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Quantitative determination of a novel insulin sensitizer and its *para*-hydroxylated metabolite in human plasma by LC–MS/MS

Kerry A. Riffel*, Michelle A. Polinko, Hengchang Song, Ronda K. Rippley, Man-Wai Lo

Merck Research Laboratories, Department of Drug Metabolism, WP75A-303, West Point, PA 19486, USA

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Abstract

I, 5-{3-[3-(4-phenoxy-2-propylphenoxy)-propoxy]-phenyl}-2,4-thiazolidinedione sodium salt, is a dual α/γ peroxisome proliferator-activated receptor (PPAR) agonist for potential use in diabetic patients. The compound has a *para*-hydroxylated metabolite, **II**, which has also been shown to exhibit PPAR activity. An LC–MS/MS method for the simultaneous determination of **I** and its active metabolite (**II**) in human plasma has been successfully developed. The method consists of treating 0.5 ml plasma with ammonium acetate (pH 9.6; 50 mM) and extracting **I**, **II** and internal standard (**III**, Fig. 2) with 5 ml ethyl acetate. The ethyl acetate is evaporated and the samples are reconstituted in 0.1 ml acetonitrile:0.1% formic acid (65:35, v/v). The entire extraction procedure, as well as sample collection, was performed in glass tubes and vials to overcome the analytes adherence to polypropylene. A linear HPLC gradient was used to separate the analyte, metabolite, internal standard, and other interfering, non-quantitated metabolites. Detection was by negative ionization MS/MS on a turbo ionspray probe. Precursor → product ion combinations were monitored in multiple reaction monitoring (MRM) mode. The linear range is 0.05–20 ng/ml for **I** and 0.1–20 ng/ml for **II**. Recoveries were 59.4, 90.1 and 56.8% for **I**, **II** and **III**, respectively. Intraday variation using this method was ≤7.0% for **I** and ≤9.2% for **II**. The method exhibits good linearity and reproducibility for each analyte and good sensitivity, selectivity and robustness when used for the analysis of clinical samples.

Keywords: Insulin sensitizer; PPAR; Negative ion LC–MS/MS; Liquid–liquid extraction

1. Introduction

I, 5-[3-(3-(4-phenoxy-2-propylphenoxy)-propoxy)-phenyl]-2,4-thiazolidinedione sodium salt, is a dual α/γ peroxisome proliferator-activated receptor (PPAR) agonist for potential use in treatment of diabetic patients [1,2]. Thiazolidinedione (TZD) insulin sensitizers, such as rosiglitazone and pioglitazone, are selective agonists for PPARγ and have been shown to be effective antihyperglycemic agents in man [3].

PPAR agonists, particularly thiazolidinedione compounds, have been analyzed in a number of different ways. LC–MS/MS with atmospheric pressure ionization was used to analyze troglitazone (Fig. 1) at

* Corresponding author. Tel.: +1-215-652-5758; fax: +1-215-652-4524.
E-mail address: kerry_riffel@merck.com (K.A. Riffel).

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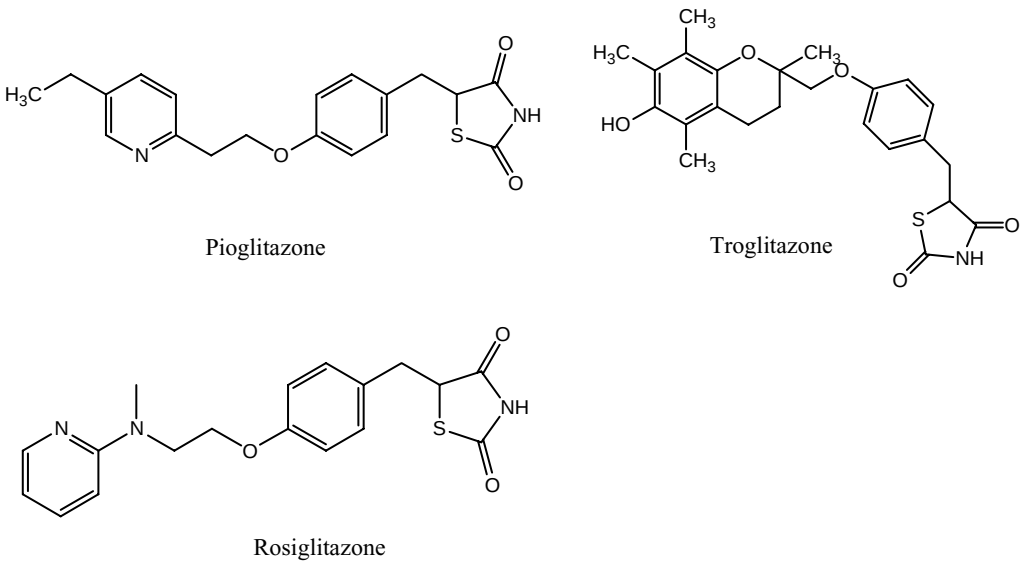


Fig. 1. Structures of several thiazolidinedione compounds.

a limit of quantitation (LOQ) of 10 ng/ml and its major metabolites in human plasma [4]. Yamashita et al. report a method for the determination of pioglitazone, another thiazolidinedione compound, and its metabolites in human plasma and urine [5]. The method utilizes solid phase extraction followed by HPLC with detection by UV. The method reports a LOQ of 0.01–0.05 μg/ml for pioglitazone and its metabolites in plasma and 0.1–0.5 μg/ml in urine. A third thiazolidinedione compound, rosiglitazone, was analyzed in human plasma samples to support clinical studies using an automated high performance liquid chromatography method. Plasma concentrations of rosiglitazone were determined using sequential automated dialysis of human plasma samples. The dialysate was then concentrated by trace enrichment on a C_{18} cartridge and eluted by mobile phase onto the HPLC column. The drug and its internal standard were detected by fluorescence detection with a LOQ of 3 ng/ml in 0.2 ml of plasma [6].

