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Stabilization and determination of a PPAR agonist in human urine using automated 96-well liquid–liquid extraction and liquid chromatography/tandem mass spectrometry

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Abstract

Two stability challenges were encountered during development of an urine assay for a proliferator-activated receptor (PPAR) agonist, **I** (2-{[5,7-dipropyl-3-(trifluoromethyl)-1,2-benzisoxazol-6-yl]oxy}-2-methyl propionic acid), indicated for the treatment of Type II diabetes. First, the analyte was lost in urine samples due to adsorption on container surface which is a common problem during clinical sample handling. Secondly, the acylglucuronide metabolite (**III**), a major metabolite of **I**, displayed limited stability and effected the quantitation of parent drug due to the release of **I** through hydrolysis. Therefore, a clinical collection procedure was carefully established to stabilize **I** and its acylglucuronide metabolite, **III**, in human urine. The metabolite was not quantitated with this method. The urine samples are treated with bovine serum albumin (BSA) equal to 1.75% of the urine volume and formic acid equal to 1% of urine volume. Compound (**I**) and internal standard (**II**) were extracted from urine with 1 mL ethyl acetate using a fully automated liquid–liquid extraction in 96-well plate format. The analytes are separated by reverse phase high-performance liquid chromatography (HPLC) with tandem mass spectrometry in multiple-reaction-monitoring (MRM) mode used for detection. The urine method has a lower limit of quantitation (LLOQ) of 0.05 ng/mL with a linearity range of 0.05–20 ng/mL using 0.05 mL of urine. The method was validated and used to assay urine clinical samples.

Keywords: Bovine serum albumin; LC–MS/MS; Urine; Stabilization of acylglucuronide metabolite; Automated liquid–liquid extraction

1. Introduction

A method was developed and validated to determine the urinary excretion of a proliferator-activated receptor (PPAR) agonist (**I**, 2-{[5,7-dipropyl-3-(trifluoromethyl)-1,2-benzisoxazol-6-yl]oxy}-2-methyl propionic acid) in clinical samples. The drug compound (**I**) is an α PPAR agonist with some λ activity which was investigated for the treatment of Type II diabetes. (Fig. 1) Sensitive and reliable human plasma and urine assays were needed to support initial Phase I human clinical trials which assess safety, tolerability and pharmacokinetics of **I** in healthy volunteers. A sensitive bioanalytical method to support clinical plasma sample analysis has been developed and validated [1]. However, human urine sample analysis presented unique method development challenges.

Two stability challenges were encountered during development of the urine assay for **I**. First, the analyte was lost during urine sample handling and transfer due to adsorption on container surface. Compound **I** was highly bound to proteins (≥99.9% in vitro in all species) and showed no absorption to plastic containers in plasma. When the plasma assay was applied to urine, the analyte had low recoveries from urine. Since **I** was stable in plasma, the results indicated that the pre-extraction sample handling and storage needed to be evaluated which is discussed within. This is a common problem during clinical sample handling of urine samples which has been previously addressed in the literature. To address this challenge, reported methods have added control human plasma [2], bovine serum albumin [2–4], or detergents such as Tween-20 and Tween-40 [5].

Secondly, the stabilization of the acylglucuronide metabolite in urine samples during the pre-extraction urine sample handling, extraction and LC–MS/MS analysis was addressed during method development. The preclinical metabolism studies indi-

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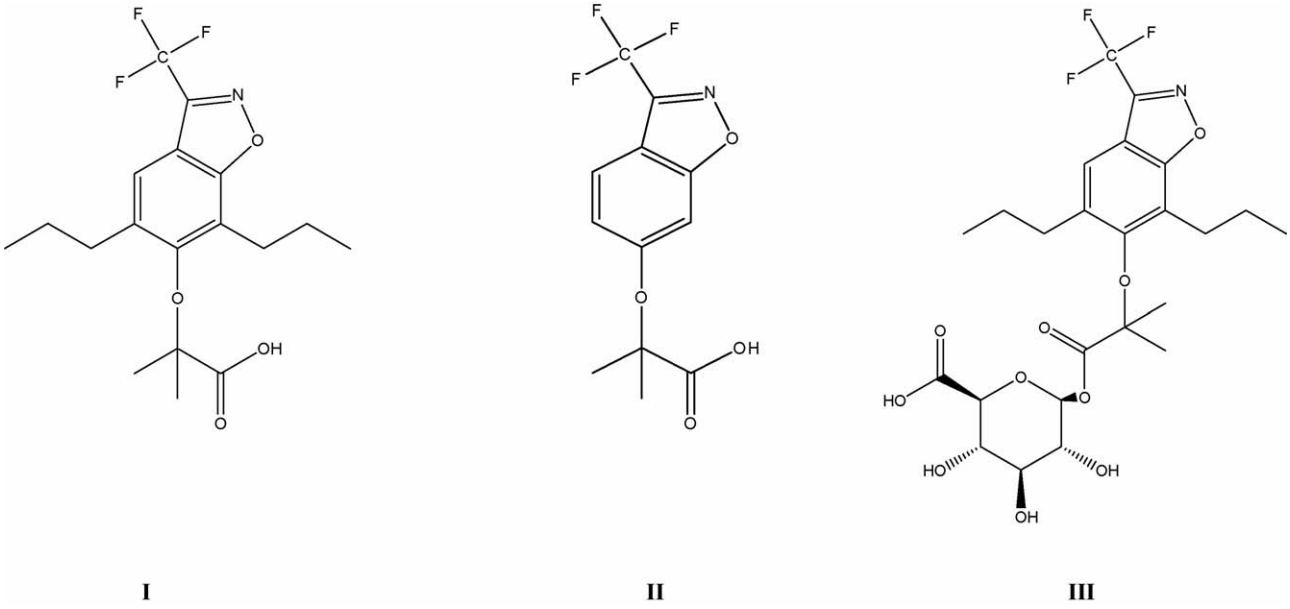

