Reversed-phase chiral liquid chromatography on polysaccharide-based stationary phase coupled with tandem mass spectrometry for simultaneous determination of four stereoisomers of MK-0974 in human plasma

Yang Xu*, Donald G. Musson[1]

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

ABSTRACT

MK-0974 (**1a**), *N*-[(3*R*,6*S*)-6-(2,3-difluorophenyl)-2-oxo-1-(2,2,2-trifuoroethyl)azepan-3-yl]-4-(2-oxo-2,3-dihydro-1*H*-imidazo-[4,5-B] pyridine-1-yl)piperidine-1-carboxamide, is a novel calcitonin gene-related peptide (CGRP) receptor antagonist with two chiral centers. Direct separation of its four stereoisomers (**1a–d**) was achieved using a cellulose chiral stationary phase, a Chiralcel OJ-RH column (150 mm × 4.6 mm), under reversed-phase condition, following the extraction of 0.2 mL plasma on Oasis μElution HLB 96-well solid-phase-extraction (SPE) plate. The tandem mass spectrometric detection was conducted in the positive-ion mode with a turbo-ion-spray (TIS) interface using multiple-reaction-monitoring on a Sciex API3000. Addition of ammonium trifluoroacetate to low-organic mobile phase improved detection sensitivity by more than 30-fold. The simultaneous quantification of the four stereoisomers in human plasma was validated over the ranges of 0.5–5000 nM for **1a** and 0.5–500 nM for its three isomers (**1b–d**). Intraday validation, conducted with five lots of human control plasma, resulted in <12.4% (% coefficient of variation, CV) precision and 96.3–105.4% accuracy for all four stereoisomers. Further evaluation indicated that the assay was specific, the samples were stable after three freeze/thaw cycles, the recovery was reasonable (above 65%) and no matrix effect was observed for all four isomers. Investigation on the chiral integrity of **1a** indicated that the diastereomer **1c**, inversion at azepinone-3 carbon, was the only isomer observed in the post-dose clinical samples and accounted for 2.4–5.2% of MK-0974 exposure in the circulatory system. The possibility of inversion during blood collection, plasma storage and sample preparation was ruled out, while inversion was observed in the clinical formulation accounting for ~0.12% of **1a** in a 100-mg capsule.

© 2008 Elsevier B.V. All rights reserved.

1. Introduction

The development of a single-enantiomer of a chiral drug has become increasingly important in the pharmaceutical industry because of the clear clinical benefits in terms of specific interaction with a biological target to produce the desired efficacy outcome, unique pharmacokinetic profile and reduced unwanted side-effects caused by other isomers [1,2]. MK-0974 (**1a**, Fig. 1), *N*-[(3*R*,6*S*)-6-(2,3-difluorophenyl)-2-oxo-1-(2,2,2-trifuoroethyl)azepan-3-yl]-4-(2-oxo-2,3-dihydro-1*H*-imidazo-[4,5-B] pyridine-1-yl)piperidine-1-carboxamide, is a calcitonin gene-related peptide (CGRP) receptor antagonist and is currently under clinical development for the treatment of migraine [3,4]. The compound is being developed as a single stereoisomer with the absolute configuration *R* and *S* at the two chiral centers at positions '3' and '6', respectively. Epimerization at the azepinone '3' carbon, **1c** (Table 1), was detected when the free base was stressed at 80 °C in 1N sodium hydroxide. The chiral integrity of **1a** in human post-dose plasma samples was a concern with oral administration of **1a** in clinical trials [5]. In order to assess the potential of inversion of the configuration at one or both chiral centers of **1a**, a method to separate and analyze the four possible stereoisomers, **1a–d** (Table 1) in human plasma was required.

