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Determination of a novel gamma-secretase inhibitor in human plasma and cerebrospinal fluid using automated 96 well solid phase extraction and liquid chromatography/tandem mass spectrometry

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Abstract

A method for determination of a gamma-secretase inhibitor, *cis*-3-[4-[(4-chlorophenyl)sulfonyl]-4-(2,5-difluorophenyl)cyclohexyl]propanoic acid (**A**), in human plasma and cerebrospinal fluid (CSF) has been developed to support the clinical investigation of compound **A** for its potential treatment of Alzheimer's disease. The method is based on HPLC with atmospheric pressure chemical ionization tandem mass spectrometric detection (APCI-MS/MS) in the negative ionization mode using a heated nebulizer interface. The addition of phosphoric acid at the ratio of 10–30 μL per milliliter of human plasma or CSF was required during clinical sample collection to stabilize an acylglucuronide metabolite (**C**), which was potentially present in human plasma and CSF. Tween 20 (10% solution) was added at the ratio of 20 μL per milliliter of CSF during CSF sample collection to prevent the loss of compound **A** during the storage of clinical samples. The compound **A** and its analog internal standard (**B**) in treated plasma or CSF were isolated from human plasma or CSF using solid phase extraction (SPE) in the 96 well format. The isolated analyte and internal standard were chromatographed on a Phenomenex Synergi® Polar RP analytical column (50 mm × 3.0 mm, 4 μm), using a mobile phase consisting of 60/40 (v/v, %) acetonitrile/water at a flow-rate of 0.5 mL/min. Tandem mass spectrometric detection was performed using a Sciex API 3000 tandem mass spectrometer operated in the multiple reaction monitoring (MRM) mode using precursor to product ion transitions of 441 → 175 for **A** and 469 → 175 for **B**, respectively. The assays were validated over the concentration range of 0.5–200 ng/mL for human plasma and CSF. Replicate analyses ($n = 5$) of spiked standards for both assays yielded a linear response with coefficients of variation less than 7% and accuracy within 5% of the nominal concentrations. In addition, the assays were automated to improve sample throughput by utilizing a Packard Multi PROBEII automated liquid handling system and a Tom-Tec Quadra 96 system. Numerous clinical studies have been supported using these assays.

Keywords: Gamma-secretase inhibitor; HPLC/MS/MS; CSF; Plasma; Acylglucuronide; Tween 20

1. Introduction

Alzheimer disease (AD) is the most common form of dementia in Western countries and the leading cause of disability in the population aged over 65 years [1,2]. Currently the only approved therapies for the treatment of AD are symptomatic treatments, which provide some modest cognitive enhancement, but are not disease modifying. Effective therapies aimed at slowing the progression of AD are a significant unmet medical need. During the past 15 years an 'amyloid hypothesis' has been developed that suggests that amyloid precursor protein (APP) in the brain is cleaved into its proteolytic peptide fragment β-amyloid (Aβ) [3]. The fragments of β-amyloid then aggregate to form amyloid plaque and neurofibrillary tangles. The plaque deposits are believed to cause neurodegeneration, leading to the development of AD [3,4]. It is believed that some enzymes, such as beta secretase and gamma-secretase, might be responsible for the APP cleavage that leads to the formation of the fragments of β-amyloid [5,6]. Compound **A**, *cis*-3-[4-[(4-chlorophenyl)sulfonyl]-4-(2,5-difluorophenyl)cyclohexyl]propanoic acid (Fig. 1), is a novel and potent gamma secretase inhibitor, which could potentially play a role in preventing APP protein cleavage and reduce the formation of amyloid plaque. In order to investigate the

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potential use of the compound **A** for the treatment of AD a series of clinical studies were conducted. The level of the fragments of β-amyloid in human cerebrospinal fluid (CSF) was used as a biomarker to assess the efficacy of **A** in the treatment of AD. During the studies it was desired to evaluate the correlation of compound **A** concentration in human plasma and CSF with the level of the fragments of β-amyloid in CSF. Therefore, assays for determination of **A** in both human plasma and human CSF were required to support clinical trails. This paper describes the development of the methods for determination of **A** in human plasma and CSF using HPLC/MS/MS. The acylglucuronide of **A** was identified as a potential metabolite of **A** during pre-clinical studies. Thus the possibility of conversion of this metabolite to **A** [7] during sample collection, preparation and analysis was investigated. A sample handling and collection procedure was developed for use at clinical sites to ensure the integrity of the plasma and CSF concentration data. The method was automated to improve sample throughput [8,9] by using a Packard Multi PROBEII automated liquid handling system and a Tom-Tec Quadra 96 system.

2. Experimental

2.1. Materials and reagents

Compound **A**, the internal standard (IS, **B**, Fig. 1), and the acylglucuronide metabolite of **A** (**C**, Fig. 1) were synthesized at Merck Research Labs (West Point, PA, USA). Tween 20 solution (10%) was obtained from Pierce (Rockford, IL, USA). Control heparinized human plasma was obtained form Biological Specialties (Lansdale, PA, USA). Control CSF was purchased from Medical Analysis Systems (Camarillo, CA, USA). Fresh control human blood was donated by healthy volunteers from the laboratory. Acetonitrile (ACN, HPLC-grade), phosphoric acid (ACS grade), formic acid (ACS grade), and other reagents were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Ansys® Spec C18 SPE 96 well format plates were purchased from Varian, Inc. (Palo Alto, CA, USA).

2.2. Instrumentation

The high-performance liquid chromatography (HPLC) system consisted of a PerkinElmer (Norwalk, CT, USA) 210 series HPLC pump, a 96 well format autosampler (HTS PAL System from Leap Technology, (Carrboro, NC, USA)), and a Sciex API 3000 triple quadrupole tandem mass spectrometer equipped with a heated nebulizer (HN) interface (Sciex, Toronto, Canada). The mass spectrometer was operated in the negative-ion mode. Data was collected and processed using Analyst® 1.4 software.