Fig. 1. Chemical structures of **I**, **II** and **III**.

cated **I** has an acylglucuronide metabolite (**III**) (Fig. 1) which circulated in animal plasma samples in low amounts. Analysis of initial clinical plasma samples indicated that **III** circulated at levels equal to or greater than drug compound. As previous studies have shown, acylglucuronide metabolites can hydrolyze to parent drug in slightly alkaline or neutral conditions [6,7]. This contribution to the drug compound would lead to inaccurate determination of analyte concentrations. Therefore, the stabilization of the **III** in urine samples was also evaluated during method development of the urine assay although **III** was not quantitated with this method.

The development of the clinical collection procedure for **I** in human urine taking into consideration challenges associated with absorption of **I** to collection containers and stabilization of the acylglucuronide metabolite is described within. Also, the development and validation of the fully automated 96-well liquid–liquid extraction with LC–MS/MS analysis is presented. By fully automating the liquid–liquid extraction [1,8], a reduction in extraction time and increase in sample throughput were possible which was essential for initial Phase I clinical studies.

2. Experimental

2.1. Materials

The compounds **I**, **II** and **III** were obtained from Merck Research Laboratories (Rahway, NJ) (Fig. 1). The internal standard (**II**, 2-{[3-(trifluoromethyl)-1,2-benzisoxazol-6-yl]oxy}-2-methyl propionic acid) was a structural analogue of **I**. Control human urine was provided by healthy male volunteers from Merck Research Laboratories (West Point, PA). Formic acid (99%) and bovine serum albumin (BSA) (35% solution) were purchased from Sigma (St. Louis, MO). All other chemicals and solvents were from Fisher Scientific (Fair Lawn, NJ).

2.2. Instrumentation

The automated liquid–liquid extraction method was performed on a Tomtec (Hamden, CT) Quadra 96, Model 320 and Tecan (Research Triangle Park, NC) Genesis RSP 150. The LC–MS/MS system consists of a PE Sciex (Thornhill, Ontario, Canada) API 4000 mass spectrometer with a Turbo IonSpray interface, a CTC PAL Leap Autosampler (Zwingen, Switzerland) and two Shimadzu (Kyoto, Japan) LC-10ADvp HPLC pumps with the SCL-10ADvp system controller and DGU-14A degasser. The column was a Phenomenex Hydro-RP 50 mm × 2 mm, 4 μm column (Torrance, CA). A Jones Chromatography (Lakewood, CO) 5795 column heater was set at 40 °C. The data were processed using PE Sciex Windows NT Analyst software (version 1.1).

2.3. Urine standards and quality controls

Using the molecular weight and purity of the standards, separate primary stock solutions of **I** and **II** were prepared at 100 μg/mL in methanol. The primary stock solution of **I** was further diluted to give a series of working standard solutions with concentrations of **I** at 0.1, 0.2, 0.8, 2.0, 8.0, 20.0, and 40.0 ng/mL in methanol/water (50/50, v/v). Urine standard curve was prepared daily by adding 0.025 mL of each working standard to 0.05 mL of human control urine with 1% formic acid and 1.75% BSA. The resulting urine standard concentrations ranged from 0.05 to 20 ng/mL. Internal standard working solution was prepared at 1 ng/mL in methanol/water (50/50, v/v). All stock solutions were stable for at least 6 months when stored at −20 °C.

Quality control (QC) primary stock solution of **I** was prepared separately at 100 μg/mL in methanol. Appropriate amount of diluted solutions from stock was placed into a 25 mL volumetric flask and then filled with human control urine with

1% formic acid and 1.75% BSA to make concentration levels of 0.15, 2 and 16 ng/mL, representing low, medium and high QCs, respectively. The QC samples were aliquoted in 1 mL portions into 2 mL polypropylene tubes and stored at −70 °C until assayed.

2.4. Sample preparation

The liquid–liquid extraction of **I** from the urine matrix was automated using a combination of a Tecan Genesis RSP 150 and a Tomtec Quadra 96. Using a Tecan Genesis RSP 150, the urine samples, QCs and urine standards (0.05 mL) were aliquoted to a 2 mL 96-well polypropylene plate. The system also aliquoted 0.025 mL of 1 ng/mL internal standard (**II**) to all wells and 0.025 mL of methanol/water (50/50, v/v) to urine samples and QCs. Using a Tomtec Quadra 96, the samples were treated with 0.1 mL of 0.5% acetic acid in acetonitrile and analytes were extracted using 1 mL of ethyl acetate. The 2 mL 96-well plate was sonicated and centrifuged for 5 min at ∼3000 rpm. Using the Tomtec Quadra 96, the organic layer (ethyl acetate) was transferred to a clean 1 mL 96-well polypropylene plate. The ethyl acetate was evaporated under nitrogen to dryness. Samples were reconstituted in 0.2 mL of methanol/0.1% formic acid (50/50, v/v) and sonicated. Sample volume of 10 μL was injected for LC–MS/MS analysis.

