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# Quantitative determination of MK-0767, a dual α/γ peroxisome proliferator-activated receptor (PPAR) agonist, in human plasma by liquid chromatography–tandem mass spectrometry

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**Abstract**

5-[2,4-Dioxothiazolidin-5-yl)methyl]-2-methoxy-*N*-[[(4-trifluoromethyl)-phenyl]methyl]benzamide (**I**, MK-0767 or KRP-297, Fig. 1), is a dual α/γ peroxisome proliferator-activated receptor (PPAR) agonist. A LC–MS/MS method for the determination of **I** in human plasma has been successfully developed, validated and applied to clinical programs. The analyte and internal standard (**II**) are extracted from 0.05 mL plasma via solid phase extraction (SPE). HPLC is used for the separation of **I** and **II** from possible co-extracted endogenous and other compounds. Detection is by MS/MS in multiple reaction monitoring (MRM) mode using a TurboIonSpray® probe. The whole sample preparation is automated by using a Packard Multiprobe liquid handling system. The linear range is 4–2000 ng/mL in plasma. Recoveries were 71.1% and 69.4% for **I** and **II**, respectively. The method exhibited good linearity, reproducibility and sensitivity, selectivity and robustness when used for the analysis of clinical samples.


*Keywords:* MK-0767; Peroxisome proliferator-activated receptor

## 1. Introduction

5-[2,4-Dioxothiazolidin-5-yl)methyl]-2-methoxy-*N*-[[(4-trifluoromethyl)-phenyl]methyl]benzamide(MK-0767 or KRP-297, **I**) is a dual α/γ peroxisome proliferator-activated receptor (PPAR) agonist previously being studied for utility in the treatment of patients with type 2 diabetes [1–4] (Fig. 1). A sensitive and reliable analytical method was needed to support the clinical program.

PPAR agonists, particularly thiazolidinedione compounds, have been analyzed in a number of different ways. Yamashita et al. reported a method for the determination of pioglitazone and its metabolites in human plasma and urine using solid phase extraction and HPLC–UV [5]. Rosiglitazone was analyzed in human plasma samples using sequential automated dialysis and HPLC–fluorescence detection [6]. High performance liquid chromatography coupled with tandem mass spectrometry (HPLC–MS/MS or LC–MS/MS) with atmospheric pressure ionization was used to analyze troglitazone [7].

LC–MS/MS has been widely used in the quantitation of drugs and their metabolites in human fluids [8]. The combination of powerful separation from HPLC and superior selectivity and sensitivity from mass spectrometer has made LC–MS/MS one of the most useful techniques in bioanalytical chemistry. Another important trend in the bioanalytical area is the automation of sample preparation procedures, typically with liquid handling systems. In this research, we report the development, validation and application of a bioanalytical method for the quantitative determination of **I**, also a thiazolidindione compound, in human plasma. The method used LC–MS/MS with turbo ionspray ionization for the separation and detection of the analytes, and a liquid handling system for the automated sample preparation. The lower limit of quantitation (LLOQ)

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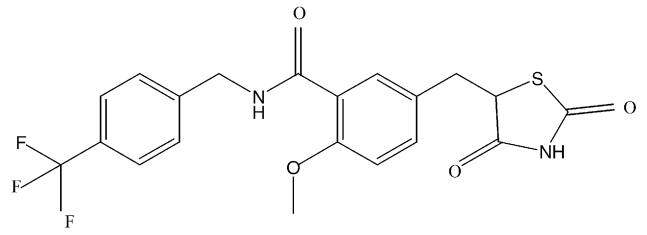
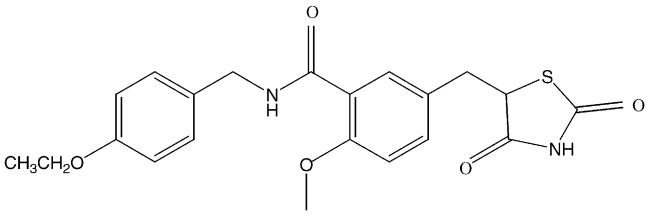


MK-0767 (***I***)



Internal Standard (***II***)

Fig. 1. Structures of **I** and **II**.

for this method was 4 ng/mL and linearity range was 4–2000 ng/mL.

