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Determination of a prostaglandin D_2 antagonist and its acyl glucuronide metabolite in human plasma by high performance liquid chromatography with tandem mass spectrometric detection—A lack of MS/MS selectivity between a glucuronide conjugate and a phase I metabolite

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Abstract

A method for the determination of a prostaglandin D_2 receptor antagonist (**I**, a compound being evaluated for the prevention of niacin induced flushing) and its acyl glucuronide metabolite (**II**) in human plasma is presented. The method utilized high performance liquid chromatography (HPLC) with tandem mass spectrometric (MS/MS) detection using an atmospheric pressure chemical ionization (APCI) interface operated in the positive ionization mode. The product ion was a radical cation generated via a homolytic bond cleavage. A chemical analog of the drug was used as internal standard (**III**). The acyl glucuronide metabolite (**II**) was detected using the same precursor-to-product ion transition used for the parent compound after chromatographic separation of **I** and **II**. Drug and metabolite were extracted using semi-automated, 96-well format solid phase extraction (SPE), and chromatography was performed using a reverse phase analytical column with an isocratic mobile phase. The chromatographic retention factor (k') of **II** was found to be highly sensitive to mobile phase formic acid concentration. An adjustment in mobile phase formic acid concentration improved the chromatographic separation between **II** and a mono-hydroxylated metabolite after an unexpected lack of MS/MS selectivity between the two molecules was observed. The dependence of retention factor on formic acid concentration (k' increased as formic acid concentration decreased) was thought to indicate polar interactions between **II** and the stationary phase. The stability of **II** in spiked human plasma was determined. The rate of hydrolysis back to parent compound was relatively low (approximately 0.1 and 0.5% per hour at room temperature and 4 °C, respectively) indicating that significant changes in analyte concentrations did not occur during sample processing. The concentration range of the assay was 10–2500 ng/mL for both drug and glucuronide metabolite.

Keywords: Prostaglandin D2 antagonist; LC–MS/MS

1. Introduction

Many physiological and pathophysiological effects of prostaglandin PGD_2 are mediated by interaction with the G-protein coupled DP receptor [1]. Binding of PGD_2 to the DP receptor is associated with the production of skin flushing observed in patients being treated with niacin, for various lipid disorders. Compound **I** [2], a high affinity reversible antagonist of the DP receptor, is currently being evaluated for the prevention of this niacin induced flushing. Reported here is a plasma HPLC–MS/MS assay for the determination of compound **I** and its acyl glucuronide metabolite, **II** (Fig. 1) used to support clinical trials.

Preclinical and in vitro studies had demonstrated that **II** was a major metabolite of compound **I** [3–5]. Significant concentrations of **II** were found in the plasma of rats, dogs, and monkeys dosed with **I**. The potential for high concentrations of **II** in clinical plasma samples created the need to assess the stability of this metabolite in human plasma, as degradation might occur via ex vivo hydrolysis or acyl migration [6]. Additionally, since a lack of MS/MS selectivity between **I** and **II** was expected due to "in source" fragmentation of **II** back to **I** [7], chromatographic separation of the two analytes was necessary, as well as careful selection of precusor-to-product ion transitions for optimal sensitivity. Finally, the potential for a lack of MS/MS selectivity between **II** and other known metabolites needed be evaluated

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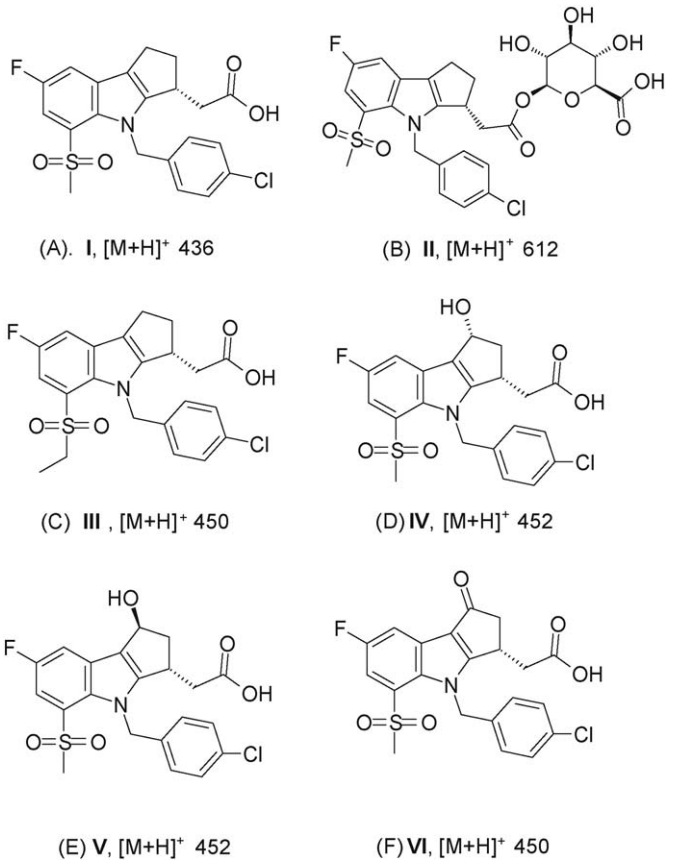

Fig. 1. Chemical structures of **I**, **II** (acyl glucuronide metabolite), **III** (internal standard), and **IV**–**VI** (phase I metabolites).

under the actual chromatographic and MS/MS conditions to be used in the assay.