Due to poor recoveries of the acylglucuronide metabolite (**III**) when applying the extraction method of **I**, an exploratory assay was developed to assess the stability of the **III** in urine. Urine samples were treated with 1% formic acid and 1.75% BSA. The acylglucuronide metabolite liquid–liquid extraction buffered 0.05 mL of human urine with 0.1 mL ammonium acetate, pH 6 and 0.05 mL of 1 ng/mL internal standard (**II**) was added. Metabolite and internal standard were extracted with 1.2 mL of ethyl acetate/isopropanol (95/5, v/v). The organic

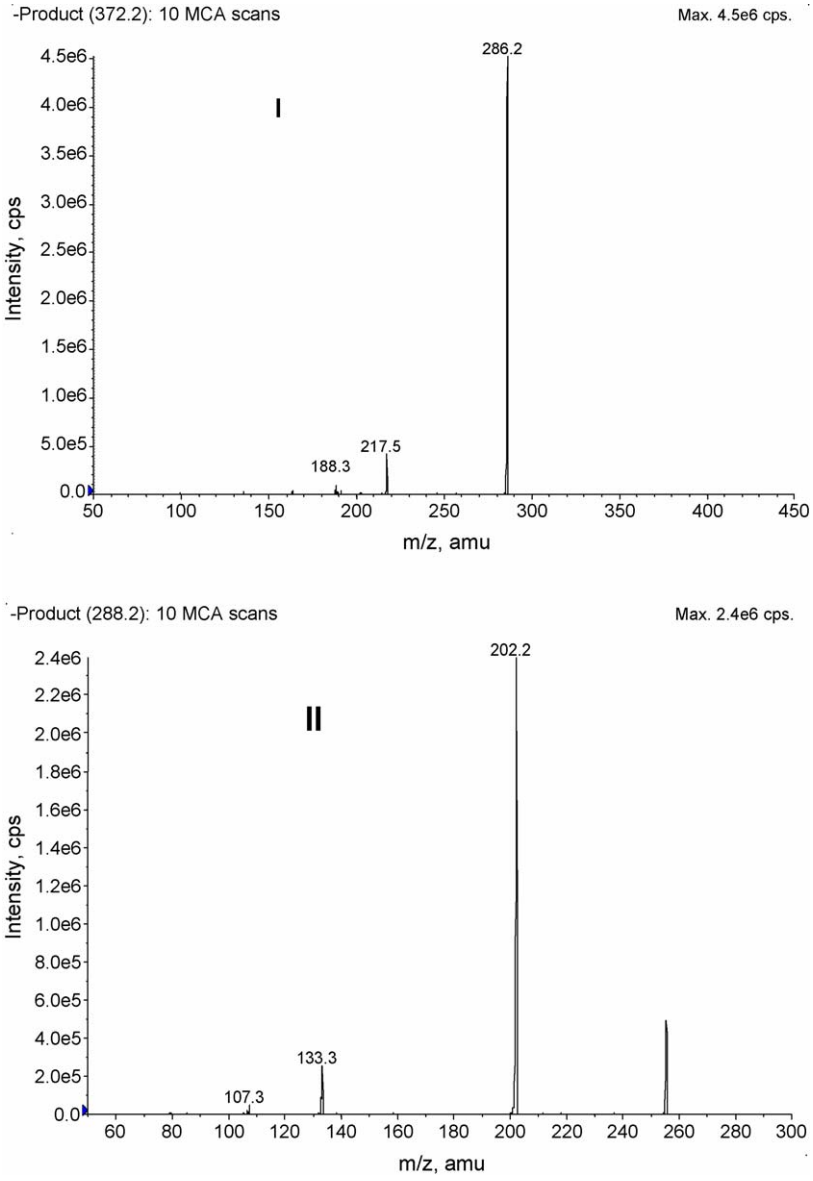

Fig. 2. Product ion scan spectra for **I** and **II** (internal standard).

layer was evaporated under nitrogen and reconstituted with 0.5 mL methanol/0.1% formic acid (50/50, v/v).

2.5. LC–MS/MS conditions

The separation of analytes was performed on a Phenomenex Hydro-RP column (50 mm × 2 mm, 4 μm) at column temperature of 40 °C. A gradient was used to separate **I**, **II**, and the acylglucuronide metabolite (**III**). The mobile phase was 0.1% formic acid and acetonitrile. The initial mobile phase composition was 45% organic. The gradient increased linearly over 3.5 min to a final mobile phase composition of 90% organic followed by a hold at 90% organic for 2.5 min. The column was then re-equilibrated at the starting conditions for 3 min.

The analytes are detected by a tandem mass spectrometer (MS/MS) with a Turbo IonSpray (TIS) interface in negative ionization mode. The negative product scan spectra for **I** and **II** are shown in Fig. 2. The precursor → product ion transitions are monitored in multiple reaction monitoring (MRM) mode. Based on the ionization of these compounds, the channel used for the quantitative determination of compound **I** was *m/z* 372.2 → *m/z* 286.2 and the internal standard (**II**) was monitored at *m/z* 288.2 → *m/z* 202.2. The acylglucuronide metabolite exploratory method used the LC–MS/MS conditions described above. The metabolite channel was added to monitor at *m/z* 548.0 → *m/z* 286.0.