## 2. Experimental

### 2.1. Reagents and materials

**I** and **II** (internal standard) were obtained from Kyorin (Japan). HPLC grade methanol was obtained from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid (minimum, 95%) and ammonium acetate (minimum, 98%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Control human plasma (heparinized) was obtained from Biological Specialty (West Point, PA, USA). ISOLUTE C18 SPE cartridges (50 mg, 1 mL, loose well configuration for 96-well) were purchased from Argonaut (Hegoed, Wales, UK).

### 2.2. Equipment

The LC–MS/MS system consisted of a PE Sciex (Thornhill, Ontario, Canada) API 365 or API 3000 mass spectrometer with a TurboIonSpray® interface and two Perkin-Elmer (Norwalk, CT, USA) Series 200 Micro Pumps. A Perkin-Elmer Series 200 autosampler was used with a temperature-controlled tray. A Model 7990 Jones Chromatography (now Argonaut, Hegoed, Wales, UK) column heater was used to control the HPLC column temperature. Data were processed using MacQuan software (Version 1.5, PE Sciex) on a Power Macintosh G3 (Apple, Cupertino, CA, USA). A Packard (now PerkinElmer, Wellesley, MA, USA) MultiProbe II was used to handle all the liquid transferring procedures including plasma samples, buffer and solvents. A Jones Chromatography SpeedDry 96 system (Hegoed, Wales, UK) was used for drying procedures in the method.

### 2.3. Instrumental conditions

The mass spectrometer was operated in the positive ion mode using a TurboIonSpray® interface. The product ion spectra for **I** and **II** are shown in Fig. 2. The monitored ion transitions were *m/z* 439.0 → 263.9 for **I** and *m/z* 414.9 → 263.9 for **II** (internal standard).

The analytical column was a Luna C18 (50 mm × 2.1 mm, 5 μm) from Phenomenex (Torrance, CA, USA). Mobile phase A was 0.1% formic acid. Mobile phase B was methanol. The analytes were separated with a composition of A/B (35:65 (v/v)) at a flow rate of 0.2 mL/min and the column temperature was 35 °C.

### 2.4. Calibration standard preparation

Primary stock solutions of **I** and **II** were prepared at a concentration of 100 μg/mL in methanol. Stock solution for **I** was further diluted in methanol:water (50:50 (v/v)) to make a series of working standard solutions. Concentrations for compound **I** working standards were 4, 10, 40, 100, 400, 1000 and 2000 ng/mL. Concentrations for compound **II** were further diluted to 50 ng/mL with methanol:water (50:50 (v/v)). All standard solutions were stored at 4 °C.

Calibration standards were prepared daily by adding 0.15 mL of 100 mM ammonium acetate buffer (pH 4.0) and 0.05 mL of each working standard of compound **I** to 0.05 mL of control human plasma. The final concentrations range is 0.8–400 ng/mL in solution, which is equivalent to 4–2000 ng/mL of **I** in human plasma.

### 2.5. Quality control sample preparation

A quality control (QC) standard solution at 100 μg/mL was generated by a separated weighing and then further diluted to 10 and 0.5 μg/mL in methanol/water (1:1 (v/v)). QC samples were prepared by adding 0.8 mL of these QC standard solutions to a 50 mL glass volumetric flask containing control human plasma. The final QC plasma concentrations were 1600, 160 and 8 ng/mL for high, medium and low concentrations. QC plasma samples were stored at −20 °C until assayed.

### 2.6. Extraction procedure

Frozen plasma samples were thawed to room temperature prior to extraction. After vortexing and centrifuging, a 0.05 mL aliquot of the sample was added to a 12 mm × 75 mm polypropylene tube. The sample tube was added by 0.15 mL of 100 mM ammonium acetate (pH 4.0) and 0.05 mL of methanol/water (1:1 (v/v)). Internal standard (0.05 mL of the 50 ng/mL solution) was then added, and the tube was vortexed. An ISOLUTE C1896-well SPE cartridge plate was