A Packard Multi PROBEII automated liquid handling system (Meriden, CT, USA) and a Tom Tec Quadra 96 workstation (Model 320, Hamden, CT, USA) were used for assay automation.

2.3. Chromatographic conditions

A mobile phase consisting of 60/40 ACN/water was delivered to a Phenomenex Synergi® Polar RP guard column (4.0 mm × 2.0 mm, 4 (m) connected to a Synergi® Polar RP

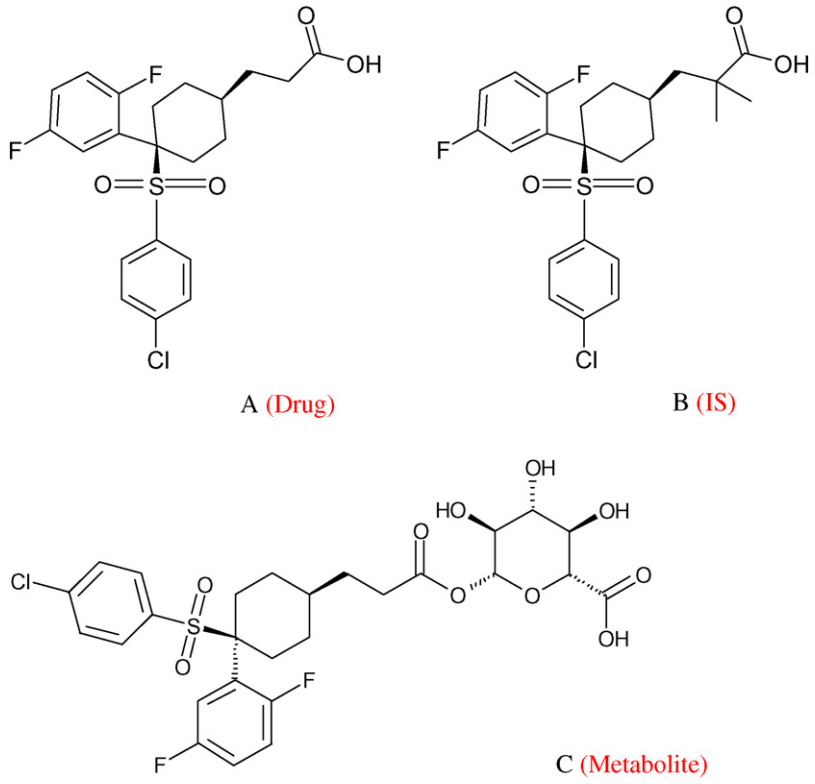

Fig. 1. Structures of compounds **A**, **B** and **C**.

analytical column (50 mm × 3.0 mm, 4 (m) at a flow-rate of 0.5 mL/min. The total run time was 5 min; **A** eluted between 1.6 and 1.9 min, **B** eluted between 2.7 and 3.0 min, while **C** eluted at the solvent front.

2.4. Mass spectrometric conditions

The precursor ion to product ion transitions of *m/z* 441 → 175 for compound **A** and 469 → 175 for **B** were selected for multiple reaction monitoring (MRM). The instrument settings were optimized during analyte infusion to maximize response. The temperature of the nebulizer probe was set at 500 °C, while nebulizer pressure (N_2) was at 70 psi. The nitrogen (N_2) flow-rates of nebulizing gas, collision gas, and curtain gas (N_2) were set at 10, 8 (CGT = 2.0×10^{15} molecules per cm^2) and 8 L/min, respectively. The optimized declustering (DP), collision cell exit (CXP), focusing (FP), entrance (EP) potentials were set at −26, −13, −190 and −10 V, respectively. The optimized collision energy (CE) was −30 V. A dwell time of 400 ms was used.

2.5. Calculations

Unknown sample concentrations were calculated from the equation $y = mx + b$, as determined by the weighted ($1/x^2$) linear least-square regression of the calibration line constructed from peak area ratios (y) of compound **A** to **B** (IS) versus compound **A** concentration (x).

2.6. Standard solutions and quality control (QC) sample preparation

A stock solution of **A** (0.1 mg/mL) was prepared in ACN/water (1:1, v/v, %). Subsequent dilutions were made in ACN/water (1:1, v/v, %) to give working standard solutions of **A** at concentrations of: 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, and 4 μg/mL. A standard stock solution of **B** at a concentration of 50 μg/mL was prepared in ACN/water (1:1, v/v, %); a subsequent dilution was made to prepare a 0.5 μg/mL working standard solution for **B**.

Standard samples were prepared by spiking 0.5 mL aliquots of treated control human plasma or CSF (30 μL of phosphoric acid to 1 mL of plasma, 10 μL of phosphoric acid and 20 μL of Tween 20 per 1 mL of CSF) with 25 μL of each of the working standards of **A**. The standard samples were then extracted as described below.

QC plasma or CSF samples at concentrations of 1.5, 15, and 150 ng/mL of **A** were prepared by diluting 1 mL of 0.15, 1.5 and 15 μg/mL solutions (from a new weighing) to a total volume of 100 mL with treated control human plasma or CSF. Aliquots (0.75 mL) of these solutions were transferred to 3.6 mL Nunc cryotubes. The tubes were stored at −20 °C.

2.7. Sample dilution integrity

Dilution integrity control plasma samples were prepared by spiking a standard solution into 5 mL of control plasma to make a plasma sample containing the analyte at the concentration of 1000 ng/mL. Aliquots (1 mL) of this plasma sample then were transferred to 4.5 mL Nunc cryotubes and stored at −20 °C for overnight. The next day these spiked samples were thawed and centrifuged at room temperature. They were analyzed with a 1:20 dilution ($n = 5$) and the analyte concentrations were calculated based on a standard curve.