Concentrations of **I** in clinical urine samples are calculated from the equation $y = mx + b$ as determined by the weighted $(1/x)$ linear least squares regression of the calibration curves. Calibration curves are constructed from peak area ratios of the analyte to the internal standard versus the concentrations of the analyte. The standard curve for **I** consisted of seven standards at the following concentrations: 0.05, 0.1, 0.4, 1.0, 4.0, 10 and 20 ng/mL.

3. Results and discussion

3.1. Clinical collection procedure

The acylglucuronide metabolite (**III**) needed to be stabilized to prevent conversion into drug **I**. Using the metabolite exploratory assay described, initial urine stability tests for **III** determined that there was some conversion of metabolite to parent during varying sample storage conditions. The conversion of **III** to **I** could lead to enhancement of calculated **I** concentration found in urine. Since acylglucuronide metabolites can hydrolyze to parent drug in slightly alkaline or neutral conditions, the urine was acidified to stabilize the metabolite. At an acylglucuronide concentration of 500 ng/mL, some conversion to parent was still observed when adding 0.1% formic acid (24% of **I** LLOQ peak area) and 1% acetic acid (21% of **I** LLOQ peak area). The optimal condition identified was 1% formic acid in urine with no conversion being detected across different lots of urine.

When initial pre-extraction condition (addition of with 1% formic acid to control urine) was applied to urine quality controls samples for acylglucuronide and drug compound, recovery of **I** and **III** were low and varied across lots of urine. (Tables 1 and 2) For **III** QC samples, no drug compound was determined chromatographically at the concentrations tested which indicated the loss of metabolite was not due to conversion to drug compound. Drug **I** is highly bound to proteins in plasma (≥99.9% in vitro in all species) and stable in human plasma. To create an environment more like plasma, BSA (35% solution) was added to urine samples. The BSA adds protein to the urine matrix which the analytes could bind to, instead of the walls of the polypropylene plates. During method development, varying percentages of BSA from 0.35 to 3.5% were tested to determine the optimum conditions to stabilize both **I** and **III** in urine. The addition of albumin equal to 1.75% of the total volume of the sample was able to increase accuracy from 28.7% to an accuracy

Table 1
Stability for quality controls of **I** in human urine with and without BSA

	1% Formic acid (lot #1)			1% Formic acid (lot #2)	1% Formic acid, 1.75% BSA (lot #2)		
Nominal concentration of **I** (ng/mL)	0.12 ($n=4$)	2 ($n=4$)	16 ($n=4$)	10 ($n=3$)	0.05 ($n=3$)	1.0 ($n=3$)	10.0 ($n=3$)
Mean (CV%)	0.05 (9.6)	1.02 (3.1)	8.78 (6.0)	2.90 (22.5)	0.05 (10.1)	0.99 (1.7)	10.53 (4.7)
Accuracy (%)[a]	42.0	50.9	54.9	28.7	104.1	99.5	105.3

Quality control samples were analyzed after being stored at −70 °C overnight and stored on the benchtop at room temperature for ∼2 h.
[a] Expressed as [(mean calculated concentration)/(nominal concentration)] × 100%.

Table 2
Stability for quality controls of **III** in human urine with and without BSA

	1% Formic acid		1% Formic acid, 1.75% BSA	
Nominal concentration of **III** (ng/mL)	50 ($n=3$)	500 ($n=3$)	50 ($n=3$)	500 ($n=3$)
Mean (CV%)	40.2 (43.8)	316.7 (4.6)	51.3 (14.7)	477.3 (6.2)
Accuracy (%)[a]	80.5	63.3	102.7	95.5

Quality control samples were analyzed after being stored at −70 °C overnight and stored on the benchtop at room temperature for ∼2 h.
[a] Expressed as[(mean calculated concentration)/(nominal concentration)] × 100%.