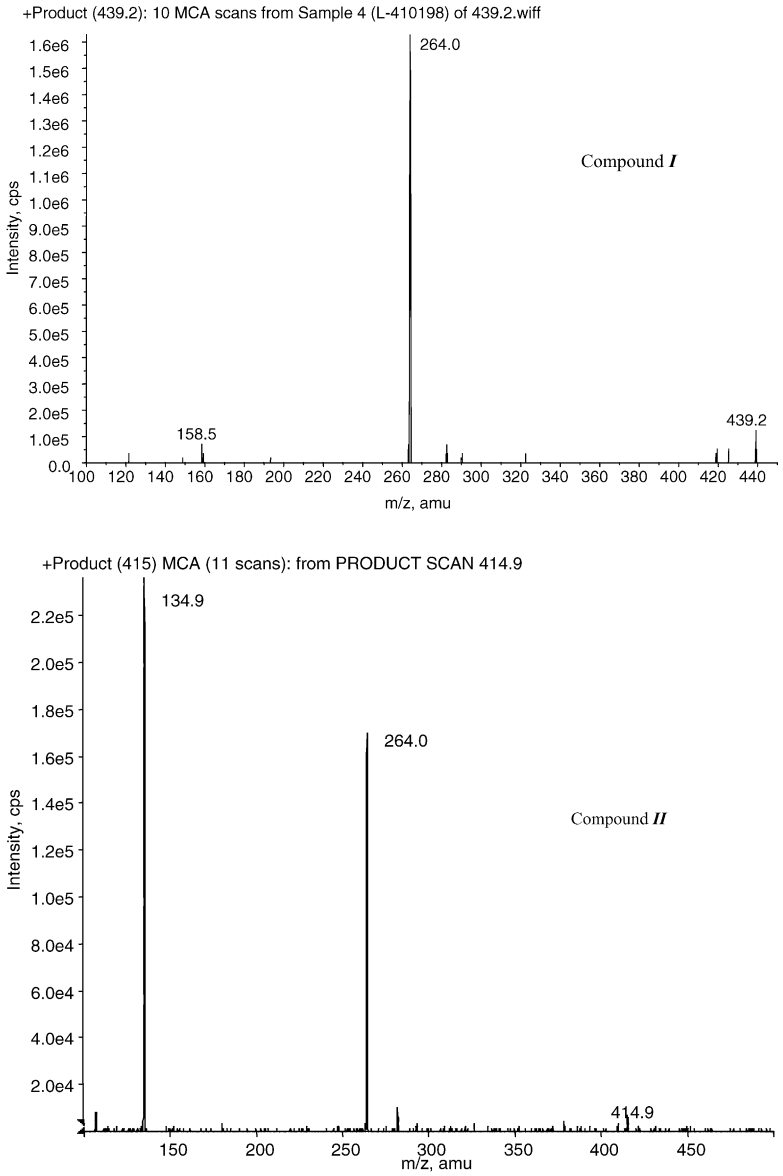



Fig. 2. Product scan spectra of **I** and **II**.

conditioned with 0.5 mL of methanol followed by pretreatment with 0.5 mL of 100 mM ammonium acetate (pH 4.0) for each well, and then the sample solution was loaded. Each well was washed by 0.5 mL of 5% methanol and the analytes were eluted with 0.20 mL of methanol. The eluent was evaporated to dryness under nitrogen on a Jones Chromatography SpeedDry 96 system at 40 °C for 20 min. The samples were reconstituted in 0.1 mL methanol:water (50:50 (v/v)) with vortexing and sonication, and 20 μL was injected for analysis. All the liquid transferring procedures were performed on a Packard MultiProbe II liquid handler system.

### 2.7. Quantitation

Calibration standard curve was prepared daily. Concentrations were determined from the linear least-squares fitted line of the peak area ratios of **I** to the internal standard (**II**) versus the concentrations of **I** with reciprocal weighting ($1/x$) on the concentration. Standards were assayed daily with quality control and unknown samples.

## 3. Results and discussion

### 3.1. Sample preparation and automation

In the early method development stage, we explored the sample preparation with conventional SPE cartridges. The method was fine-tuned by introducing the 96-well SPE plate with similar retention phase and employing automation in sample preparation. A Packard MultiProbe II liquid handling system was used for all the liquid transferring procedures,

including plasma transferring and the 96-well plate SPE extraction steps.