This paper reports the analysis of a novel PPAR α/γ insulin sensitizer, also a thiazolidinedione compound, and an active metabolite in human plasma using liquid–liquid extraction and LC–MS/MS on a PE Sciex turbo ionspray source in negative ion mode. The LOQ for this method was 0.05 ng/ml for the parent compound and 0.1 ng/ml for the metabolite when extracting 0.5 ml of plasma. Several factors considered during method development were the stability of the analyte and metabolite in solution, chromatographic separation of the metabolite from other minor hydroxylated metabolites, adherence of the metabolites to polypropylene and the need for assay sensitivity.

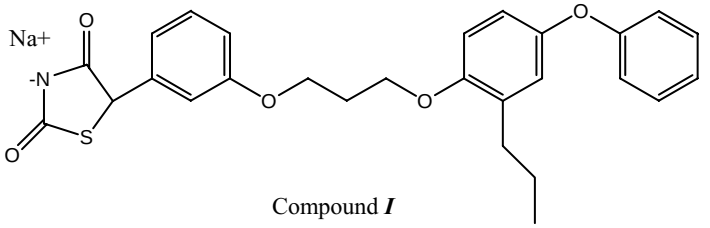
2. Experimental

2.1. Reagents and materials

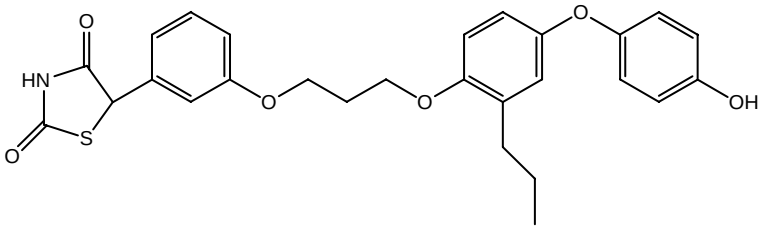
I, **II** and **III** (internal standard) were obtained from Merck Research Laboratories (Rahway, NJ, USA, Fig. 2). Purity of the analytes was 99.3% for **I** and 99.5% for **II**. Optima grade ethyl acetate, methanol, acetonitrile and ammonium hydroxide were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid (99%) and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Control human plasma (heparinized) was obtained from Sera-Tec Biologicals (New Brunswick, NJ, USA).

2.2. Equipment

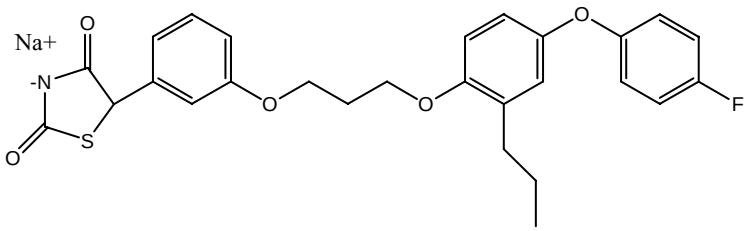
The LC–MS/MS system consisted of a PE Sciex (Thornhill, Ont., Canada) API 3000 mass spectrometer with a turbo ionspray interface and two Perkin-Elmer (Norwalk, CT, USA) Series 200 high pressure



Compound ***I***



Compound ***II***, *Para*-Hydroxylated Metabolite



Compound ***III***, Internal Standard

Fig. 2. Structures of the compound, metabolite and internal standard investigated in this study.

mixing pumps. A Perkin-Elmer Series 200 autosampler was used with a temperature-controlled tray set to 4 °C. A Model 7990 Jones Chromatography (Hegoed, Wales, UK) column heater was set to 40 °C. Data was processed using MacQuan software (Version 1.5, PE Sciex) on a Power Macintosh G3.

2.3. Instrumental conditions

The mass spectrometer was operated in the negative ion mode using the turbo ionspray interface. The monitored ion transitions were m/z 476.1 → m/z 433.2 for **I**, m/z 492.1 → m/z 449.2 for **II** and m/z 494.1 → m/z 451.2 for the internal standard (**III**). The MRM signal was optimized by co-infusion of a 500 ng/ml standard for each analyte with mobile phase. Mass spectrometer parameters including temperature, curtain and collision gases, ionspray voltage, and Q1 and Q3 voltages were optimized to obtain the highest sensitivity for the monitored transitions. The negative product ion spectra for **I**, **II** and **III** are shown in Fig. 3.

The analytical column used to separate the two analytes and the internal standard was an Xterra MS C8 (50 mm × 2.1 mm, 3.5 μm) from Waters (Milford, MA, USA). Mobile phase A consisted of ammonium acetate (pH 9.6; 0.1 mM) and mobile phase B contained acetonitrile:methanol (50:50, v/v). The analytes were separated using a gradient consisting of a starting composition of A/B (60:40, v/v) for 4 min, followed by a linear gradient to A/B (10:90, v/v) for 4 min ending with a 2 min hold at A/B (10:90, v/v). The column was equilibrated for 3.5 min before each injection. Data was collected for 10 min. The flow rate was 0.2 ml/min and the column temperature was 40 °C.