With regard to sample preparation, we set out to determine if a single rapid solid phase extraction (SPE) scheme could provide satisfactory recovery of both **I** and **II**. Our experimental approach utilized disk based 96-well format SPE and monitoring of extraction recovery by HPLC with ultraviolet (UV) detection. The SPE procedure could then be quickly and simply optimized using plasma drug and metabolite concentrations suitable for UV detection. A final objective was to automate the sample preparation procedure (including addition of standards, internal standard, and pipetting of all plasma samples) using a robotic sample processor.

2. Experimental

2.1. Materials

Compound **I** was obtained from the Chemical Data Department of Merck Research Laboratory (Rahway, NJ, USA). The acyl glucuronide **II** was synthesized by Dr. M. Braun (Drug Metabolism, Rahway, NJ, USA). Internal standard **III** and phase I metabolites **IV**–**VI** (Fig. 1) were supplied by Merck Frosst (Canada). Drug free heparinized plasma was purchased from Biological Specialties (Colmar, PA, USA). All other reagents were of American Chemical Society (ACS) grade and were used as received. SPEC-C8 disk-based, 96-well SPE plates were purchased from Varian Inc. (Lake Forest, CA, USA).

2.2. Instrumentation

An Agilent (Wilmington, DE) 1100 HPLC equipped with a binary pump, well plate autosampler, and ultraviolet detector was used to optimize the chromatographic conditions and plasma SPE extraction procedure. HPLC–MS/MS analysis was performed on a Sciex (Toronto, Canada) API 3000 tandem mass spectrometer equipped with Analyst software and connected to a series 200 Perkin-Elmer (Norwalk, CT, USA) autosampler and micro pump via an atmospheric pressure chemical ionization (APCI) interface. The SPE procedure was executed by a Packard HT sample processor (Perkin-Elmer, Shelton, CT, USA) equipped to use fixed or disposable pipet tips without hardware change (Versa-tips). Ultraviolet absorption and fluorescence spectra were recorded using a HP 8452 diode-array spectrophotometer and a Hitachi (San Jose, CA, USA) F-4500 fluorescence spectrophotometer, respectively.

2.3. Chromatographic conditions

2.3.1. HPLC–MS/MS analysis

A Waters C-8 Symmetry column (2.1 mm × 50 mm, 3.5 μm) was employed for the HPLC–MS/MS analysis (injection volume, 10 μL). The mobile phase contained 0.05% formic acid in 50/50 (v/v) acetonitrile:water and the flow rate was 0.3 mL/min. Column temperature was 35 °C.

2.3.2. HPLC–UV analysis

The HPLC–UV analysis used to optimize chromatographic and solid phase extraction conditions employed a 4.6 by 50 mm Waters C-8 Symmetry column and UV detection at 246 nm. The mobile phase formic acid concentration was varied from 0.005 to 0.1% formic acid (in 50/50 (v/v) acetonitrile/water) in experiments aimed at determining the effect of formic acid concentration on column selectivity. The flow rate was 1.5 mL/min, and the column temperature was 35 °C.

2.4. Mass spectrometric conditions

The APCI interface was operated at a temperature of 475 °C, using nitrogen as nebulizing and auxiliary gas. Declustering and focusing voltages were 35 and 225 V, respectively. The optimal collision energy was 19 V, using nitrogen as collision gas. Compounds **I** and **II** were detected using the same precursor-to-product ion transition (*m/z* 436 → 357, dwell time-500 ms, inter-scan pause-5 ms), after chromatographic separation. The internal standard (**III**) was detected using a *m/z* 450 → 357 transition.

2.5. Preparation of standard and quality control (QC) samples

Separate standard curve and quality control samples were prepared for analytes **I** and **II**. Stock standards (1 mg/mL free acid)

of **I**, **II**, and **III** were prepared using sample solvent (60/40 (v/v) acetonitrile:water) and stored at −70 °C. Quality control samples were prepared using stock and working standards other than those used for the preparation of the standard line samples. Internal standard working solution was prepared by diluting the stock standard with sample solvent to a concentration of 1 μg/mL. The calibration curves consisted of seven plasma standards, 10, 25, 100, 250, 500, 1000, and 2500 ng/mL. The 2500 ng/mL plasma standard was prepared by adding the appropriate volume (50 μL) of a 12.5 μg/mL working standard (in sample solvent) to human plasma (250 μL). The other plasma standards were prepared using dilutions made from the 12.5 μg/mL working standards. Quality control samples for compound **I** were made at concentrations of 2000, 200, and 25 ng/mL. Compound **II** quality control samples were prepared at concentrations of 500 and 50 ng/mL (a third, higher concentration QC was not prepared due to limited availability of analytical standard). All plasma QC samples were stored at −70 °C. Working standards of compounds **I** and **II** were stored at 4 and −70 °C, respectively.