2.8. Pre-treatment of plasma and CSF clinical samples

Phosphoric acid at the ratio of 10–30 μL per milliliter of plasma was added to clinical plasma samples containing compound **A**. The procedure followed at the clinical sites for the addition of phosphoric acid to subject plasma was as following: Five milliliters of blood was drawn into a sodium heparin (anticoagulant) containing tube and placed on the ice immediately. Blood samples were centrifuged within 20 min of collection at 1500 × *g* for 10 min at 2–4 °C. The plasma was transferred to a labeled, 4.5 mL Nunc cryotube to which 30 μL of concentrated phosphoric acid (85%, certified ACS grade) was previously added. The plasma was well mixed and then frozen (−20 °C) for storage/shipment.

For CSF sample collection the following procedure was carried out: CSF was collected, via lumbar puncture, directly into a polypropylene conical tube and gently mixed. The tube was then immediately centrifuged at 1500 × *g* for 10 min at 2–4 °C. A 1.5 mL of CSF was transferred to a labeled, 4.5 mL Nunc cryotube to which 30 μL of 10% Tween 20, and 15 μL of concentration phosphoric acid had been previously added. The CSF was well mixed and then frozen (−20 °C) for storage/shipment.

2.9. Extraction procedure for plasma and CSF

A 0.5 mL aliquot of treated plasma or CSF samples was pipetted into a 13 mm × 85 mm polypropylene tube. A 25 μL aliquot of 0.5 μg/mL working IS solution was then pipetted into each of the tubes containing the clinical samples (unknown), QC samples, and the previously prepared standards. Tubes containing clinical samples and QC samples received an additional 25 μL aliquot of ACN/water (1:1, v/v, %) to make these samples equivalent in organic content to the standards. The tubes were then vortexed. A 0.7 mL aliquot of 10% formic acid was added into each tube. The resulting solution was transferred into a 96 deep well plate (2 mL) by a Packard Multi PROBEII automated liquid handling system. The solid phase extraction of samples was performed using a Tom-Tec Quadra 96 system. The extraction protocol was as follows: the sample mixture was loaded onto an Ansys® Spec C18 SPE plate which had been previously conditioned with 0.4 mL of methanol and 0.4 mL of 2% formic acid. The plate was washed with 1 mL of water, followed by 1 mL of 20/80 acetonitrile/2% formic acid (v/v,%). Compounds **A** and **B** were eluted with 250 μL of 90/10 ACN/water (v/v, %). The elutent was then diluted with 125 μL of water. A 25 μL aliquot of the solution was injected onto the HPLC/MS/MS system.

2.10. Assessment of the stability of acylglucuronide metabolite of A (C) in plasma and CSF

A 25 μL aliquot of compound **C** standard was spiked into test tubes containing untreated fresh or frozen human plasma or acidified fresh or frozen human plasma. These test tubes were then either placed in a water bath (37 °C), on the ice (4 °C) or on the lab bench (room temperature) for periods up to 60 min. The hydrolysis of **C** was terminated by addition of a 0.7 mL of 10% formic acid to the plasma samples at the end of the evaluation periods. The concentration of compound **A**, hydrolyzed from compound **C** in plasma sample mixtures was then determined in accordance with the assay method.

A similar method was used to evaluate the stability of compound **C** in CSF.

3. Results and discussion

3.1. Chromatography and MS/MS detection

Precursor ions of compounds **A** and **B** (IS) were determined from Q1 scans during the infusion of neat solutions under negative mode ionization conditions. The precursor ions, $(M-H)^-$ at *m/z* 441 for **A** and *m/z* 469 for **B**, were subjected to collision-induced dissociation (CID) to determine the resulting product ions. One major product ion (*m/z* 175) was present in each of the product ion scans, as shown in Fig. 2. Therefore, the mass transitions of, *m/z* 441 → 175 for **A** and 469 → 175 for **B**, were selected to monitor these analytes.

Initially, compound **A**, which contains a carboxyl group, was found to chromatograph best, with respect to peak shape and retention, under acidic conditions where the carboxyl group