To evaluate the method with and without automation, a test was conducted by performing all the steps in the method manually. The result showed that both automated and manual procedures need approximately 3 h to finish the preparation of one plate of 96 samples. Automation in 96-well SPE format did not significantly save sample preparation time in comparison with manual procedure. Several factors could have contributed to the fact that the fully automated procedure was not as fast as anticipated, including: (1) a special procedure was needed to inspect the tubes of plasma to ensure that there was no bubble or floating protein on the surface of the plasma samples. Any bubble or floating protein present would require manual removal before starting the automated procedure. The same procedure was not needed when handling manually since a skilled analyst could easily pipet the plasma without touching the bubbles and floating plasma protein; (2) in the automated SPE extraction, a Packard liquid handling system was controlling all eluting steps with vacuum. To ensure all the SPE cartridge cells in the 96-well plate were eluted properly, the vacuum was set to less than 5 psi and the vacuum procedure was broken into several steps. This was very critical to minimize the variation among 96-well cartridge cells and among the sample runs. The actual time for each vacuum step could vary slightly depending on the plasma sample sources and the 96-well cartridge manufacturing lot; (3) the Packard system we used was a four-tip system which is slower than an eight-tip system; (4) due to the nature of the compound, we have to use disposable tips for most of the fluid transferring steps to eliminate sample carryover. As a result of all these factors, the sample preparation time in the automated method was not reduced significantly compared to the manual procedure. However, an automated method was still favored because (1) it minimized possible human error in sample preparation such as misplacing, skipping or switching samples; (2) it reduced man-power needed, thus increasing productivity and (3) it reduced the possibility of ergonomic or occupational health problems of analysts.

### 3.2. MS/MS conditions

Turbo ionspray and atmospheric pressure chemical ionization (APCI) ion sources were tested for the ionization efficiency of compound **I**. Both sources produced significant signal for quantitation. However, Turbo ionspray was chosen for this method since it provided better sensitivity for compound **I**.

Fig. 2 is the product scan spectrum of **I** and **II**. The predominant fragment for both compounds was *m*/*z* 264, the thiazolidinedione moiety of both **I** and **II**. The fragment was formed by breaking the amide bond between the carbonyl group and the nitrogen. The transition of *m*/*z* 439 → 264 was chosen as the detection channel for **I**. Similarly, *m*/*z* 415 → 264 was selected for detecting the internal standard (**II**).

### 3.3. HPLC conditions

Although detection by a tandem mass spectrometer is very selective, it is possible that some other compounds such as metabolites of the parent compound might interfere with the detection of the selected mass transitions used for quantification of the parent compound. So, a very important task in the method development process is to obtain a chromatographic condition, which will provide the necessary separation of parent compound and its metabolites.

In order to test the chromatographic condition of the method, eight potential human metabolites were obtained. They are derivatives of MK-0767 in the forms of methyl sulfoxide, phenylacetic acid, methyl sulfone, benzoic acid, *O*-demethylated, detrifluorobenzyl and two sulfinyl carboxylic acids. The eight compounds plus the parent compound were mixed in a solution. A series of tests were conducted using this solution as a probe to find a suitable HPLC condition including column and mobile phase selection. After careful comparison of many columns and HPLC conditions, an acceptable HPLC condition was established with a Luna C18 column (50 mm × 2.1 mm, 5 μm) from Phenomenex. The mobile phase consisted of 35% of 0.1% formic acid and 65% of methanol delivered by two PE Micro pumps isocratically at 0.2 mL/min. Under this condition, there was baseline separation of the parent peak from all other compounds (Fig. 3). The chromatograms also showed that the transition channel of *m*/*z* 439 → 264 was highly selective for **I**. A baseline separation of **I** from other metabolites indicated that further investigation of possible cross-talk and ion suppression/enhancement between the parent and these potential metabolites was not necessary.

### 3.4. Sensitivity, linearity, accuracy and precision

Sensitivity, or the lower limit of quantification (LLOQ), was defined as the lowest concentration of the standard curve that could be measured with acceptable precision and accuracy. The lower limit of quantitation was 4 ng/mL for **I** using 0.05 mL of plasma. The linear dynamic range was from 4 ng/mL to 2000 ng/mL. The correlation coefficient (*r*) using weighted (1/*x*) linear least-squares regression was >0.997 for all the experimental runs.

The intra-day accuracy and precision for the method was determined from the analysis of 5 replicates of QC samples. Inter-day precision and accuracy were tested also with QCs at three different days. Table 1 summarized intra- and inter-day data.

### 3.5. Stability

Table 2 lists data for bench top, autosampler, freeze/thaw and storage stability.