Chiral liquid chromatography has been widely used in enantiomeric separations with different types of chiral stationary phases (CSPs) under normal-phase or reversed-phase chiral HPLC

* Corresponding author. Tel.: +1 215 652 8606; fax: +1 215 652 4524.
  E-mail address: yang_xu@merck.com (Y. Xu).
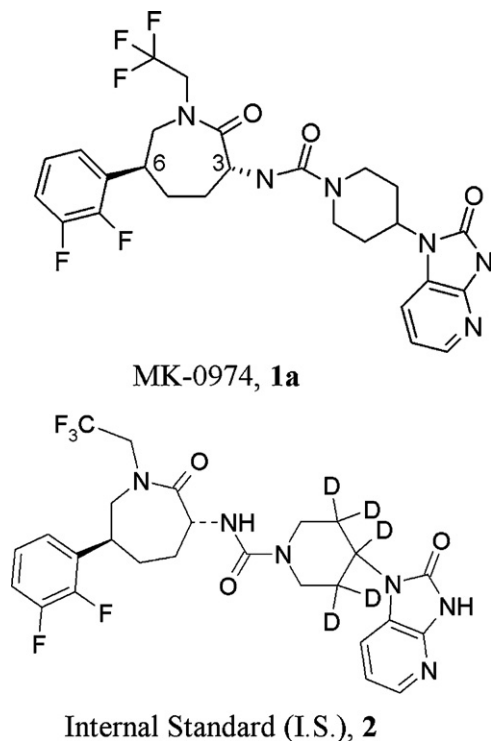[1] Current address: BioMarin Pharmaceutical, 105 Digital Drive, Novato, CA 94949, United States.

Fig. 1. Chemical structures of MK-0974 (**1a**) and its Internal standard (I.S., **2**).

conditions. A direct reversed-phase chiral assay has a number of advantages, such as easy sample handling, compatible mobile phase with mass spectrometry (MS) detection and more buffer choices (buffer components and pHs) to control selectivity by maximizing the interaction between analyte and CSPs. Given these benefits, our focus was on developing a chiral assay under reversed-phase conditions for **1a–d**. Due to the availability of a variety of CSPs, there is usually a chiral column available that can resolve the specific target enantiomers of interest that contains one [6–9] or two chiral centers [10–12]. However, direct reversed-phase chiral separation for the simultaneous determination of four stereoisomers is a challenge due to insufficient chemo-selectivity or limited peak efficiencies of chiral stationary phases.

This paper describes: (1) the method development for a reversed-phase chiral assay to allow direct separation of the four stereoisomers of **1a**, (2) the simultaneous determination of all four isomers in human plasma with increased sensitivity by using appropriate additives in the mobile phase for LC–MS/MS analysis, (3) assay validation to evaluate sensitivity, precision, linearity, recovery, matrix effect and compound stability, and (4) investigation on the chiral integrity of **1a** in clinical plasma samples.

Table 1
The absolute configurations, enantiomeric pairs and diastereomeric relationships of **1a** and its isomers

Abbreviation	Configuration	CGRP[a] binding (nM)	Enantiomeric pairs
1a	(3R, 6S)	Ki = 0.8	
1b	(3S, 6R)	Ki = 86	#1 (**1a**/**1b**)
1c	(3S, 6S)	Ki = 380	
1d	(3R, 6R)	Ki = 145	#2 (**1c**/**1d**)

[a] Calcitonin gene-related peptide.

2. Experimental

2.1. Materials

Compounds **1a–d** were synthesized at the Merck Research Laboratories, Merck & Co. (West Point, PA, USA) [3]. Compound **2** (Fig. 1), containing five deuterium labels, was obtained from Merck Research Laboratories (Rahway, NJ, USA), and used as an internal standard (I.S.) in the assay. HPLC grade acetonitrile, ammonium trifluoroacetate, and ACS grade acetic acid were obtained from Sigma–Aldrich Co. (Milwaukee, WI, USA). Human control blood and plasma (sodium heparin as anticoagulant) were purchased from Biological Specialty Co. (Colmar, PA, USA). Water was purified by a Milli-Q ultra-pure water system from Millipore (Bedford, MA, USA). Oasis®HLB μElution plate (96-well) was purchased from Waters Corporation (Milford, MA, USA) and used for solid-phase extraction (SPE) of plasma samples.