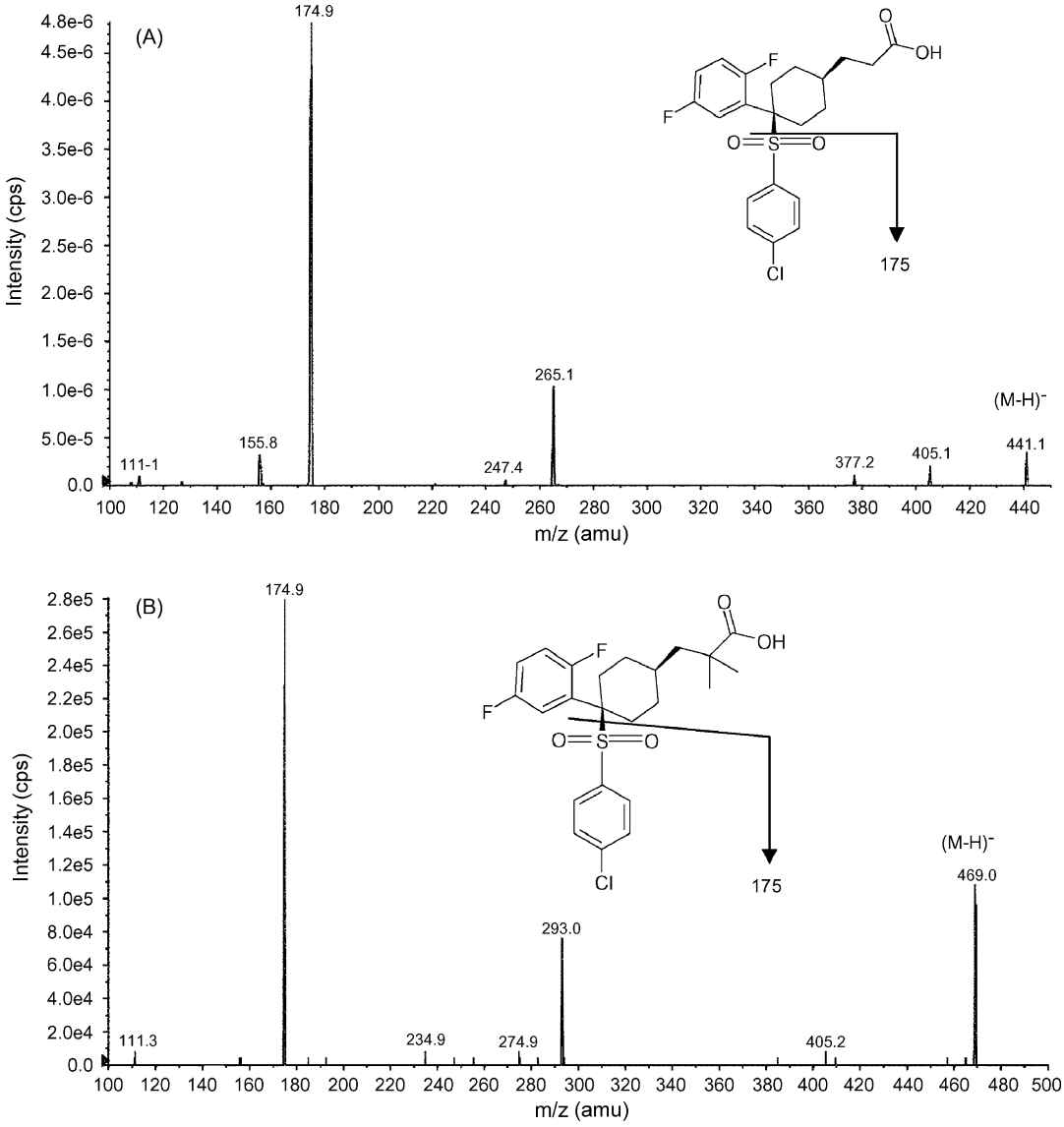

Fig. 2. MS/MS product ion mass spectra of precursor ion $(M-H)^-$ of compounds **A** and **B**.

was fully protonated. Under acidic mobile phase conditions the negative mode ionization of compound **A** was surpressed; a 50-fold decrease in compound **A** sensitivity was observed when formic acid was used as a modifier in the HPLC mobile phase. To maintain assay sensitivity it was desired to identify suitable chromatographic conditions that did not require an acidic modifier. After investigation of many columns and various mobile phase modifiers it was found that a Phenomenex Synergi® Polar RP analytical column (50 mm × 3.0 mm, 4 (m) with a mobile phase consisting of 60/40 (v/v, %) acetonitrile/water gave best results in terms of peak shape, retention time and sensitivity. Even though there were no modifiers in the mobile phase, the method has been proven to be very rugged and reliable as is demonstrated by its successful application to the analysis of samples from 3 different clinical studies over a period of 15 months (Table 3).

3.2. Sample preparation, extraction recovery and matrix effects

Under acidic conditions **A** is fully protonated, hence reverse phase SPE plates were evaluated for its extraction. It was found that a Varian Ansys® C18 SPE plate gave best results in terms of recovery and sample cleanliness. To obtain a consistent recovery, the volume of eluent during the elution step was critical. It was found that 250 μL of 90/10 ACN/H_2O delivered the most consistent recovery for **A** and **B** in plasma and CSF, indicated by the precision of recovery (%CV) in Table 1. The SPE sample preparation was readily automated using a Tom-Tec Quadra 96 system, an automated 96 well format liquid transfer workstation [8].

Extraction recovery for **A** was evaluated at nominal concentrations of 1, 5, 50 and 200 ng/mL ($n = 5$ at each concentration level). Recovery of **B** was determined at its working standard of 25 ng/mL ($n = 5$). Recovery of the extraction was determined by comparing the absolute peak areas of the pre-spiked analyte standards with those of the post-spiked analyte standards. The pre-spiked analyte standards were prepared as specified in the extraction procedure. The post-spiked standards were extracts of drug free matrix to which **A** or **B** was added post-extraction. Results are shown in Table 1. The average recovery of **A** and **B** in plasma and CSF was 87% and 92%, respectively.

Table 1
Extraction recovery and assessment of matrix effects on ionization during the determination of compound **A** in treated human plasma or CSF

Assay matrix	Nominal concentration (ng/mL)	% mean[a], recovery[b] ($n = 5$)	% mean[a], matrix effect[c] ($n = 5$)
Plasma	1	82.5 (8.6)	111.6 (7.0)
	5	89.0 (6.5)	107.5 (3.9)
	50	87.9 (1.8)	114.7 (1.9)
	200	89.1 (1.2)	117.1 (2.1)
	25[d]	83.5 (1.5)	121.9 (3.2)
CSF	1	90.2 (10.1)	103.3 (1.3)
	5	91.8 (5.1)	103.3 (5.1)
	50	94.2 (3.1)	104.0 (2.2)
	200	93.7 (2.0)	106.3 (2.0)
	25[d]	96.6 (1.6)	128.9 (3.1)

[a] Numbers in parentheses are coefficients of variation (%CV).
[b] Expressed as [(pre-spiked standard peak area/post-spiked standard peak area) × 100].
[c] Expressed as [(post-spiked standard peak area/neat standard peak area) × 100].
[d] Internal standard concentration.

Matrix enhancement/suppression of ionization was assessed by comparing the absolute peak areas of post-spiked analyte standards to neat standards. The neat standard was prepared in 40/60 ACN/0.1% formic acid solution to prevent peak tailing. For plasma, there was slight matrix enhancement (Table 1). The lack of a relative matrix effect, however, was demonstrated based on the fact that the slopes of standard curves prepared in five different lots of control plasma resulted in a precision of 2.2% during intra-day assay validation [10]. The lack of a relative matrix effect indicates that B is adequately compensating for the slight matrix enhancement of A. For CSF a slight matrix enhancement was observed as well (Table 1).

3.3. Assay selectivity, linearity, precision and accuracy

The selectivity of the plasma assay was assessed in 6 lots of human control plasma treated with phosphoric acid. No interfering peak was observed in the retention time window of the analyte and its internal standard (Fig. 3). No interfering peaks were observed in all predose CSF samples analyzed using the assay (Fig. 4).