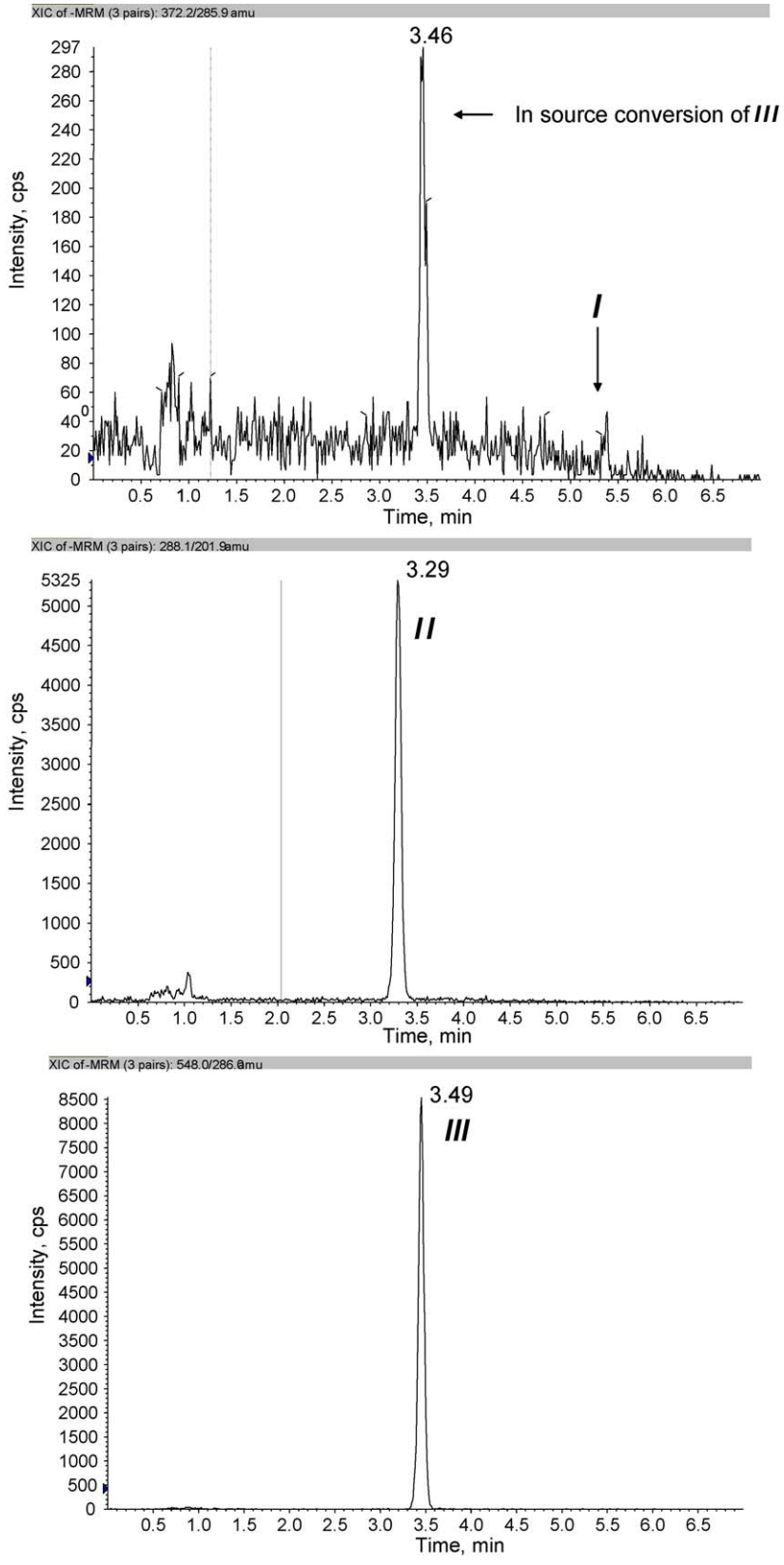

Fig. 3. Representative chromatogram of acylglucuronide sample with internal standard (500 ng/mL of **III** and 1 ng/mL **II**).

range of 99.5–105.3% for **I**. (Table 1) The addition of albumin also improved the stability of **III** in urine with accuracy of 95.5–102.7% (Table 2).

Fig. 3 demonstrates that at 500 ng/mL of **III** no conversion is determined during the storage and extraction procedure. Although in-source conversion of **III** was observed at the given LC–MS/MS conditions, the metabolite was separated chromatographically and does not interfere with quantitation of **I**. Also, the in-source conversion was decreased by detecting analytes with a Turbo IonSpray interface source instead of the harsher ionization of the heated nebulizer source.

The final clinical collection procedure which was used for initial Phase I clinical trials stabilizes both **I** and **III** in human urine added: (a) BSA equivalent to 1.75% urine volume and (b) formic acid equivalent to 1% urine volume.

3.2. Extraction method

The acylglucuronide conversion during the extraction method was considered during development. Conditions were evaluated to stabilize **III** during the extraction method. With regard to acylglucuronide stability, the most significant step of the extraction process was the reconstitution step. By reconstituting in methanol/0.1% formic acid (50/50, v/v), **III** is stabilized in the extracted samples which could be stored on the autosampler for up to 48 h.

3.3. Method validation and specificity

The validated urine method is a sensitive method from a limited sample size. The lower limit of quantitation (LLOQ) was 0.05 ng/mL for **I** using 0.05 mL of urine with a linear range of 0.05–20 ng/mL. Representative standard curve parameters have a slope of 0.599 and *y*-intercept of 0.00313 with a correlation coefficient of greater than 0.997 for analytical runs completed.

Human control urine from five different sources were treated with formic acid and BSA (35% solution). Double blank and blank samples with internal standard were extracted and analyzed to assess the specificity of the method. No significant interferences were detected at the retention times of **I** and **II**. Representative chromatograms for blank control urine (A), control urine spiked with **II** (1 ng/mL) (B), and control urine spiked with **I** at LOQ (0.05 ng/mL) and **II** (1 ng/mL) (C) are given in Fig. 4.

Method was validated by analyzing five replicates of calibration standards at all concentrations. The precision (%CV, $n = 5$) at LOQ (0.05 ng/mL) was 9.42% for **I** with an accuracy (percentage of nominal value) of 94.8% for **I**. For all concentration levels in the standard curve, the precision varied from 3.67 to 9.42% for **I**, and the accuracy ranged from 93.0 to 106.5% for **I**. The data is summarized in Table 3.

3.4. Accuracy and precision

The precision and accuracy of intraday QCs were determined by analyzing five replicates of QC samples at low, medium, and high concentrations. Table 4 summarizes the means, precision and accuracy. The precision (%CV) was ≤5.03% for **I** with accuracy range (percentage of nominal value) of 88.5–95.3% for **I** over three QC concentrations.