2.2. Equipment

A Packard MultiPROBE II automated liquid handling system (Meriden, CT, USA) and a TomTec Quadra 96 workstation (Model 320, Hamden, CT, USA) were used to perform sample transferring and automated solid-phase extraction process respectively. LC–MS/MS was performed on a LC micro pump (Series 200 from PerkinElmer, Ont., Canada) and 96-Well Plate Autosampler (HTS PAL System from Leap Technology, Carrboro, NC, USA), coupled to a Sciex API 3000 triple quadrupole mass spectrometer with a Sciex Turbo IonSpray (TIS) Interface (Sciex, Toronto, Canada). The data were collected and processed through Analyst software.

2.3. Chromatographic conditions for the chiral assay

The chiral separation was performed on a Chiralcel OJ-RH (150 mm × 4.6 mm) column with 5 μm particle size from Chiral Technologies Inc. (Exton, PA, USA) with a 10-μL sample injection. The mobile phase was composed of acetonitrile and 0.05% ammonium trifluoroacetate aqueous solution at a ratio of 35/65 (v/v), and its flow rate was 0.5 mL/min. The column temperature was maintained at 40 °C and the autosampler temperature was set at 5 °C.

2.4. MS/MS detection and calculation for plasma assay

A PE Sciex API 3000 with a TIS ionization source operated in a positive-ion mode was used to quantitate the analytes. The ion pairs (precursor ion → product ion), m/z 567 → 219 for isomers and m/z 572 → 224 for I.S. were selected for multiple reaction monitoring (MRM). The instrument setting was adjusted to maximize the response for the analyte and I.S., respectively using t-shaped infusion in the presence of mobile phase. A high voltage of 5 kV was applied to the sprayer and the turbo gas temperature was set at 450 °C. The flow rates of nebulizing gas (nitrogen), collision gas (nitrogen) and curtain gas (nitrogen) were at 14, 7 and 8 L/min, respectively. The optimized declustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit potential (CXP) were set at 48, 29, 12 and 14 V for both isomers and I.S. Both Q1 and Q3 quadrupoles were set at unit resolution. For each injection, the total acquisition time was 20 min. The calibration curve was obtained by weighed ($1/x^2$) least squares linear regression on the peak area ratio of each analyte to the internal standard versus the nominal concentration (x) of each analyte. Quality Control (QC) and unknown sample concentrations were determined by interpolation from the calibration curve.
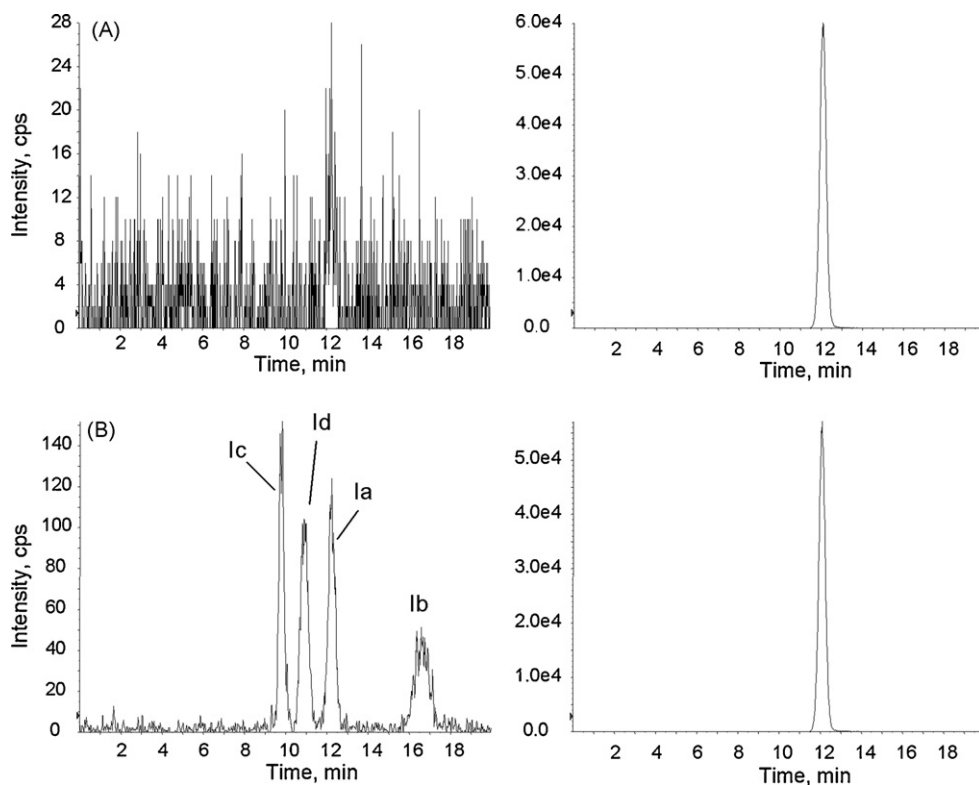
Fig. 2. Representative extracted ion chromatograms of standard human plasma samples (A) single blank, (B) lower limit of quantification (LLOQ), 0.5 nM of **1a–d** (left panel: isomers, right panel: I.S.).