Bench top stability was investigated to ensure that ***I*** was not degraded in plasma samples at room temperature for a time period to cover the sample preparation. Three sets of

Table 1
Intra- and inter-day precision and accuracy of **I** in plasma

| QCs (ng/mL) | Intra-day ($n$ = 5) | | | Inter-day ($n$ = 3) | | |
|---|---|---|---|---|---|---|
| | Found concentration (ng/mL) | Accuracy (%) | CV (%) | Found concentration (ng/mL) | Accuracy (%) | CV (%) |
| 8 | 8.93 | 111.6 | 14.5 | 8.47 | 105.8 | 7.1 |
| 160 | 173.3 | 108.3 | 6.2 | 175.4 | 109.6 | 5.0 |
| 1600 | 1691 | 105.7 | 2.6 | 1736 | 108.5 | 4.7 |

plasma samples at concentrations of 12, 160 and 1600 ng/mL were left at room temperature for 15 h. The samples were then processed and analyzed. The results indicated that **I** was stable during the exposure period.

Due to the need for occasional delayed injection or re-injection of extracted samples, stability of **I** in the final reconstituted extraction fluid was evaluated in the 96-well plate in the HPLC autosampler at 4 °C. A group of QC samples were extracted, loaded onto the autosampler and kept in the autosampler for 48 h before injection. The quantitative results

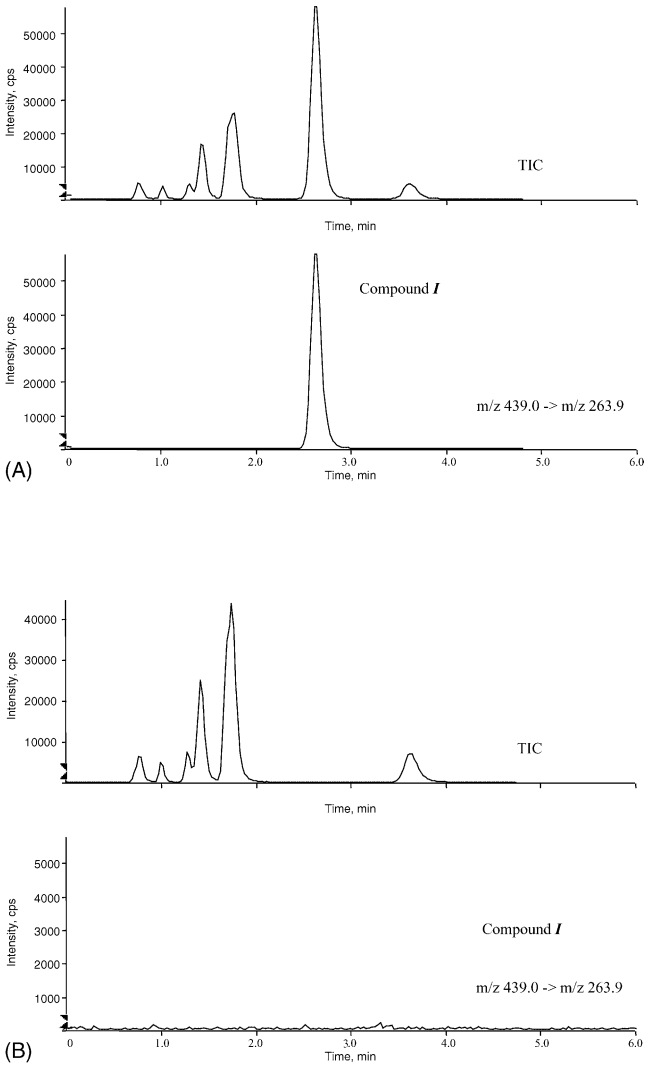



Fig. 3. Separation of **I** and eight potential human metabolites. Mixed sample with (A) and without **I** (B).