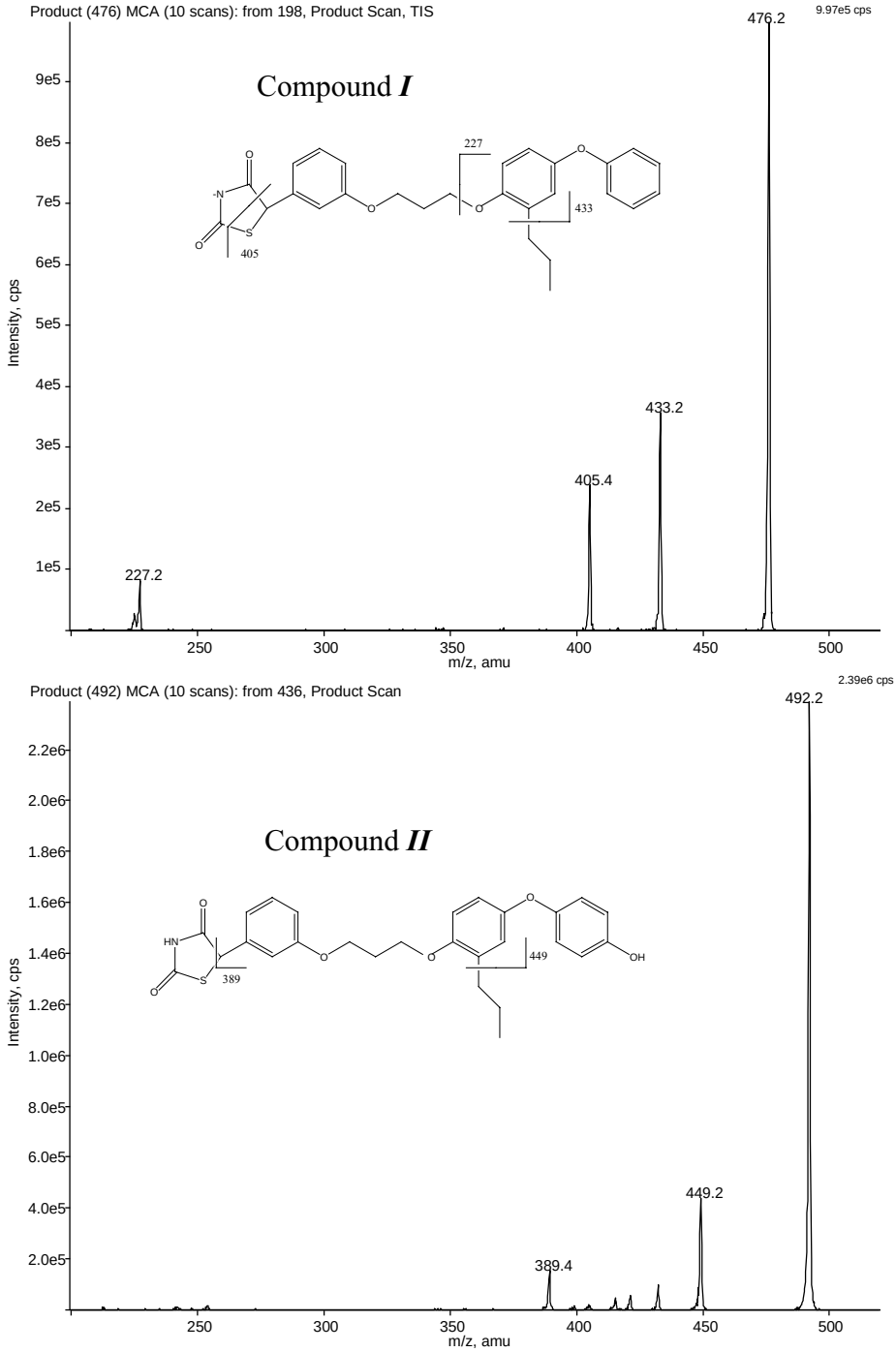


Fig. 3. Product ion mass spectra for **I**, **II** (metabolite) and **III** (internal standard).

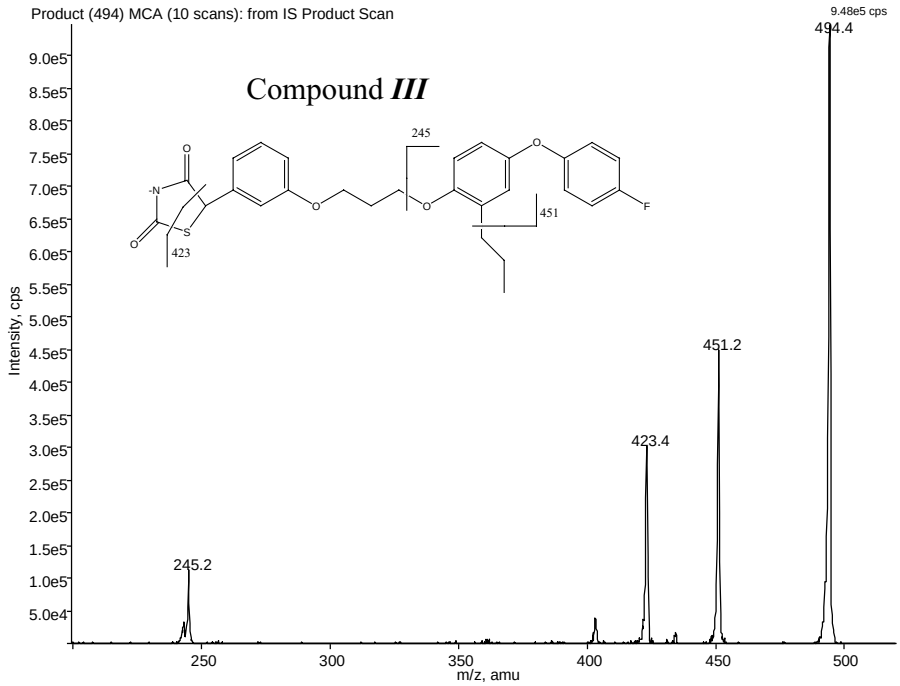


Fig. 3. (*Continued*).