The precision and accuracy of interday standards and QCs from three sample runs of a clinical study are given in Table 5. The interday QC precision (%CV) was ≤4.23% for **I** with the

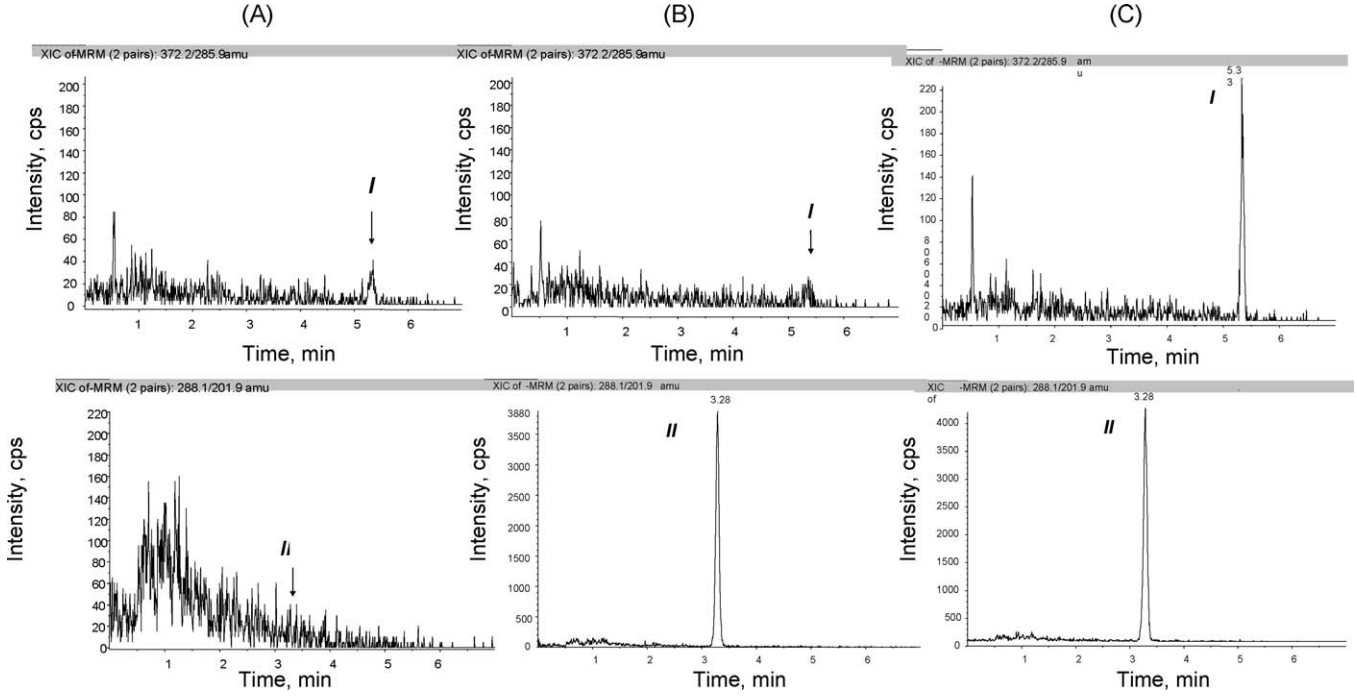

Fig. 4. Representative chromatograms for **I** in human urine: (A) blank control urine; (B) control urine spiked with **II** (1 ng/mL); and (C) control urine spiked with **I** at LLOQ (0.05 ng/mL) and **II** (1 ng/mL).

Table 3
Intraday reproducibility for standards of **I** in human urine

Nominal concentration (ng/mL)	**I** in human urine		
	Mean[a] calculated concentration (ng/mL)	Accuracy[b] (%)	Precision[c] (CV%)
0.05	0.047	94.8	9.4
0.1	0.093	93.0	6.2
0.4	0.397	99.2	5.0
1.0	1.065	106.5	5.4
4.0	4.202	105.1	5.8
10.0	10.575	105.7	4.9
20.0	19.171	95.9	3.7

[a] Mean concentrations are the averages of $n = 5$.
[b] Expressed as [(mean calculated concentration)/(nominal concentration)] × 100%.
[c] Precision was calculated as CV% from peak area ratios.

QC accuracy range (percentage of nominal value) of 89.7–95.0% for **I** over three QC concentrations.

3.5. Extraction recovery and matrix effects

Extraction recoveries for **I** in urine were determined at three concentrations over the standard curve range ($n = 5$ lots of urine). At each concentration, post-spiked samples were prepared by extracting blank control urine and spiking it with **I** and **II** after the extraction. The post-spiked samples were compared to extracted samples that were at the same concentrations. Results were calculated by comparing mean peak areas of the post-spiked samples with the mean peak areas for the corresponding pre-spiked samples. The recoveries for **I** at 0.1, 1.0, and 20 ng/mL were 74.4, 76.7, and 78.6%, respectively. The overall mean recovery was 76.6%. Recovery of **II** at the working standard concentration (1 ng/mL) was 79.1%.