Table 2
MK-0767 stability data

| Concentration (ng/mL) | Found concentration (ng/mL) | CV (%) | Accuracy (%) |
|---|---|---|---|
| Bench top stability ($n$ = 5)[a] | | | |
| 12 | 13.20 | 2.6 | 110.0 |
| 160 | 171.4 | 3.0 | 107.1 |
| 1600 | 1705 | 1.7 | 106.6 |
| Autosampler stability ($n$ = 6)[b] | | | |
| 8 | 7.90 | 5.3 | 98.8 |
| 160 | 169.0 | 2.7 | 105.6 |
| 1600 | 1749 | 8.6 | 109.3 |
| Freeze/thaw stability ($n$ = 6)[c] | | | |
| 8 | 7.90 | 2.9 | 98.8 |
| 160 | 165.8 | 1.0 | 103.6 |
| 1600 | 1672 | 1.2 | 104.5 |
| 6-week storage stability ($n$ = 2)[d] | | | |
| 8 | 7.80 | | 97.5 |
| 160 | 174.7 | | 109.2 |
| 1600 | 1685 | | 105.3 |

[a] Exposed at room temperature (22 °C) for 15 h.
[b] Kept at 4 °C for 48 h.
[c] After three freeze/thaw cycles.
[d] Stored at −20 °C.

indicated that **I** was stable in the autosampler up to at least 48 h.

Freeze–thaw stability was evaluated for **I** using QC samples. The QCs were exposed to three freeze–thaw cycles, each cycle consisted of removing the QCs from the freezer, thawing them unassisted to room temperature, kept at room temperature for 4 h and re-freezing at −20 °C. The samples were processed along with a standard curve and concentrations were determined. This result indicated that **I** had an acceptable stability after three freeze–thaw cycles in human plasma.

The storage stability at −20 °C was also tested using QC samples. The stability has been closely monitored during validation and sample analysis periods, and no degradation of the compound was observed. The 6-week stability data is listed in Table 2. The result indicated that **I** was stable in plasma for at least 6 weeks.

### 3.6. Recovery and matrix effect

Extraction recovery of the analytes was determined by analyzing extracts of five replicate plasma samples containing **I** in human control plasma at three different concentrations

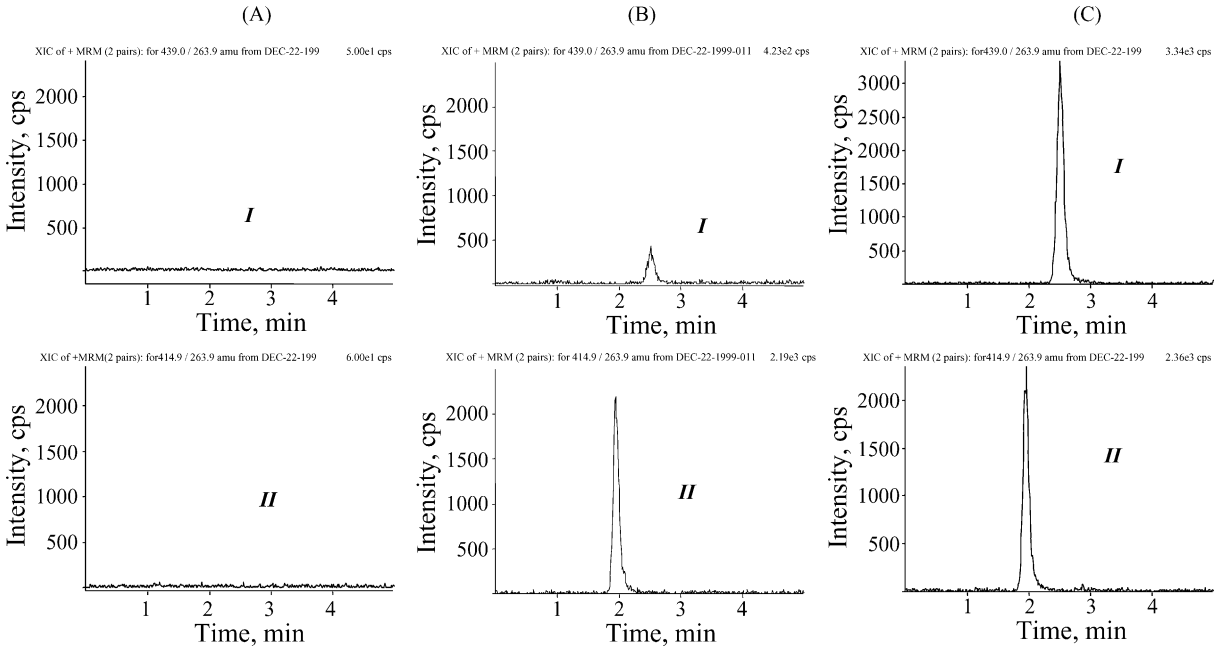



Fig. 4. Representative chromatograms: (A) control blank plasma; (B) plasma standard at LLOQ (**I**, 4 ng/mL: internal standard **II**, 50 ng/mL) and (C) plasma sample from a healthy subject following an oral dose of 2 mg of **I** (0.5 h post-dose, analyzed concentration was 36.2 ng/mL).