2.6. Plasma extraction procedure

The plasma solid phase extraction was performed using a Packard multiprobe HT sample processor (see next section for details). A 0.250 mL aliquot of plasma was mixed with 50 μL **I** working standard, 50 μL internal standard working solution, and 250 μL 2% formic acid. The resulting solution was transferred to a conditioned C-8 SPE plate and allowed to flow through under slight vacuum. The plate was then washed with 0.5 mL water and 0.5 mL 30/70 (v/v) methanol/water solution under full vacuum (20 in. of mercury). At this point, the SPE plate was removed from the manifold, placed on top of a clean deep well plate (2 mL/well) and the wells eluted using 0.3 mL 80/20 (v/v) methanol/water. In order to maximize recovery, elution was performed using centrifugation (1500 × *g* for 3 min at room temperature). Following centrifugation, an additional 200 μL of water was added to each well of the collection plate.

2.7. Acyl glucuronide stability studies

The stability of **II** in human plasma was determined using fresh and previously frozen heparinized control human plasma. Plasma, spiked with **II** to a final concentration of 1 μg/mL, was kept at 25 and 4 °C for up to 6 h. Zero, 0.5, 1, 2, 4, and 6 h samples were assayed for compounds **I** and **II** by HPLC–MS/MS.

2.8. Automation

An eight channel Packard Multiprobe HT sample processor was used to perform the solid phase extraction. Working standards of **I**, **II**, and **III** (50 μL) were pipetted using the fixed tips. Plasma (250 μL) was pipetted using disposable, conductive, 1 mL tips. Plasma, standard, internal standard, and buffer were mixed by multiple aspirate/dispense cycles in 12 mm × 75 mm tubes and transferred to conditioned SPE wells.

2.9. Validation

Intraday validation was performed by the analysis of five sets of standard line samples made in five different lots of control human plasma. Using Sciex Analyst software, the plasma standards were fitted to a $1/x$ weighted linear regression where x was the concentration of the analyte and y was the analyte to internal standard peak area ratio. Assay precision was calculated as percent coefficient of variation (%CV) of drug to internal standard peak area ratios. Accuracy was calculated relative to nominal concentrations. Interday precision and accuracy was determined using data from the daily analysis of quality control samples. The assays for **I** and **II** were validated separately using separate standard lines and quality control samples.

2.10. Extraction recovery and assessment of the matrix effect on ionization

Extraction recovery and the effect of the sample matrix on ionization efficiency were evaluated for **I**, **II**, and **III** (internal standard) at all concentrations on the standard line. This was done in five lots of plasma as part of the intraday variability experiment described above. Recovery was determined by comparing the absolute peak areas of standards spiked into five lots of human control plasma and extracted by solid phase extraction to the same five lots of control plasma extracted in the same way and then spiked post extraction with analyte and internal standard ("spiked extracts"). Matrix enhancement/suppression of ionization was evaluated by comparing the absolute peak areas of standards in the "spiked extracts" to those of neat standards.

2.11. Clinical plasma collection

Plasma was collected in heparinized tubes and kept on ice prior to centrifugation at 4 °C. Plasma was stored at −70 °C and thawed at room temperature prior to analysis.

3. Results and discussion

3.1. Absorption and fluorescence spectra

UV absorption and fluorescence spectra of **I** were recorded to determine the feasibility of using UV absorption or fluorescence detection for monitoring **I**. The UV spectrum contained absorption bands with maxima at 246 and 314 nm with molar absorption coefficients of 28,000 and 7000 $M^{-1}\,cm^{-1}$, respectively. At an excitation wavelength of 246 nm, no fluorescence emission bands were observed between 260 and 400 nm. Based on these data, the development of an HPLC method with UV detection at 246 nm was feasible, but it would most likely require an extensive sample clean-up both off- and on-line before HPLC chromatographic separation. Instead, it was decided to utilize MS/MS detection for the quantification of the analytes.

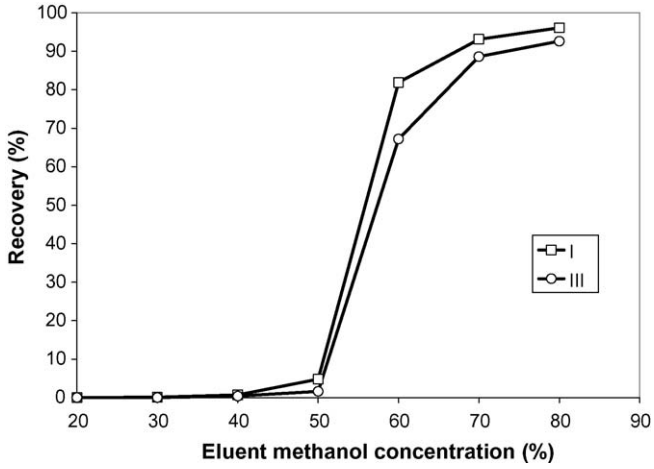

Fig. 2. The effect of eluent methanol concentration on the recovery of **I** and **III** (internal standard) from C-8 solid phase extraction plates.