2.4. Plasma standard preparation

Primary stock solutions of **I**, **II** and **III** were prepared at a concentration of 100 μg/ml in methanol. Salt factors for **I** and **III** were used so that concentrations of the analytes were determined as the free base. Stock solutions for **I** and **II** were further diluted in methanol:0.1% formic acid (50:50, v/v) to make a series of working standard solutions. Concentrations for compound **I** working standards were 0.25, 0.5, 1, 2.5, 10, 25, 50 and 100 ng/ml. Concentrations for compound **II** working standards were 0.5, 1, 2.5, 5, 10, 25, 50 and 100 ng/ml. The working solution for the internal standard (**III**) was prepared at a concentration of 20 ng/ml in methanol:0.1% formic acid (50:50, v/v). All of the standard solutions were stored at −20 °C. Primary stock solutions were stable for up to 1 month and working solutions were stable for up to 1 week. Plasma standards were prepared daily by adding 0.1 ml of each working standard to 0.5 ml of control human plasma, resulting in concentrations ranging from 0.05 to 20 ng/ml for **I** and 0.1 to 20 ng/ml for **II**.

2.5. Quality control sample preparation

Primary quality control (QC) standard solutions were prepared separately for **I** and **II** at concentrations of 100 μg/ml in methanol. For **I**, the solution was diluted in methanol:water (50:50, v/v) to concentrations of 1500, 300 and 15 ng/ml for high, medium and low QCs, respectively. The primary solution for **II** was diluted in methanol:water (50:50, v/v) to concentrations of 1500, 300 and 30 ng/ml for high, medium and low QCs, respectively. QC samples were prepared by adding 0.5 ml of the appropriate working solution to a 50 ml glass volumetric flask containing control human plasma. QC plasma concentrations were 15, 3 and 0.15 ng/ml for **I** and 15, 3 and 0.3 ng/ml for **II**. QC plasma samples were stored in amber glass vials at −70 °C until assayed.

2.6. Extraction procedure

Frozen plasma samples were thawed at room temperature prior to extraction. A 0.5 ml aliquot of plasma was added to a 13 mm × 100 mm borosilicate,

screw-top, glass tube. Internal standard (0.05 ml of the 20 ng/ml solution) and 0.2 ml methanol:0.1% formic acid (50:50, v/v) was added, followed by 0.5 ml of ammonium acetate (pH 9.6; 50 mM). Finally, ethyl acetate (5 ml) was added and the tubes were capped. The samples were vortexed for 1 min on a multi-tube vortexer on the highest setting and then centrifuged for 5 min at 2060 × *g*. The aqueous layer was frozen in a dry ice/acetone bath and the organic layer transferred to clean 13 mm × 100 mm borosilicate glass culture tubes. The ethyl acetate was evaporated to dryness under nitrogen in a Zymark Turbovap (Hopkinton, MA, USA) at 35 °C for 45 min. The samples were reconstituted in 0.1 ml acetonitrile:0.1% formic acid (65:35, v/v), transferred to amber glass autosampler vials with 0.2 ml glass inserts, and 20 μl was injected for analysis.

2.7. Quantitation

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

3. Results and discussion

3.1. LC–MS/MS conditions

Preclinical drug metabolism studies determined that the *para*-hydroxylated metabolite (**II**) was a major metabolite in various species (data not published). Preclinical studies also indicated the formation of other minor hydroxy metabolites, including those formed by the placement of hydroxy groups at the *ortho*- or *meta*-position of the benzene ring. The amount of the *ortho*- and *meta*-hydroxy metabolites present in clinical samples was unknown, but their presence could lead to interference during analysis. The mass spectrometric fragmentation pattern could not differentiate the placement of the OH group on the benzene ring (see Fig. 3), thus the separation had to be performed chromatographically. Synthetic standard material was only available for the hydroxy metabolite formed on the *para*-position of the ring. As an alternative to pure standards, **I** was incubated in human liver microsomes to produce all the metabolites of interest. When the incubation was complete, the sample was extracted using the method described in Section 2.6. Chromatographic gradients were evaluated based on the separation achieved between the various metabolites in the sample that were formed during the incubation. Fig. 4 illustrates the resulting chromatogram using the gradient procedure outlined in Section 2.3. As observed in the top chromatogram in Fig. 4, all hydroxy metabolites were separated from **II**, and there was no apparent interference from the *ortho*- and *meta*-hydroxy metabolites during integration and quantitation of **II**.

3.2. Sensitivity and linearity

Due to the low doses projected for the clinical study, a sensitive method was imperative. Sensitivity, or the LOQ, was defined as the lowest concentration of the standard curve that could be measured with acceptable precision and accuracy. The limit of quantitation was 0.05 ng/ml for **I** and 0.1 ng/ml for **II** using 0.5 ml plasma. For each analyte, the linear dynamic range was from LOQ to 20 ng/ml. For all standard curves, the correlation coefficient using weighted (1/*x*) linear least-squares regression was >0.999 for both analytes.