The linearity of the standard curve was assessed based on a plot of the peak area ratio of the drug to IS versus drug concentration. Use of a $1/x^2$ weighted linear regression resulted in better agreement between nominal and measured standard concentration than unweighted regression models.

The precision (coefficient of variation, %CV) of the assay was determined based on the replicate analyses ($n = 5$) of human plasma or CSF containing **A** at all concentrations utilized for the construction of calibration curves. The accuracy of the assay, expressed by [(mean observed concentration)/(nominal concentration)] × 100, was within 96.6–102.7% for plasma and 96.0–104.0% for CSF. The coefficient of variation (%CV) of the assay, was under 6% and 7% for plasma and CSF, respectively, at all concentration within the standard curve range (Table 2).

Sample dilution integrity was assessed by the replicate ($n = 5$) analysis of dilution integrity samples. These samples were analyzed following a 1:20 dilution with control matrix. The mean calculated concentration was within 98.9% of nominal, with a CV of 5.4%.

3.4. Assay inter-day variability

Inter-day variability of the plasma/CSF assays was evaluated using sets of low, middle and high QC samples analyzed daily along with clinical unknown samples. The overall inter-day accuracy and precision data for plasma and CSF QC samples is presented in Table 3.

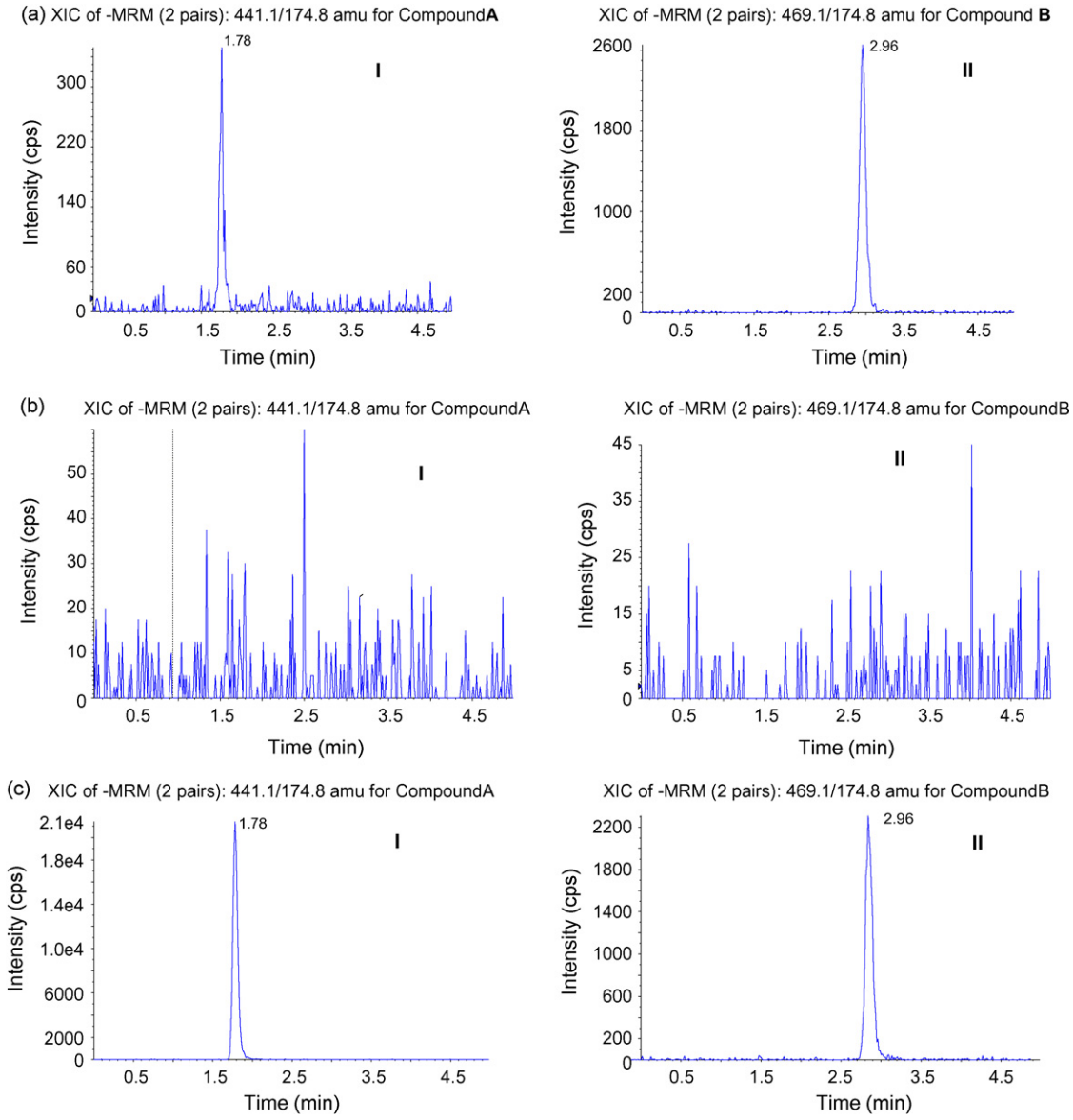

Fig. 3. Representative chromatograms for plasma assay: (a) LLOQ (low limit of quantitation), 0.5 ng/mL of compound **A** and 2.5 ng/mL of compound **B** (IS); (b) predose plasma sample from a clinical study; (c) 2 h post-dose plasma samples from a clinical study after the oral dose of 40 mg of compound **A**. (The responses in the compound **A** and compound **B** (IS) channels are shown in **I** and **II**, respectively).

*3.5. Stability of the acylglucuronide of **A** (compound **C**) in human plasma and CSF*

The acylglucuronide of compound **A**, was identified to be a potential metabolite of the analyte during pre-clinical studies. Hydrolysis of the acylglucuronides to parent compound following plasma sample collection was thus possible [7].