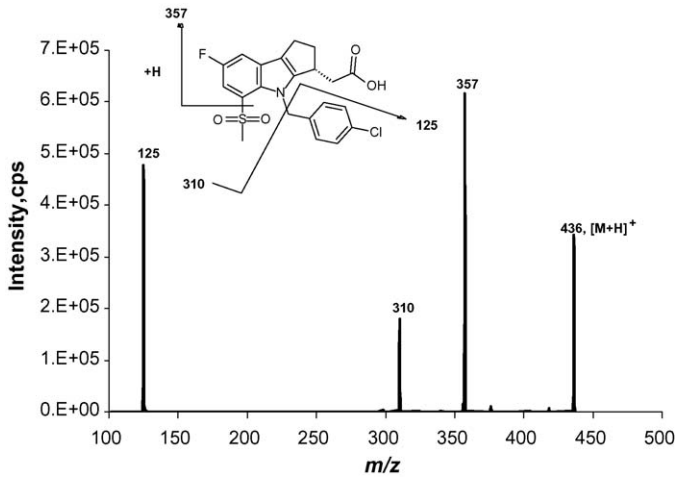

Fig. 3. The positive ionization, product ion mass spectrum of compound **I.** The *m/z* 357 fragment is thought to be a radical cation generated via homolytic cleavage of the carbon–sulfur bond.

3.2. Optimization of the solid phase extraction procedure

The solid phase extraction procedure was based on the use of 96-well format, silica impregnated fiberglass disks and was optimized using HPLC–UV analysis of spiked plasma extracts. Disk based solid phase extraction was advantageous because well-to-well flow rates were more reproducible and faster than those of dry packed SPE plates. Recovery was measured after loading acidified, spiked control plasma onto conditioned SPE plates and washing the wells with water. Individual wells were eluted with solutions containing increasing concentrations of methanol (0.4 mL). SPE recovery of **I** and **III** was calculated and is shown in Fig. 2. Based on these results, 30/70 (v/v) methanol/water and 80/20 (v/v) methanol/water were chosen as the optimal reagents for the washing and elution of the SPE wells, respectively.

In order to minimize SPE elution volume and avoid a potential evaporation step, the effect of elution volume on the recovery of drug and internal standard from spiked control plasma was tested. After loading the plasma and washing the SPE wells, the disks were eluted with 100–400 μL 80% methanol. Maximal recovery was obtained using as little as 300 μL eluent and this volume was selected for the assay.

3.3. Q1 and product ion mass spectra

The full scan, positive ion APCI mass spectrum of **I** (infused in a solution of 0.1% formic acid in 50/50 (v/v) acetonitrile/water) contained an intense protonated molecule $[M+H]^+$ at *m/z* 436. The MS/MS product ion mass spectrum of the $[M+H]^+$ precursor ion contained major fragments at *m/z* 357, 310, and 125 (Fig. 3). The *m/z* 436 → 357 precursor-to-product ion transition was selected for MS/MS detection in the multiple reaction monitor (MRM) mode. The *m/z* 357 ion was thought to be a radical cation generated via homolytic cleavage of the carbon–sulfur bond. The $[M+H]^+$ precursor ion of the internal standard (**III**, *m/z* 450) fragmented in an analogous way, producing prominent fragment ions at *m/z* 357, 324, and 125. It was detected using the *m/z* 450 → 357 transition.

3.4. Selection of MS interface and chromatographic conditions

The APCI interface was selected for the analysis after preliminary experiments demonstrated that it provided adequate sensitivity and was not significantly affected by plasma-to-plasma matrix effects. These observations agreed with previously published reports that the APCI interface was less subject to ion suppression effects than the TurboIonSpray (TISP) source, particularly when the plasma extracts being assayed were not highly purified and the internal standards employed were not stable isotope labeled [8–10]. We also found that peak tailing related to the use of the APCI interface was minimized by the use of a 2.1 mm diameter analytical column operated at an appropriate flow rate (0.2–0.3 mL/min). The use of chromatographic conditions commonly employed with the APCI source (a 4.6 mm column operated at a flow rate of about 1 mL/min) resulted in unacceptable peak tailing.

3.5. MS/MS detection of acyl glucuronide metabolite (*II*)

The full scan, positive ion APCI mass spectrum of **II** contained a weak protonated molecule (*m/z* 612) and a more intense fragment ion at *m/z* 436 corresponding to the loss of glucuronic acid. This suggested that the same precursor-to-product ion transition employed for detection of parent drug (*m/z* 436 → 357) might be suitable for the monitoring of **II** as well. HPLC–MS/MS experiments demonstrated that the *m/z* 436 → 357 transition was optimal for the detection of **II** and was used for monitoring this compound. HPLC–MS/MS analysis of neat **II** performed using the TISP interface demonstrated that deglucuronidation also occurred in this source, although not to the extent observed with the APCI interface.

3.6. Evaluation of HPLC–MS/MS selectivity for acyl glucuronide metabolite *II* in the presence of other metabolites of *I*

Three phase I metabolites of compound **I** were identified from the in vitro incubation of drug with hepatic microsomes and hepatocytes [5]. Two were hydroxy epimers (**IV** and **V**, Fig. 1) and the third was a keto metabolite (**VI**, Fig. 1). The chromatographic conditions initially developed for the analysis separated **V** and **VI** from all the other analytes, but failed to separate **II** from the hydroxy metabolite **IV**. For this reason, it was necessary to establish that **IV** did not produce a significant response in the MS/MS channel used for monitoring **II** (*m/z* 436 → 357). Unexpectedly, metabolite **IV** did generate a response in this channel. The lack of selectivity was thought to occur because the precursor ion used to monitor **II** was isobaric with a $^{37}Cl^-$ containing fragment of metabolite **IV** generated in the APCI source via loss of a water molecule (or possibly lactonization). Both molecules produced isobaric product ions (*m/z* 357) in the collision cell via homolytic cleavage of a carbon–sulfur bond (Fig. 4).