3.3. Accuracy and precision

Intraday accuracy and precision for the method was determined from the analysis of five standard curves containing both **I** and **II**. Peak area ratios of **I** or **II** to the internal standard (**III**) were used for the determination of the coefficient of variation (CV, %). CVs ranged from 1.52 to 6.92% for **I** and 1.58 to 9.13% for **II**. Accuracy was determined by the comparison of mean back-calculated concentrations to nominal concentrations. Accuracy ranged from 90.0 to 104.5% for **I** and 94.0 to 104.0% for **II**. Intraday precision and accuracy are summarized in Table 1.

Inter- and intra-assay precision and accuracy were also evaluated by the analysis of quality control samples. Intra-assay precision was determined by the replicate ($n = 5$) analysis of high, medium and low QC samples. Precision and accuracy for the intraday analysis of QC samples was assessed by comparison

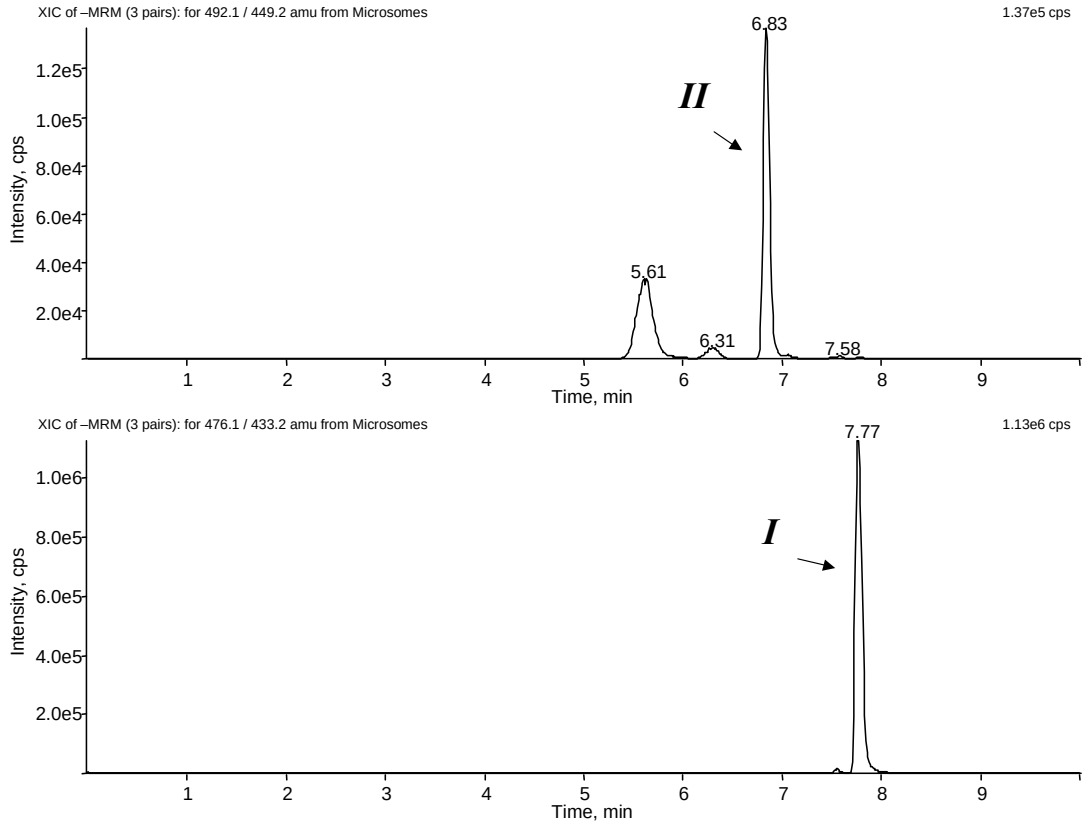


Fig. 4. Separation of **I** and **II** from other potentially interfering hydroxy metabolites in a human liver microsome sample.

of the mean calculated concentrations to nominal concentrations. Accuracy ranged from 101.0 to 107.3% for **I** and 97.4 to 113.3% for **II**. CVs were calculated for the intra-assay analysis and found to be $\leq$2.53% for **I** and $\leq$5.88% for **II**. Results from the intra-assay analysis indicate that the method is accurate and reliable. Intra-assay QC data are summarized in Table 2.

Table 1
Intraday precision and accuracy for **I** and **II** in human plasma

Compound **I**				Compound **II**			
Nominal concentration (ng/ml)	Mean concentration (ng/ml)	Precision[a]	Accuracy[b]	Nominal concentration (ng/ml)	Mean concentration (ng/ml)	Precision[a]	Accuracy[b]
0.05	0.045	6.92	90.0	0.1	0.10	8.76	100.0
0.1	0.099	3.53	99.0	0.2	0.20	9.13	100.0
0.2	0.209	4.02	104.5	0.5	0.47	7.92	94.0
0.5	0.518	2.41	103.6	1	0.98	6.93	98.0
2	2.025	2.41	101.3	2	2.01	2.85	100.5
5	5.152	2.10	103.0	5	5.05	3.18	101.0
10	10.053	1.52	100.5	10	9.83	1.58	98.3
20	19.750	2.66	98.8	20	20.15	2.00	100.8

[a] Expressed as CV (%).
[b] Accuracy (%) $= \frac{\text{mean found concentration}}{\text{nominal concentration}} \times 100$.

Table 2
Intraday precision and accuracy for human plasma quality control samples containing **I** and **II**

	Nominal concentration (ng/ml)	Mean found concentration (ng/ml)	Precision[a]	Accuracy[b]
Compound **I**				
High	15	15.155	1.25	101.0
Medium	3	3.219	2.33	107.3
Low	0.15	0.158	2.53	105.3
Compound **II**				
High	15	14.61	1.85	97.4
Medium	3	3.05	3.61	101.7
Low	0.3	0.34	5.88	113.3

[a] Expressed as CV (%).
[b] Accuracy (%) $= \frac{\text{mean found concentration}}{\text{nominal concentration}} \times 100$.