(10, 100 and 1000 ng/mL) in five different lots of human plasma. The internal standard (**II**) was evaluated only at the concentration used during extraction (50 ng/mL). For the determination of recovery, blank control human plasma was extracted. The blank extracts were reconstituted using 0.1 mL of the neat standards at concentrations corresponding to the final concentration of the extracted plasma samples. These spike-after-extraction samples represented 100% recovery. The extraction recovery was determined by comparing mean peak areas of **I** from the spike-before-extract plasma samples to the mean peak areas of the corresponding spike-after-extract samples at the same concentrations. Overall recovery for **I** in human plasma was 71.1%, and for internal standard, 69.4%.

The possibility of a matrix effect caused by competition between the ionization of the analyte and ionization of co-eluents exists when using LC–MS/MS for analysis [8–10]. To evaluate the matrix effect in our method, chromatographic peak areas of **I** from the spike-after-extraction samples were compared to the neat standards at the sample concentrations. No quantitatively significant difference was found after evaluating as many as five different lots of plasma. The same evaluation was performed on internal standard and no significant peak area differences were observed. Thus, we concluded that ion suppression or enhancement from plasma matrix was not significant for this method.

### 3.7. Application of the method

The method has been applied plasma samples from many clinical studies, and nearly ten thousands of samples have been analyzed. Fig. 4 shows representative chromatograms from plasma samples as control blank, LLOQ and a sample from a healthy subject following an oral dose of 2 mg of **I** (0.5 h post-dose). QC samples were run in duplicate at each level with the clinical samples to monitor daily performance

Table 3
Precision and accuracy for plasma quality control samples in clinical sample analyses ($n$ = 12, over a period of 6 weeks)

| QC | Nominal concentration (ng/mL) | Mean found concentration (ng/mL) | Accuracy (%) | Precision, CV (%) |
|---|---|---|---|---|
| High | 1600 | 1651 | 103.2 | 4.2 |
| Medium | 160 | 169.9 | 106.2 | 6.3 |
| Low | 8 | 8.54 | 106.8 | 6.2 |

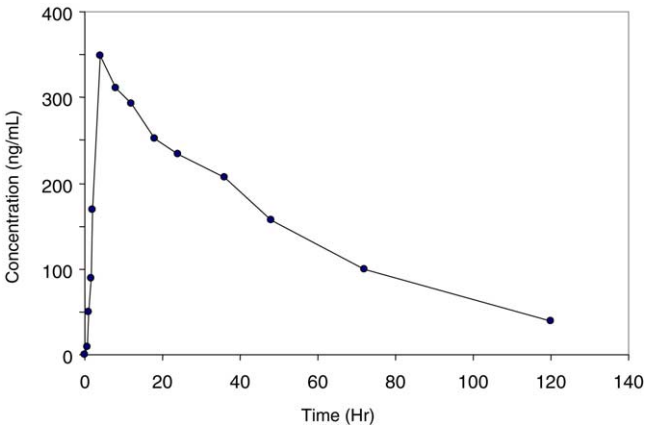



Fig. 5. A representative plasma concentration vs. time profile of **I** following a single oral 5 mg dose of **I**.

of the method. Table 3 shows the summarized QC information from one clinical study. The sample analysis for this study was finished in twelve experimental runs that spread over a period of six weeks. For all QCs analyzed in this study, the precision (CV) for the QC samples were ≤6.3%. The accuracy ranged from 103.2% to 106.8%. Results from the analysis of QC samples illustrate the robustness of the method. Fig. 5 is a representative plasma concentration versus time profile obtained from a healthy male subject following a single, 5 mg oral dose of **I**.

## 4. Conclusions

A fully automated LC–MS/MS method for the determination of **I** in human plasma has been successfully developed and validated. The linear range is from 4 to 2000 ng/mL. The method exhibits good selectivity and reproducibility; sensitivity is adequate for the doses studied. The method was successfully used to analyze thousands of clinical samples and produced satisfactory results.

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