3.7. Chromatographic separation of metabolites *II* and *IV*

The lack of APCI–MS/MS selectivity between the metabolites **II** and **IV** necessitated that they be well separated chromatographically prior to MS/MS detection. In experiments aimed at optimizing the separation, the effect of mobile phase formic acid concentration on the chromatographic retention of all analytes was tested. Decreasing formic acid concentrations significantly increased the chromatographic retention of acyl glucuronide **II**, but had relatively little effect on the other analytes**.** Metabolite

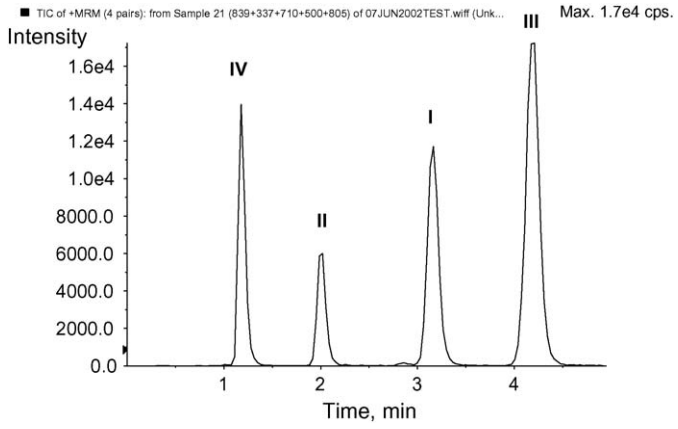

Fig. 5. Total ion chromatograph demonstrating the separation of compounds **I–IV**. The chromatographic conditions are described in the text (Section 2.3).

II was so strongly retained at the lowest mobile phase formic acid concentration tested (0.005%, Fig. 6), that it eluted after the parent drug. This surprising observation suggested that **II** was retained by both reverse phase and polar type chromatographic interactions and that the polar interactions predominated at low formic acid concentration. It also provided a means to easily optimize chromatographic selectivity. Using an isocratic mobile phase containing 0.05% formic acid in 50/50 (v/v) acetonitrile/water, **II** was well separated from **IV**, parent drug and internal standard (Fig. 5).

The data presented in Fig. 6 also clearly indicate that the chromatographic retention of a glucuronide conjugate (**II**) may be highly dependent on small differences in the composition of the mobile phase and the common assumption of the relatively easy

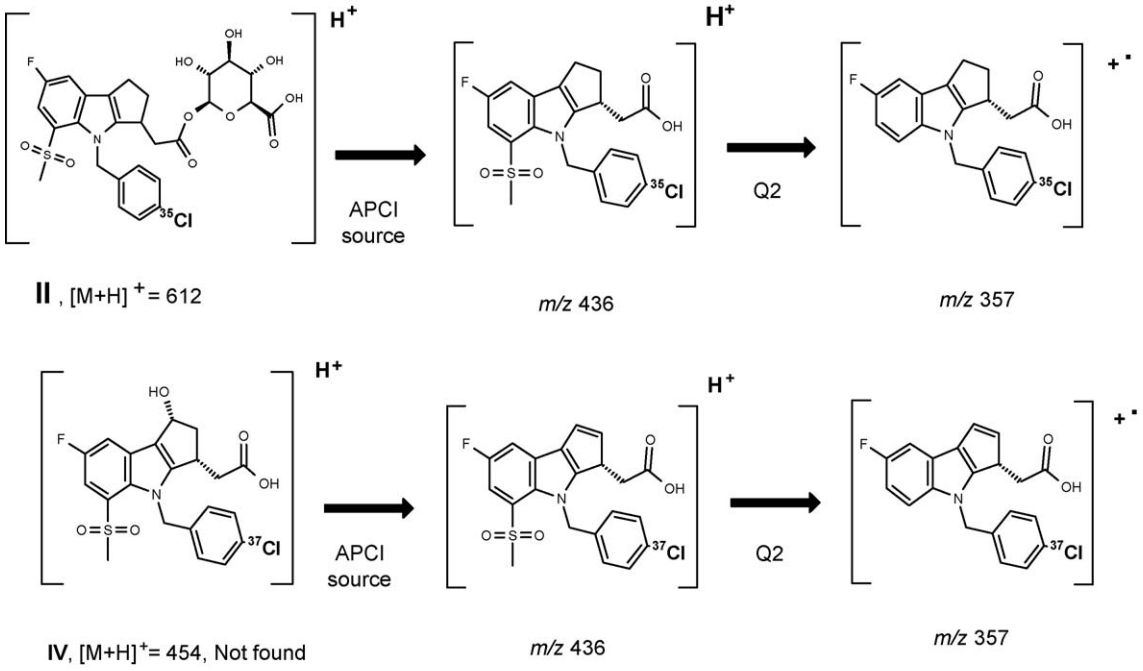

Fig. 4. Mechanism proposed to mediate the lack of MS/MS selectivity between **II** and hydroxyl metabolite **IV** observed using the *m/z* 436 → 357 transition. "In source" fragmentation generates isobaric precursor ions (*m/z* 436) from both molecules. The precursor and product ions generated from compounds **II** and **IV** contain ^{35}Cl and ^{37}Cl, respectively.