Acylglucuronides are reported to be chemically stable in solutions whose pH is between 2 and 4 [11]. Hence, acidification of human plasma was evaluated as a means to stabilize compound **C**. Various acids have been added to human plasma to stabilize acylglucuronides [12–14]. It was found that when concentrated phosphoric acid was added to plasma at the ratio of 30 μL to 1 mL of plasma, the pH of plasma was adjusted to ∼3 without denaturing the proteins in plasma. Therefore concentrated phosphoric acid was used for plasma acidification.

Based on the results of the experiment described in Section 2.10, it was found that compound **C** was stable in acidified fresh or frozen plasma stored between 4 and 37 °C for up to 60 min (Fig. 5). In contrast, significant formation of **A** was observed in non-acidified fresh or frozen plasma, especially in samples kept at elevated temperatures (Fig. 5). Based on the temperature dependence of the hydrolysis, clinical sites were instructed to keep blood samples on ice following collection for a period not to exceed 20 min. Additionally, a refrigerated centrifuge (2–4 °C) was used to separate plasma prior to acidification.

In order to simplify the sample collection procedure at clinical sites the effect of varying phosphoric acid amounts was also evaluated. It was found that addition of at least 10 μL of concentrated phosphoric acid per milliliter plasma was required to prevent glucuronide hydrolysis. Up to 30 μL of concentrated phosphoric acid per milliliter of plasma could be added without

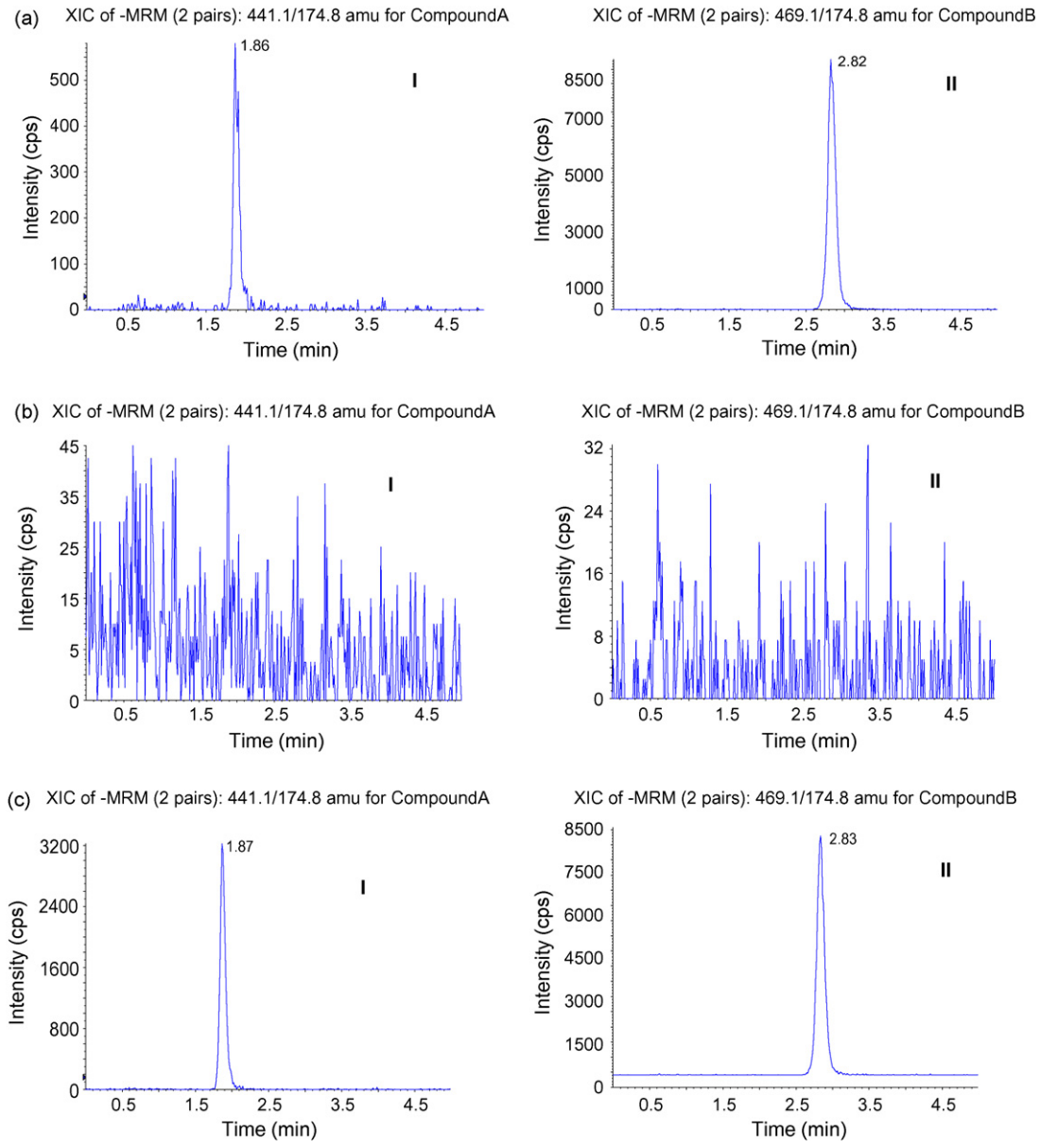

Fig. 4. Representative chromatograms for CSF assay: (a) LLOQ (low limit of quantitation), 0.5 ng/mL of compound **A** and 2.5 ng/mL of compound **B** (IS); (b) predose CSF sample from a clinical study; (c) 1 h post-dose CSF samples from a clinical study after an oral dose of 110 mg of compound **A**. (The responses in the compound **A** and compound **B** (IS) channels are shown in **I** and **II**, respectively).

denaturing plasma proteins. The acceptable range of 10–30 μL per milliliter of acid provides flexibility at clinical sites; the recommended sample acidification procedure was to add the plasma (typically 1–3 mL) resulting from a 5 mL blood drawn to a cryotube containing 30 μL of concentrated phosphoric acid.