2.5. Preparation of calibration standards and QC samples

Internal standard and each diastereomer (**1a–d**) were weighed and dissolved in acetonitrile/water (50/50, v/v) to make stock solutions at 100 μM (free base form) for each compounds. Working standards of stereoisomers were prepared as a mixture of **1a–d** at the concentration levels from 0.5 to 500 nM through serial dilutions and stored at 4 °C. Solutions of **1a** at the levels of 1000 and 5000 nM were also prepared. An internal-standard working solution (80 nM) was obtained by dilution of 100 μM I.S. stock solution in acetonitrile/water (50/50, v/v). Plasma calibration standards were prepared daily by adding 50 μL of working standard and 50 μL of 80 nM I.S. into 200 μL of control plasma and 200 μL of 0.1 M acetic acid.

Plasma quality control (QC) samples containing **1a–d** were prepared at 1.5, 20 and 400 nM. In addition, a QC containing **1a** only was prepared at 4000 nM. Aliquots of 0.6 mL were dispensed into 3-mL polypropylene vials and then stored at −70 °C.

Blood QC containing **1a** at 4000 nM was prepared by spiking **1a** into human blood and dividing into six tubes. After incubation of triplicates at either 4 °C or 35 °C for 1 h, the blood QCs were centrifuged at 3000 rpm at 4 °C for 10 min. The plasma from the blood QCs was tested along with plasma standards.

2.6. Sample preparation

Control plasma, QCs and clinical samples were thawed, mixed and centrifuged at about 1000 g RCF (relative centrifugal force), 10 °C for 5 min. An aliquot of 200 μL of each sample (or control plasma for standards) was transferred from the vial into a 2-mL 96-well deep-well plate (Matrix Technologies Corp., Hudson, NH, USA). 50 μL of working standard (for calibration standards) or solvent (for single blank, QC and clinical samples), 50 μL of 80 nM I.S. working

solution and 200 μL of 0.1 M acetic acid were sequentially added to each well to make a total volume of 500 μL of acidified sample mix. The acidified sample mix was loaded onto the Oasis HLB uElution solid-phase extraction plate under applied vacuum after conditioning the plate with 200 μL of acetonitrile and 200 μL of 0.1 M acetic acid sequentially. The sample wells were then washed with 400 μL of 0.1 M acetic acid, followed by 400 μL of 10% acetonitrile. The analytes were eluted with 2× 0.1 mL of 100% acetonitrile into a new 1-mL deep-well collection plate (MicroLiter Analytical Supplies, Suwanee, GA, USA). The collected eluent was evaporated under a stream of nitrogen at 35 °C on a SPE Dry-96 (John Chromatograph, Lakewood, CO, USA). The residues were reconstituted into 200 μL of acetonitrile/water (30/70, v/v), and the samples were ready for injection to LC–MS/MS.