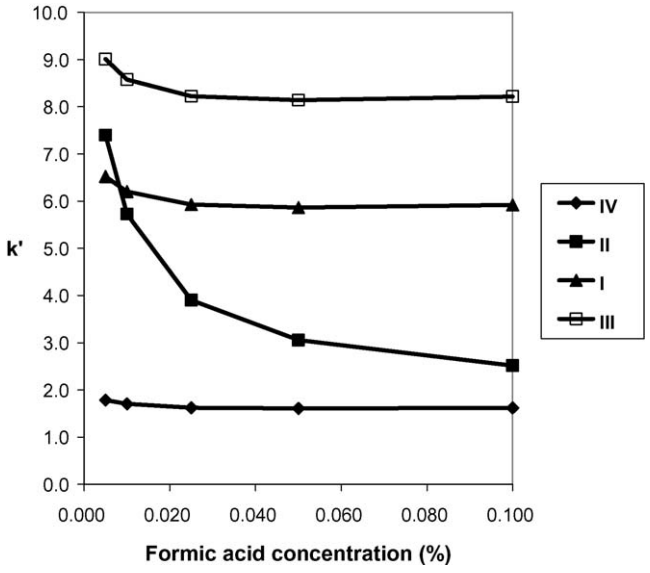

Fig. 6. The effect of mobile phase formic acid concentration on chromatographic retention factor. The mobile phase contained formic acid (0.005 to 0.1%) in 50/50 (v/v) acetonitrile/water. The experiment was performed using a Waters Symmetry C-8 column (see text for details).

separation of a glucuronide from other, non-conjugated metabolites may not be valid. The selectivity of MS/MS detection of a metabolite in the presence of other conjugated metabolites needs to be carefully evaluated and experimentally confirmed irrespective of the interface (APCI, TISP) utilized. The example provided is to our knowledge, one of the first examples of non-selectivity due to contributions from a hydroxy metabolite (**IV**) containing an isotope of chlorine (^{37}Cl) to the quantification of a phase II metabolite (glucuronide conjugate **II**) via dehydration of **IV** and thermal conversion of **II** to a common MS/MS fragment, as depicted in Fig. 4.

3.8. Stability of acyl glucuronide metabolite *II* in human plasma

The stability of **II** in fresh (never frozen) and control human plasma was determined at room temperature and at 4 °C. This was done to insure that significant hydrolysis of the acyl glucuronide metabolite back to parent drug did not occur during sample collection or processing. The results of these studies are shown in Table 1. In control plasma, **II** hydrolyzed to the parent compound at a rate of about 0.5 and 0.1% per hour at room temperature and on ice, respectively. The rate of hydrolysis in fresh plasma was slightly slower. Based on this data, it was recommended that clinical blood samples be placed on ice, centrifuged at 4 °C and frozen promptly. The low rate of hydrolysis observed in this experiment indicated that a significant change in the concentrations of **I** and **II** would not occur during the period of time required for sample processing.

3.9. Assay validation

3.9.1. Assay selectivity

Extracted ion chromatograms of control plasma spiked with 200 ng/mL **III** (internal standard) are shown in Fig. 7A. Chromatograms of plasma standards containing 200 ng/mL **III**, with 10 ng/mL **I** and 10 ng/mL **II** are shown in Fig. 7B and C, respectively. Extracted ion chromatograms of a 2 h post dose clinical plasma sample are shown in Fig. 7D.

Table 1
The stability of **II** in control and fresh human plasma at room temperature and at 4 °C

Time (h)	Percent hydrolysis to **I**[a]			
	Room temperature		4 °C (on ice)	
	Fresh	Control	Fresh	Control
0	0.38[b]	0.43[b]	0.37[b]	0.43[b]
0.25	0.06	0.12	0.03	0.04
0.5	0.17	0.32	0.03	0.16
1	0.26	0.51	0.03	0.19
2	0.63	0.90	0.08	0.26
3	1.09	1.50	0.13	0.38
4	1.41	1.84	0.22	0.44

[a] [**I** area/(**II** + **I** area) × 100] − time 0.
[b] **II** standard.

3.9.2. Intraday validation

Standard lines for **I** and **II** were constructed separately because the analytical standard used for compound **II** contained a small amount (about 0.4%) of **I** as an impurity (see Table 1, 0 h)**.** The use of separate standard lines and quality control samples also allowed us to confirm that significant hydrolysis of **II** back to the parent drug did not occur during sample processing. The intraday validations were performed using five standard lines made from five different lots of human control plasma. The range of the standard lines was 10–2500 ng/mL for both analytes. The results are summarized in Table 2. Assay precision was satisfactory (between 2.0 and 7.0% coefficient of variation) for both analytes across the range of the standard lines. Accuracy was between 96.8 and 105.8% of nominal.

3.9.3. Freeze/thaw stability assessment

The freeze/thaw stabilities of **I** and **II** in human plasma were tested separately. No freeze/thaw instability was observed for either compound (Table 3).