Inter-assay precision and accuracy were determined by the replicate ($n = 4$) analysis of high, medium and low quality control samples on five different days. CVs for interday analysis were ≤4.79% for **I** and ≤7.33% for **II**. Accuracy ranged from 102.6 to 111.9% for **I** and 98.1 to 107.2% for **II**. The results of interday analysis indicate that the method is accurate and reproducible from day-to-day. The data are summarized in Table 3.

3.4. Sample stability

During method development, very poor recoveries were observed when polypropylene tubes were used during the extraction procedure. The results suggested that both **I** and **II** adhered to polypropylene, leading to the use of glass tubes during the extraction procedure and glass vials for sample collection and storage. Additionally, standard solutions of **I** and **II** in methanol were stable for 1 month but stable for only 1 week in methanol:0.1% formic acid (50:50, v/v). Stability of the analytes in plasma and reconstitution solutions was determined under various conditions including prolonged time in the autosampler, exposure to at least three freeze-thaw cycles, and storage at room temperature.

Due to the need for occasional delayed injection or re-injection of extracted samples, stability of **I** and **II** in the final reconstituted extract was evaluated. Twelve replicates of each QC level were extracted. Four replicates were stored at 4 °C for 24 h and four replicates were stored at room temperature for 24 h. The last four replicates were not analyzed immediately after extraction but were placed in a refrigerated (4 °C) autosampler tray and analyzed after 48 h. In order to determine stability, calculated concentrations of the QCs stored for 24 and 48 h at 4 °C on the autosampler tray and 24 h at room temperature were compared to calculated concentrations of QCs extracted on the same day of analysis. The results indicate that **I** was stable for up to 48 h at 4 °C. However, **II** was only stable for 24 h at 4 °C. **I** was stable for 24 h at room temperature but the results indicated that **II** was not stable in reconstitution solution for longer than 24 h at room temperature. As a result, all clinical samples were analyzed within 24 h of the extraction and a temperature-controlled autosampler tray was set at 4 °C.

Table 3
Interday accuracy and precision of **I** and **II** in human plasma

	Compound **I**			Compound **II**		
	High	Medium	Low	High	Medium	Low
Nominal concentration (ng/ml)	15.000	3.000	0.150	15.00	3.00	0.30
Day 1[a]	14.552	3.014	0.178	14.35	2.92	0.32
Day 2[a]	15.155	3.289	0.171	13.46	2.77	0.30
Day 3[a]	15.722	3.429	0.164	14.82	3.11	0.31
Day 4[a]	15.614	3.295	0.163	15.84	3.29	0.35
Day 5[a]	15.892	3.353	0.164	15.14	3.28	0.33
Mean found concentration (ng/ml)	15.387	3.276	0.168	14.72	3.07	0.32
CV (%)	3.51	4.79	3.86	6.04	7.33	6.75
Accuracy (%)[b]	102.6	109.2	111.9	98.1	102.5	107.2

[a] $n = 4$ replicates per sampling day, mean concentrations of the samples is reported.
[b] Accuracy (%) $= \frac{\text{mean found concentration}}{\text{nominal concentration}} \times 100$.

Freeze-thaw stability was evaluated for **I** and **II** using replicate ($n = 4$) QC samples from each level exposed to three freeze-thaw cycles. Each cycle consisted of removing the QCs from the freezer, thawing the replicate samples at room temperature for up to 4 h and re-freezing at −70 °C. The samples were processed along with a standard curve and a control set of QCs that had been thawed just prior to extraction. Stability was determined by comparison of the mean calculated concentrations of the freeze-thaw samples to the mean calculated concentrations of the control QCs. The results indicate that **I** and **II** have acceptable stability after three freeze-thaw cycles in human plasma.

Stability of **I** and **II** in plasma at room temperature for 24 h was evaluated using replicate ($n = 4$) QC samples. QCs were removed from the freezer and left at room temperature for 24 h. The samples were then processed along with a standard curve and a control set of QCs that was thawed just prior to the extraction. Stability was determined by comparing the mean calculated concentrations of the 24 h room temperature plasma sample to the mean concentrations of the control QCs. The results indicated variable stability for **I** and moderate stability for **II** after 24 h in plasma at room temperature. The results suggest that plasma samples should not be stored at room temperature for 24 h. Stability data are summarized in Table 4.

3.5. Recovery and matrix effect

Extraction recovery of the analytes was determined by analyzing extracts of five replicate plasma samples containing **I** and **II** in human control plasma at three different concentrations (0.1, 2 and 20 ng/ml). The internal standard (**III**) was evaluated only at the concentration used during extraction (2 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. Recovery was determined by comparing mean peak areas of **I**, **II** and **III** from the spiked plasma samples to the mean peak areas of the corresponding spiked extract samples. Overall recovery for **I** in human plasma was 59.4%, overall recovery of **II** was 90.1%, and recovery of **III** was 56.8%. Recovery data are summarized in Table 5.

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 [7–9]. The matrix effect for this method was evaluated by comparing the peak areas of analytes from neat standard to those of neat standard added to control blank plasma extracts ($n = 5$ lots of control plasma). The results indicated a signal enhancement for **I** and a moderate amount of signal suppression for **II** when comparing the spiked plasma extracts to the neat standards. Attempts to diminish the matrix effect for **I** or **II** resulted in increased suppression or enhancement of the other analyte. However, the experimental conditions described in Sections 2.3 and 2.6 produced the lowest amount of matrix effect for each compound when simultaneously determining the concentrations of the analytes in plasma.