Sample extraction during the entire sample preparation procedure was performed under acidic conditions to prevent the hydrolysis of **C**. The stability of any co-extracted **C** in prepared samples after they were set at room temperature for 12 h was evaluated as a component of assay validation. Hydrolysis of **C** was not observed when plasma samples spiked with **C** were extracted and reinjected after remaining at room temperature for 12 h, thus demonstrating that **C** is stable in extracted samples for up to 12 h at room temperature.

Sample handling and storage can have a dramatic effect on the ability to accurately measure analyte concentrations in biological fluids. Thus a further evaluation was performed to determine the effect of storage (−20 °C) and freeze–thaw cycles on the hydrolysis of **C** in acidified plasma. A standard solution of **C** was spiked into the acidified plasma. The resulting **A** concentration was determined following multiple freeze–thaw cycles. The analyzed concentration of **A** in these samples practically did not change following up to three freeze–thaw cycles, which demonstrates that **C** is stable in acidified plasma samples subjected to multiple freeze–thaw cycles.

As was the case for plasma, **C** could potentially hydrolyze to **A** in CSF [7]. Thus it was necessary to evaluate the stability of **C** in human CSF. The procedure used to assess the stability of **C** in CSF was the same as what was used for plasma. In con-

Table 2
Intra-day precision and accuracy data for the determination of compound **A** in five different lots of treated human plasma or CSF

Assay matrix	Nominal concentration (ng/mL)	Mean ($n=5$) determined concentration (ng/mL)	Accuracy[a] (%)	Precision[b] (%)
Plasma	0.50	0.50	100.0	5.5
	1.00	1.02	102.0	3.5
	2.50	2.41	96.6	4.1
	5.00	4.89	97.8	3.0
	10.00	9.93	99.3	2.5
	25.00	24.7	98.9	3.0
	50.00	50.48	101.0	2.9
	100.00	101.77	101.8	4.9
	200.00	205.33	102.7	3.2
CSF	0.50	0.52	104.0	6.9
	1.00	1.02	102.0	3.1
	2.50	2.40	96.0	5.8
	5.00	4.96	99.9	4.7
	10.00	9.88	102.1	4.3
	25.00	24.58	99.0	2.7
	50.00	49.22	101.4	3.2
	100.00	106.82	99.3	3.3
	200.00	201.24	100.1	1.7

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

Table 3
Assessment of inter-day variability of compound **A** by coefficient of variation (%CV) of low, medium, and high quality control samples

Assay matrix	Nominal concentration (ng/mL)	Mean found concentration[a] (ng/mL)	Inter-day accuracy[b] (%)	Inter-day precision[c] (%)
Plasma QCs	1.5	1.47	97.7	5.4
	15	14.6	97.0	6.0
	150	146.8	97.9	6.2
CSF QCs	1.5	1.45	96.4	7.2
	15	14.5	96.8	7.3
	150	144.4	96.2	5.5

[a] $N=50$ for plasma, 50 analytical runs over a 15-month period (two replicates of quality control samples at each concentration were analyzed in each run). $N=18$ for CSF, 18 analytical runs over a 27-month period (two replicates of quality control samples at each concentration were analyzed in each run).
[b] Expressed as [(mean observed concentration)/(nominal concentration)] × 100.
[c] Coefficient of variation (%CV) of daily QC mean value.

trast to plasma, **C** was stable in un-acidified CSF at 37 °C, room temperature and on ice (−4 °C) up to 60 min. Although hydrolysis of **C** in CSF was not observed, CSF was acidified (10 μL phosphoric acid to 1 mL of CSF) to minimize the possibility of acyl migration [7,11] in the event that later analysis of **C** was required.

3.6. Investigation of the loss of compound A in CSF during sample storage

QC plasma samples of compound **A** were found to be stable through 3 freeze–thaw cycles (Table 4). However, it was found that the CSF QC samples containing **A** analyzed at

Table 4
Assessment of freeze–thaw (F/T) stability of compound **A** in treated human plasma or CSF

Assay matrix	Nominal concentration (ng/mL)	Control found concentration[a], mean[b] (ng/mL, $n=3$)	3 F/T cycles found concentration[c], mean[b] (ng/mL, $n=3$)	Stability[d]
Plasma	1.5	1.44 (3.3)	1.43 (4.0)	99.3
	15	14.2 (2.8)	14.4 (1.8)	101.4
	150	144.7 (1.4)	142.7 (2.4)	98.6
CSF	1.5	1.43 (4.0)	1.47 (3.9)	102.8
	15	14.5 (2.4)	14.6 (1.2)	100.7
	150	139.8 (1.1)	143.6 (2.4)	102.7

[a] Quality control samples concentration determined after 1 freeze–thaw cycle.
[b] Numbers in parentheses are coefficients of variation (%CV).
[c] Quality control samples concentration determined after 3 freeze–thaw cycles.
[d] Expressed as [(mean 3 F/T cycles found concentration)/(mean control found concentration)] × 100.