Table 2
Intraday precision and accuracy data for the determination of **I** and **II** in five different lots of human plasma

Standard concentration in plasma (ng/mL)	**I**		**II**	
	Accuracy[a] (%)	Precision[b] (%)	Accuracy [a] (%)	Precision[b] (%)
10	105.8	6.0	98.1	3.9
25	99.7	4.5	100.9	7.0
100	96.8	3.9	99.8	5.8
250	98.2	4.3	102.3	6.0
500	99.9	3.4	99.4	6.4
1000	98.7	2.0	100.6	4.3
2500	100.7	3.7	99.8	4.7

[a] Expressed as [(mean observed concentration)/(nominal concentration)] × 100.
[b] Coefficient of variation of peak area ratios.

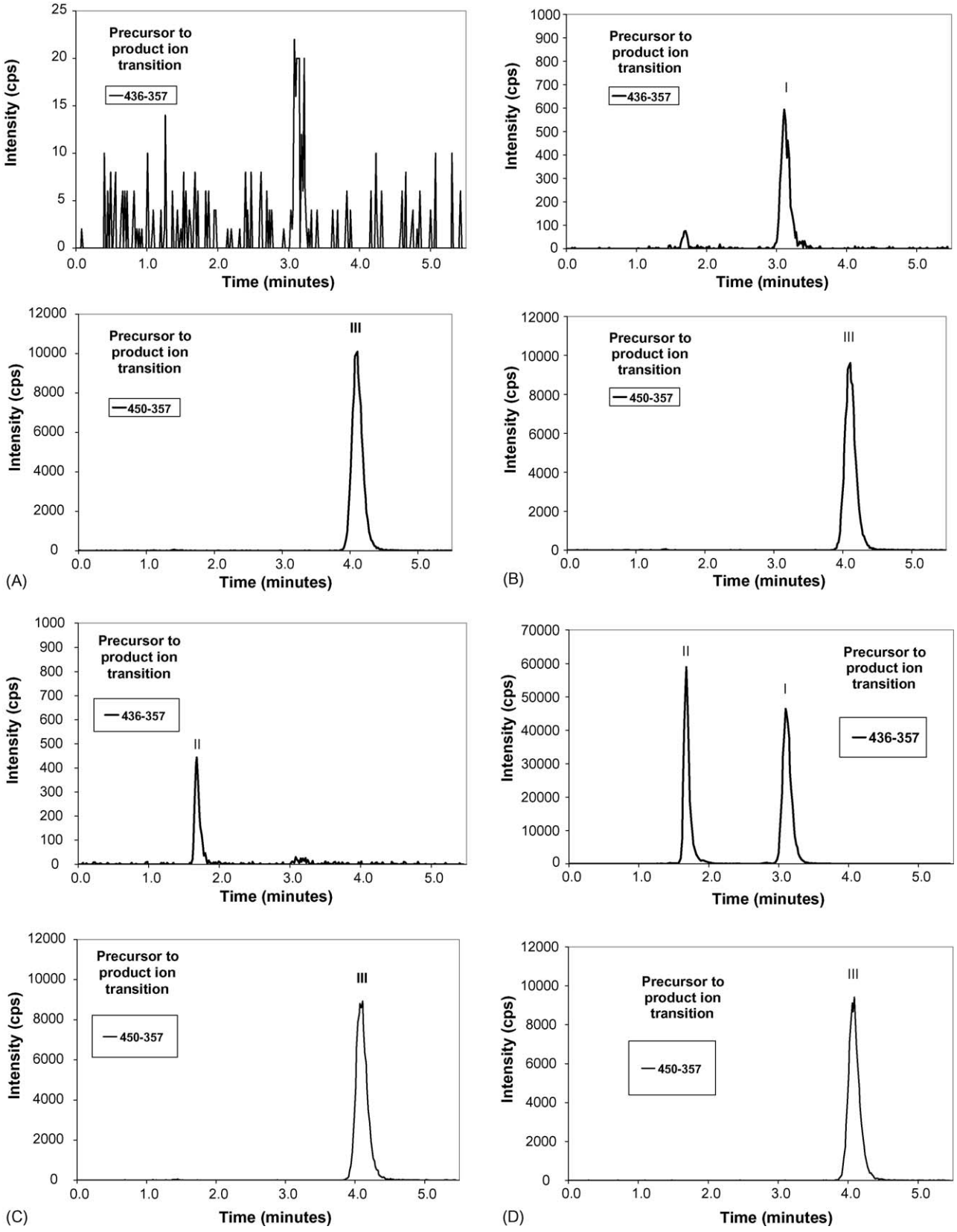

Fig. 7. Representative extracted ion chromatograms of **I**, **II**, and **III** (internal standard) in spiked and post dose human plasma. (A) Control plasma containing 200 ng/mL **III**. (B) Control plasma containing 10 ng/mL **I** (lower limit of quantification) and 200 ng/mL **III**. (C) Control plasma containing 10 ng/mL **II** (lower limit of quantification) and 200 ng/mL **III**. (D) Two hour post 60 mg oral dose human plasma containing 200 ng/mL **III**; concentrations of **I** and **II** are equivalent to 922 and 1280 ng/mL, respectively.