Table 4
Stability of compounds **I** and **II** in human plasma and extracts from human plasma under various conditions

QC level	Compound **I**			Compound **II**		
	High[a] 15 ng/ml	Medium[a] 3 ng/ml	Low[a] 0.15 ng/ml	High[a] 15 ng/ml	Medium[a] 3 ng/ml	Low[a] 0.3 ng/ml
Autosampler stability of plasma extracts						
24 h 4 °C	100.4	100.3	101.2	96.7	95.8	96.8
48 h 4 °C	103.4	95.1	99.4	99.2	92.4	91.4
24 h room temperature	106.3	101.7	107.9	103.8	96.8	103.2
Plasma at ambient temperature						
24 h	98.8	98.3	82.6	93.7	92.1	90.6
Freeze-thaw cycles						
Three cycles	102.9	100.5	107.9	104.3	104.5	109.7

[a] Stability was determined by the accuracy of mean QC calculated concentrations under experimental conditions to the mean QC concentrations under normal conditions.

Table 5
Recovery of **I**, **II** and **III** in human plasma

Nominal concentration	Compound **I**			Compound **II**			Compound **III** (IS)
	0.1 ng/ml	2 ng/ml	20 ng/ml	0.2 ng/ml	2 ng/ml	20 ng/ml	2 ng/ml
Blank extracted control plasma spiked with neat standards							
Mean peak area[a]	2224	45507	417450	1986	25298	264570	92674
CV (%)	8.9	5.9	3.2	8.2	9.9	8.6	7.5
Control plasma spiked with neat standards and extracted							
Mean peak area[a]	1351	25861	252561	2239	19971	208109	52652
CV (%)	23.8	14.3	11.2	6.4	3.2	4.6	17.1
Recovery[b] (%)	60.8	56.8	60.5	112.7	78.9	78.7	56.8

[a] Mean peak area determined from $n = 5$ samples at the noted concentrations for compounds **I** and **II** and $n = 15$ samples at the concentration used in sample analysis for compound **III** (IS).
[b] Recovery (%) $= \frac{\text{mean peak area spiked plasma}}{\text{mean peak area spiked extract}} \times 100$.

The matrix effect was thought to be due to the presence of residual protein in the extracted plasma samples. **I** had been shown to be highly bound to protein in preclinical studies (data not published). An experiment was performed where a 5% bovine serum albumin solution in 0.85% sodium chloride (Sigma, St. Louis, MO, USA) was added to the neat standards to equal 1% of the sample volume (final albumin concentration

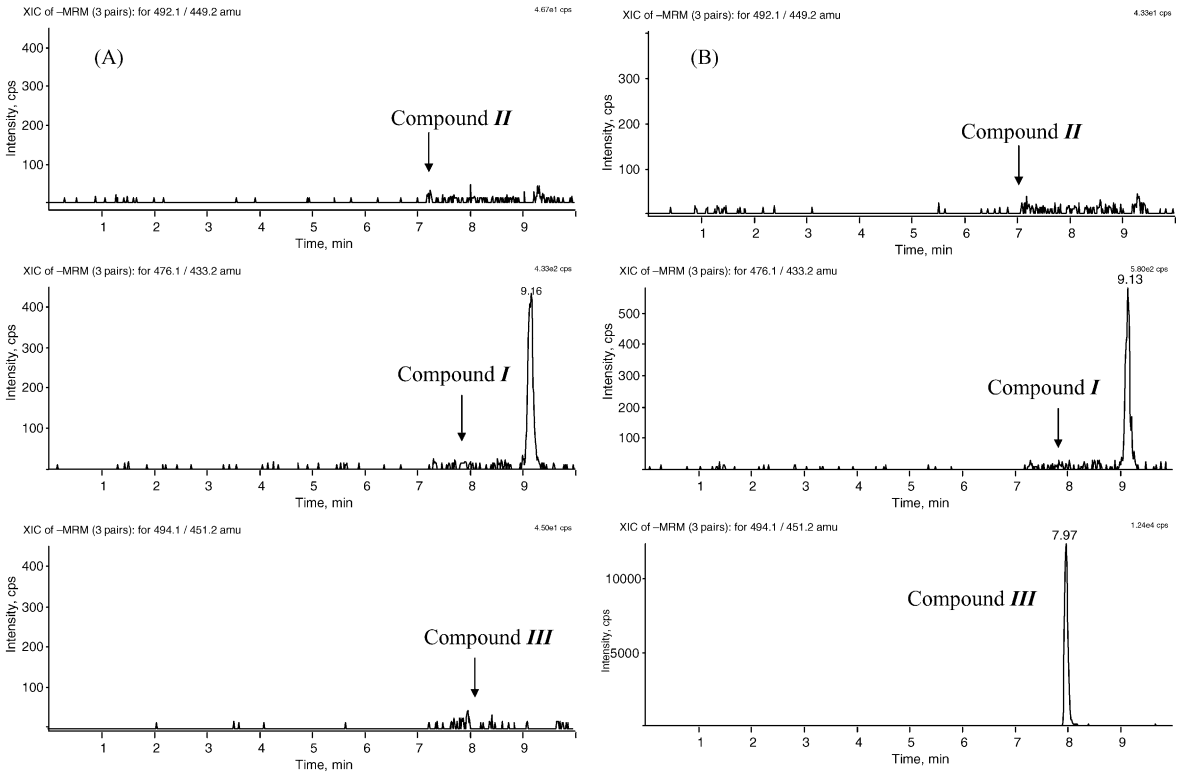


Fig. 5. Representative chromatograms of **I** and **II** in control human plasma. (A) Blank control human plasma, (B) control human plasma with internal standard (**III**, 2 ng/ml), (C) control human plasma with **I** (0.05 ng/ml, LOQ), **II** (0.1 ng/ml, LOQ) and internal standard (**III**, 2 ng/ml).