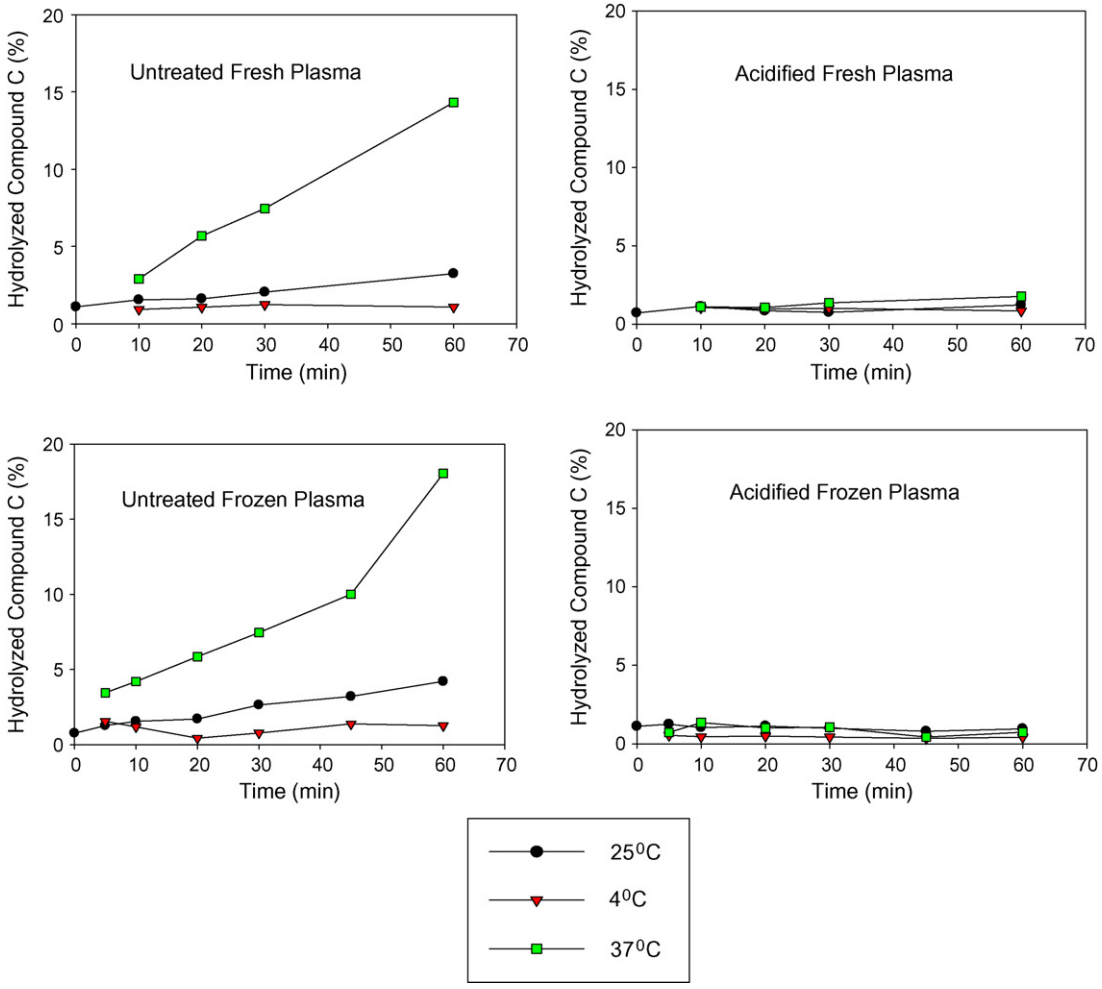

Fig. 5. Stability evaluation of the acylglucuronide metabolite (**C**) of compound **A** in acidified or untreated fresh or frozen plasma. Note: hydrolyzed compound **C** (%) = [(found compound **A** concentration)/(compound **A** concentration equivalent calculated from nominal compound **C** concentration)] × 100.

approximately 73–85% of their nominal concentration after one freeze–thaw cycle. The major difference between plasma and CSF in term of their composition is that the former contains 6–8% proteins [15] the latter only has 0.3% proteins [16]. The trace amount of protein in CSF may contribute to the loss of **A** in CSF during freeze–thaw cycle. The addition of Tween 20, a non-ionic surfactant, to biological matrices that contain a little or no protein has been found to prevent the loss of the compounds that are prone to adsorption losses [15]. Tween 20 addition to biological matrices has been found not to interfere with SPE [15]. Therefore, Tween 20, at a ratio of 20 μL per milliliter of CSF [15], was used to treat CSF prior to QC sample preparation. The QC samples prepared in Tween 20 treated CSF analyzed at concentrations near nominal (Table 4).

3.7. Application to clinical studies

The described method has been successfully applied to multiple clinical studies to determine compound **A** concentrations in plasma or CSF in support of pharmacokinetic analysis during phase I and II clinical trials. Representative chromatograms of human clinical plasma and CSF samples obtained from subjects dosed with **A** are shown in Figs. 3 and 4. A representative mean plasma concentration–time profile of compound **A** following a 40 mg dose to healthy elderly male and female subjects, is shown in Fig. 6.

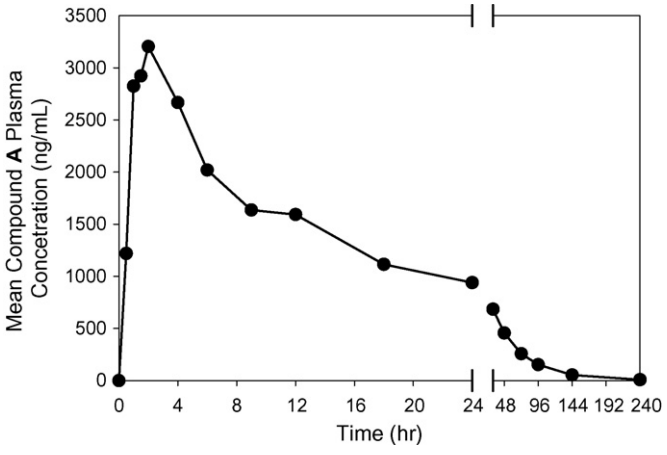

Fig. 6. Mean compound **A** plasma concentration (ng/mL) vs. time (h) after the administration of single oral dose of 40 mg of **A** in healthy elderly subjects ($n = 6$).

4. Conclusion

A highly selective and sensitive method for the determination of compound **A** in human plasma and CSF using LC–MS/MS has been developed and validated. To ensure the integrity of clinical sample data, the stability of a potential acylglucuronide metabolite (**C**) in human plasma were investigated. In addition a method to prevent loss of **A** in CSF was developed. Appropriate sample collection procedures were implemented at clinical sites. Over all, the methods have been proved to be accurate, precise and suitable for analysis of plasma and CSF samples collected during clinical pharmacokinetic studies. The ruggedness of the methods has been demonstrated by the successful analysis of several thousand clinical samples by multiple analysts over a 3-year period.

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