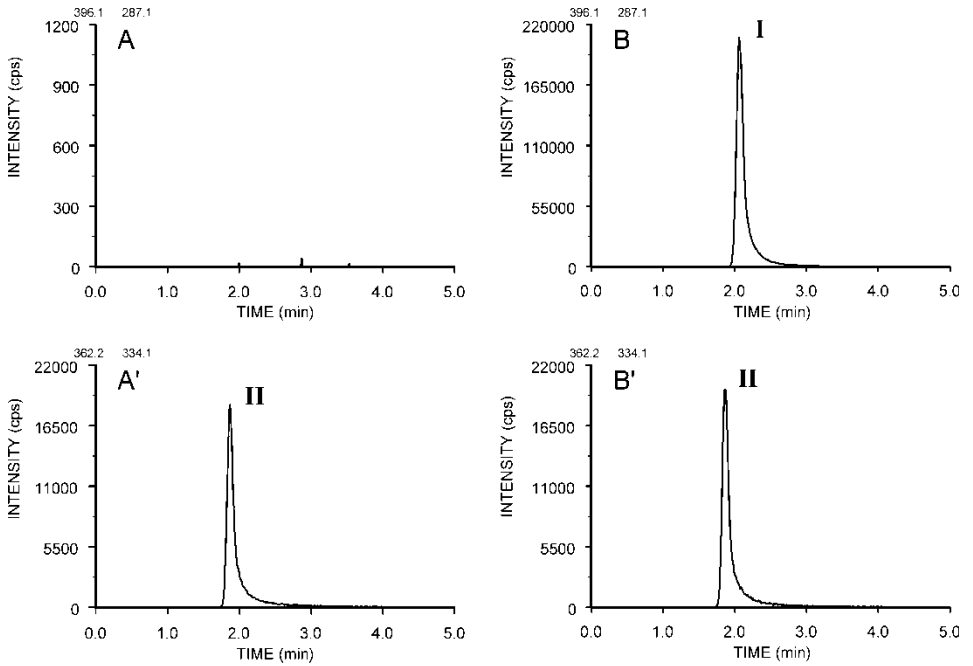


Figure 4. Representative extracted ion chromatograms of pre- and post-dose clinical samples. Ion chromatograms of clinical plasma extracts were obtained by multiple reaction monitoring at m/z 396 → 287 for *I* and m/z 362 → 334 for **II** (Internal Standard). (A, A′) Pre-dose clinical plasma sample spiked with 2.5 ng/mL of **II**. (B, B′) Post-dose clinical plasma sample spiked with 2.5 ng/mL of **II**. Calculated analyte (**I**) concentration was 20.87 ng/mL.

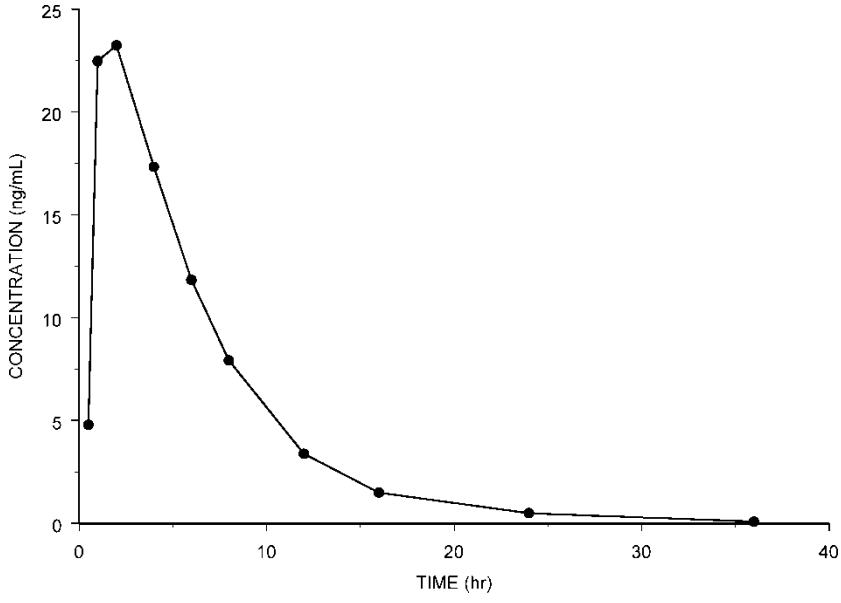


Figure 5. A representative plasma concentration vs. time profile following oral administration of I to a human subject.

Verification of a lack of any significant absolute matrix effect between different plasma lots 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 %CV), indicating very little relative (i.e., inter-lot) matrix effect for either compound. 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 no significant absolute matrix effect, indicating only a 10% signal enhancement in plasma extracts vs. neat reference standards. As a result of these studies, sample matrix effects were shown to have no impact on the accurate quantification of **I**.

Table 4 presents inter-day precision results based upon the analyses of QC samples over a 6 week period. The data show the inter-day precision for all QC concentrations was $<4\%$.

The validated method has been successfully applied to the analysis of over 4000 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 Figure 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 Figure 5.

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 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 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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