The matrix effect for the assay was determined at 0.1, 1.0, and 20 ng/mL of **I**, and the working standard concentration 1 ng/mL of **II** in urine ($n = 5$ lots of urine). At each concentration, the mean peak area of the post-spiked extracted samples was compared to the mean peak area of neat standards prepared to the same concentrations with reconstitution solution. The absolute matrix effect for **I** at 0.1, 1.0, and 20 ng/mL was 104.0, 104.2 and 104.6%, respectively. Matrix effect was not significant for **I** with an overall enhancement of 4.3%. Matrix effects were consistent for **I** over the concentration range of the drug compound in varying lots of urine. For the internal standard, suppression was negligible at 99.2% at the working standard concentration.

3.6. Sample stabilities

Clinical urine samples were stored at −70 °C. Freeze–thaw stability was evaluated by freeze–thawing QC samples for three cycles which consists of thawing the QC samples for 4 h at room temperature and re-freezing at −70 °C. Room temperature stability was also evaluated by thawing QC samples to room temperature and storing them on the benchtop for ∼24 h. Results in Table 4 indicate **I** was stable in human urine with formic acid equal to 1% of the total volume and 1.75% BSA of the total volume after three freeze/thaw cycles and after being stored at room temperature for ∼24 h.

Table 4
Initial intraday QC, freeze/thaw QC and room temperature QC stability for **I**

	Initial intraday ($n = 5$)			Freeze/thaw[a] ($n = 4$)			Room temperature/24 h[b] ($n = 4$)		
	High	Medium	Low	High	Medium	Low	High	Medium	Low
	16[c]	2[c]	0.15[c]	16[c]	2[c]	0.15[c]	16[c]	2[c]	0.15[c]
Mean	14.158	1.896	0.143	14.218	1.894	0.144	13.613	1.744	0.141
CV%	5.03	3.12	3.89	4.27	6.16	7.46	1.39	4.78	4.64
Accuracy (%)	88.5	94.8	95.3	88.9	94.7	96.0	85.1	87.2	94.2
Stability (%)[d]				100.4	99.9	100.7	96.2	92.0	98.8

[a] Freeze/thaw QC analyzed after three freeze/thaw cycles.
[b] Room temperature QC analyzed after being stored on benchtop for ∼24 h.
[c] Nominal concentration of **I** (ng/mL)
[d] Expressed as [(mean measured concentration)/ (mean initial concentration)] × 100%.

Table 5
Interday precision and accuracy of urine standards and QCs for **I**

	Standard curve							QC[a]		
	0.05[b]	0.1[b]	0.4[b]	1.0[b]	4.0[b]	10.0[b]	20.0[b]	16[b]	2[b]	0.15[b]
Mean[c]	0.057	0.092	0.387	0.966	4.069	9.715	20.264	14.348	1.899	0.142
CV %	5.26	6.52	7.75	3.93	4.62	1.89	0.63	2.25	0.74	4.23
Accuracy (%)[d]	114.0	92.0	96.8	96.6	101.7	97.2	101.3	89.7	95.0	94.7

[a] Two QC replicates were included in each clinical run.
[b] Nominal concentration of **I** (ng/mL)
[c] Mean of data collected in three clinical runs.
[d] Expressed as [(mean measured concentration)/(mean nominal concentration)] × 100.

Table 6
Storage stability for quality controls of **I** and **III** in human urine

I	−70 °C/14 h (n = 3)			4 °C/14 h (n = 3)			−70 °C/1 month (n = 2)		
	16[a]	2[a]	0.15[a]	16[a]	2[a]	0.15[a]	16[a]	2[a]	0.15[a]
Mean	16.667	2.117	0.139	16.933	2.200	0.153	14.528	1.912	0.145
CV %	3.90	5.20	12.85	4.51	1.64	1.65	2.89	3.88	4.40
Accuracy (%)	104.2	105.8	92.9	105.8	110.0	101.8	90.8	95.6	96.3
Stability (%)[b]							102.6	100.8	101.0

III	−70 °C/14 h (n = 3)		4 °C/14 h (n = 3)	
	120[c]	500[c]	120[c]	500[c]
Mean	121.3	469.0	125.0	470.0
CV%	7.20	2.93	7.11	1.82
Accuracy (%)	101.1	93.8	104.2	94.0

[a] Nominal concentration **I** (ng/mL).
[b] Expressed as [(mean measured concentration)/ (mean initial concentration)] × 100%. Initial QC concentrations are given in Table 4.
[c] Nominal concentration **III** (ng/mL).

Clinical urine samples were collected predose on Day 1 and at 0–12 h and 12–24 h on Day 1 and Day 14. During the 12 h sample collection timeframe, the urine aliquots (treated with BSA and formic acid) are combined and stored at 4 °C. At the end of the collection timeframe, the urine samples are then transferred to −70 °C and kept frozen until time of sample analysis. Therefore, the stability of **I** and **III** at 4 °C for ∼12 h needed to be established to verify clinical collection procedure. QC samples for **I** at 0.15, 2, and 16 ng/mL and **III** at 120 and 500 ng/mL were prepared and replicates were stored at −70 °C and 4 °C for approximately