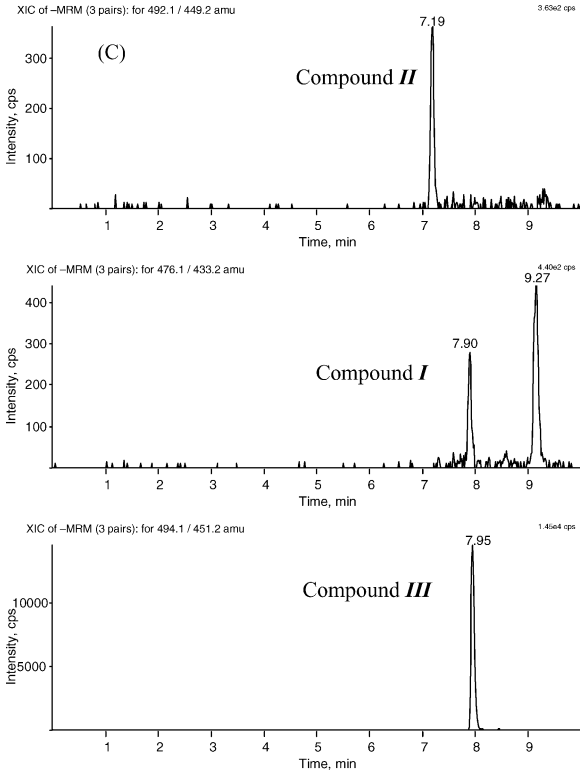


Fig. 5. (*Continued*).

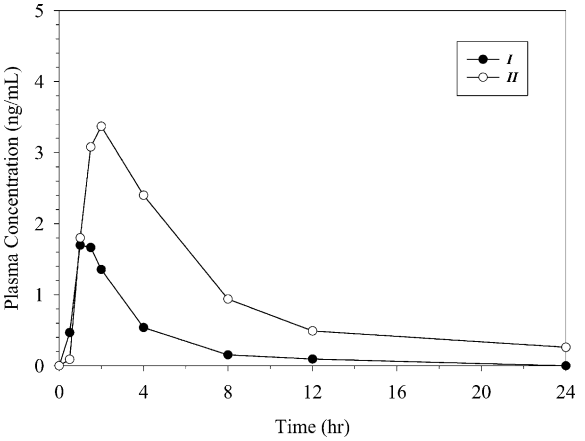


Fig. 6. Representative plasma concentration profiles of **I** and **II** following a single oral 0.2-mg dose of **I**.

was 0.05%). No apparent matrix effect was observed when peak areas of neat standards containing albumin were compared to peak areas of neat standards added to blank extracted plasma. Five different lots of control plasma were used for the experiment. Furthermore, results obtained from the analysis of QC samples prepared from various lots of control plasma showed that the matrix effect did not interfere with the accurate quantitation of the analytes.

3.6. Specificity

The specificity of the method was determined by extracting and analyzing control human plasma from five different sources. Blank samples containing no standard and blank samples containing the internal standard (**III**) were analyzed. Chromatograms indicate that the assay was selective and specific for **I**, **II** and the internal standard. There was no detectable interference in any of the plasma samples at the retention times of **I**, **II** and **III**. The unidentified peak at 9.2 min appears to be an artifact from the extraction procedure (see Fig. 5). This peak did not interfere with the integration of **I** but was present in all lots ($n = 5$) of blank control plasma that were tested as well as in the predose plasma samples from study subjects. Representative chromatograms of plasma spiked with no analytes, plasma spiked with internal standard and plasma spiked with **I** and **II** at the LOQ are shown in Fig. 5.

3.7. Application of the method

The method was used to analyze plasma samples from a clinical study. Representative plasma concentration profiles of **I** and **II** following a 0.2-mg single oral dose of **I** are shown in Fig. 6. QC samples were analyzed with the clinical samples to monitor daily performance of the method. Results from the analysis of QC samples illustrate the robustness of the method. CVs for the QC samples were ≤8.1% for **I** and ≤5.7% for **II**. For all QCs analyzed, accuracy averaged 108.1% for **I** and 109.3% for **II**.

4. Conclusions

Reproducibility, specificity and sample stability were assessed under various conditions for the analysis of **I** and its *para*-hydroxylated metabolite, **II**, in human plasma. Turbo ionspray LC–MS/MS operated

in negative ion mode was successfully used to quantify the analytes at low levels (0.05 ng/ml for **I** and 0.1 ng/ml for **II**), making it possible to accurately analyze clinical samples from subjects receiving doses of only 0.1 or 0.2 mg of compound **I**. Stability of the analytes in solution was accounted for by preparing fresh working solutions weekly. Samples were collected in glass Vacutainers and vials in the clinic and extracted in glass tubes to prevent loss of the analytes to polypropylene. Experimental results show that liquid–liquid extraction followed by LC–MS/MS analysis is sensitive, accurate, selective and reproducible for the analytes of interest. This method provides good sensitivity and selectivity for the analytes using a chromatographic gradient and a relatively short run time.

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