Table 3
Assessment of freeze/thaw stability of **I** and **II** in human control plasma

Analyte	Nominal concentrations (ng/mL)	Mean determined concentrations ($n=5$)		Change (%)
		Control	Freeze/thaw	
I	25	25.2[a]	25.4[b]	+0.8
I	250	246.4[a]	247.0[b]	+0.2
I	2000	2026.0[a]	2030.0[b]	+0.2
II	50	46.7[c]	49.0[d]	+0.9
II	500	480.8[c]	493.4[d]	+1.0

[a] Frozen and thawed once.
[b] Frozen and thawed at room temperature three times.
[c] Fresh.
[d] Frozen and thawed at room temperature once.

3.9.4. Extraction recovery and assessment of the matrix effect on ionization

Extraction recovery across the range of the standard line and the effect of sample matrix on MS/MS response to analytes **I** and **II** are shown in Table 4. The mean recovery of the internal standard (**III**, $n=40$) was 82.2%. Although significant absolute matrix enhancements were observed, the intraday variability data demonstrated that the precision and accuracy of the assay was not adversely affected by the use of five different lots of control human plasma. In addition, the values of the slopes of the compound **I** standard lines in five different lots of human plasma were similar, ranging in value from 0.00474 to 0.00514 with a coefficient of variation of 3.1%. The slopes of the five standard lines of compound **II** were between 0.00256 and 0.000290 with a %CV of 4.6%. Since a similar MS/MS response (slope) was derived from extracts of five plasma lots, a significant "between plasma" or "relative" matrix effect was not observed. Absolute matrix enhancements using the APCI interface with plasma extracts have been previously reported [9], although the mechanism is not understood.

3.9.5. Interday validation

Interday variability was assessed using human plasma quality control samples assayed in duplicate during daily runs. The daily mean values were used to calculate overall interday accuracy and precision. Separate human plasma QC samples were made for **I** (25, 250, and 2000 ng/mL) and **II** (50 and 500 ng/mL) prior to the start of clinical studies and stored at −70 °C. Interday precision for the determination of **I** was between 4.0 and 5.9% (coefficient of variation). Accuracy was better than 10% of nominal (Table 5). Interday precision for the analysis of metabolite **II** quality controls was 9.1 and 11.8% for the 50 and 500 ng/mL QCs, respectively. Accuracy was about 10% of nominal for both analytes.

Table 5
Interday precision and accuracy data for the determination of **I** and **II** in human plasma quality control samples

	I (ng/mL)			**II** (ng/mL)	
	25	250	2000	50	500
Mean ($n=16$)[a]	23.2	233.7	2075.9	45.1	447.6
% CV	5.9	5.2	4.0	9.1	11.8
Accuracy	92.9	93.5	103.8	90.2	89.5

[a] Over a 4 month period.

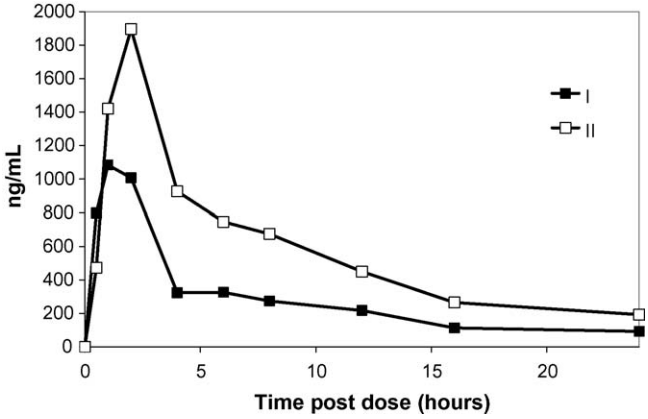

Fig. 8. Mean ($n=6$) concentrations of **I** and **II** in clinical plasma samples obtained from healthy subjects administered a 50 mg oral dose of **I**.

3.9.6. Drug and metabolite concentrations in post-dose clinical plasma samples

Plasma samples obtained from six healthy subjects who had received a 50 mg oral dose of **I** were assayed for **I** and **II**. Mean concentration versus time profiles are shown in Fig. 8. The plasma concentrations of **II** were generally higher than those of the parent drug.

Table 4
Extraction recovery and assessment of matrix effects on ionization during the determination of **I** and **II**

Standard concentration in plasma (ng/mL)	**I**		**II**	
	Mean extraction recovery[a] (%, $n=5$)	Matrix effect[b] (%, $n=5$)	Mean extraction recovery[a] (%, $n=5$)	Matrix effect[b] (%, $n=5$)
10	87.4	134	85.3	150
25	86.1	141	90.3	157
100	87.5	139	90.1	160
250	87.8	138	94.9	176
500	91.0	139	92.9	161
1000	89.5	142	92.1	162
2500	89.2	135	87.4	161

[a] Extraction recovery was calculated by dividing the mean peak area of analyte spiked into plasma before extraction by the peak area of the corresponding "spiked extract".
[b] Matrix enhancement was calculated by dividing the analyte peak area of the "spiked extract" by that of the corresponding neat standard.

4. Conclusions

A plasma assay suitable for the support of clinical trials was developed and validated for the determination of drug **I** and major acyl glucuronide metabolite **II**. The assay utilized 96-well format solid phase extraction and an analog as internal standard. Relative (plasma-to-plasma) matrix effects were minimized by use of an APCI interface. Metabolite **II** was detected using the same precursor-to-product ion transition used for **I**. A hydroxyl metabolite (**IV**) that was detected by the same MS/MS transition and initially interfered with the quantification of **II** was chromatographically separated from **I** and **II**.

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