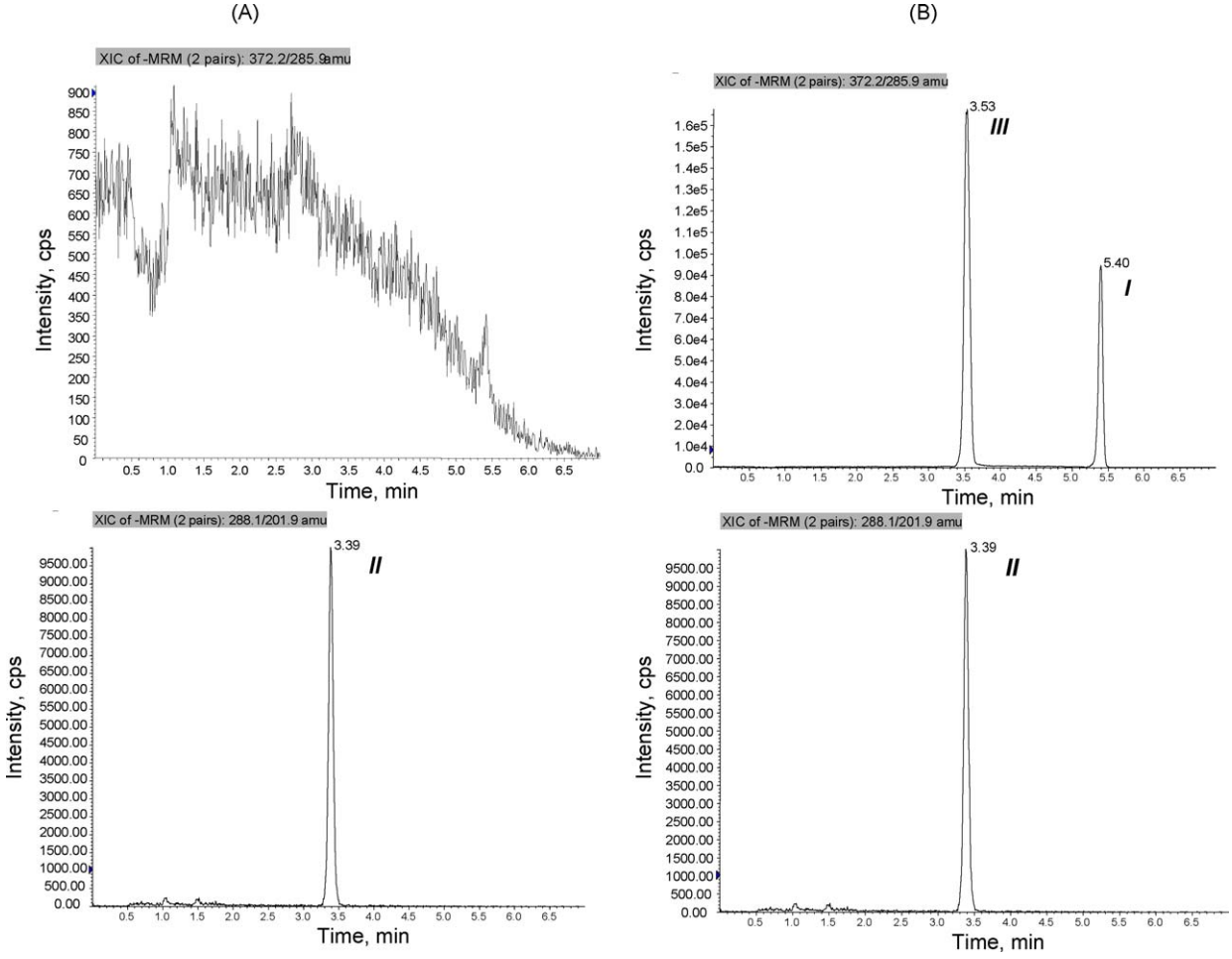

Fig. 5. Representative chromatograms from Phase I clinical urine samples: (A) predose urine sample and (B) 0–12 h urine sample following a 400 mg oral dose of **I**.

14 h. Replicates ($n = 3$) of QC samples stored at both −70 °C and 4 °C were then extracted to determine stability of analytes. Table 6 indicates that **I** and **III** are stable when stored at −70 °C or 4 °C for approximately 14 h.

Long term storage stability of **I** in urine that had been treated with formic acid equal to 1% of the total volume and 1.75% BSA of the total volume was determined by analyzing replicate QCs ($n = 2$) that had been stored at −70 °C for 1 month. The results in Table 6 indicate that **I** was stable at the given collection conditions for at least 1 month.

3.7. Clinical applications

The clinical collection procedure and validated urine method have been applied successfully to the analysis of samples from Phase I clinical studies. Representative chromatograms from one subject on Day 1 at predose and 0–12 h are given in Fig. 5. Results indicate there are no significant interferences seen in predose urine at the retention time for **I**. There was acylglucuronide metabolite detected in high concentrations in the Day 1 0–12 h sample which was consistent with results from plasma samples. The metabolite is separated chromatographically from drug compound and does not interfere with quantitation. The calculated concentration of **I** in Day 1 sample from 0 to 12 h was 13.34 ng/mL.

4. Conclusions

Determining the appropriate clinical collection procedure for urine studies was a critical step in the method development process of the assay. The urine clinical collection procedure identified for **I** added 1% formic acid to stabilize an acylglucuronide metabolite and added 1.75% BSA to stabilize the drug compound and metabolite in urine matrix. A fully automated bioanalytical method to accurately determine the concentrations of compound **I** from human urine with limited urine sample size was developed and validated. The urine method has a LLOQ of 0.05 ng/mL and linear range is 0.05–20 ng/mL. The clinical collection procedure and urine assay have been successfully applied to Phase I clinical sample analysis and produced satisfactory results. The automated method has been proven fast, specific, and sensitive.

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