2.7. Method validation

The selectivity of the assay was confirmed by processing control plasma from six different lots. Intraday precision and accuracy were determined by analyzing five standard curves, each prepared in a different lot of control plasma. Assay accuracy was calculated from a least-squares regression curve constructed using all five replicate values at each concentration, and the intraday precision (%CV) was calculated from the peak area ratio of parent versus I.S. for each concentration used to construct the standard curve. The lower limit of quantification (LLOQ) was confirmed after observing <20% CV from five different lots of plasma at the selected concentration (0.5 nM). QC samples were analyzed after first freezing and thawing, and the measured concentrations were considered as the initial values. Freeze–thaw stability was evaluated using QC samples that went through three cycles of freezing and thawing, with at least 1-day storage at −70 °C between each thaw.
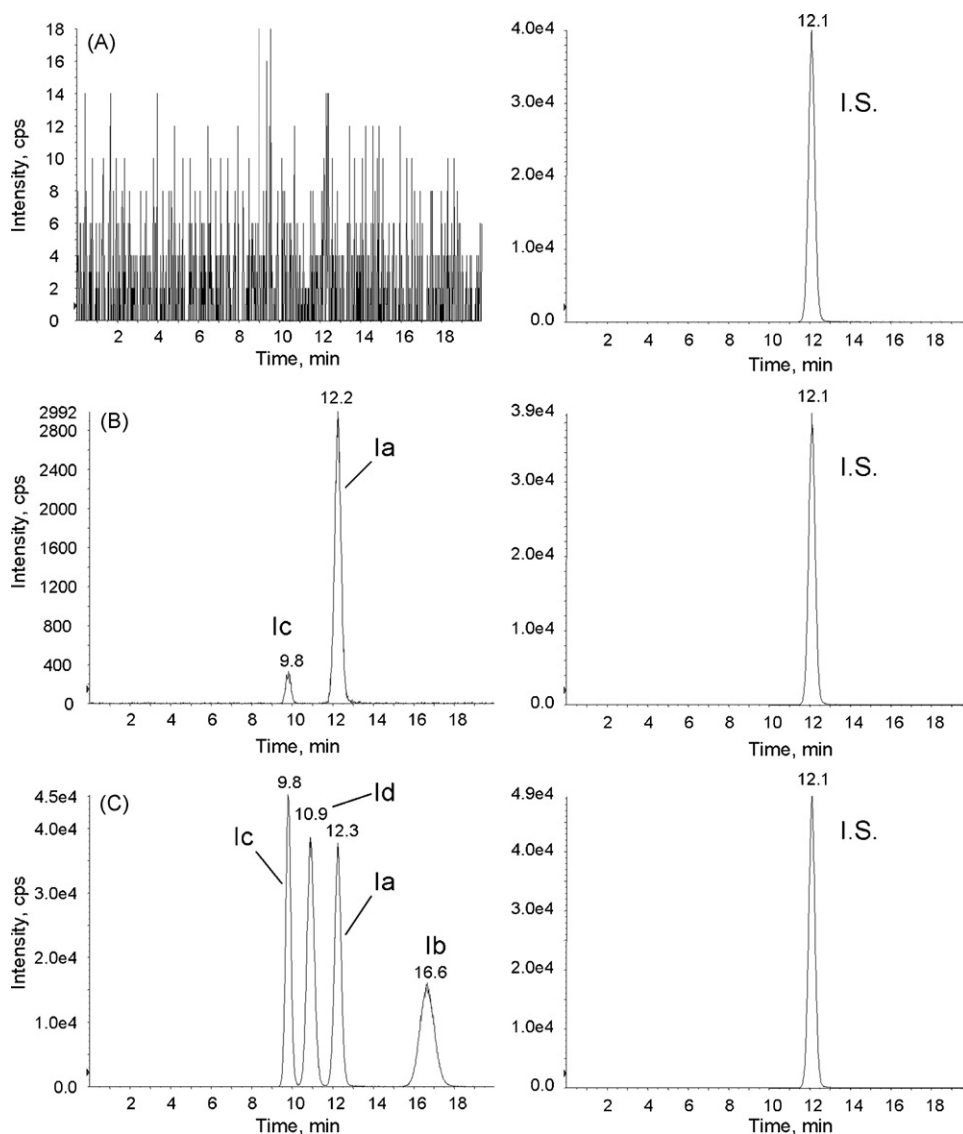
Fig. 3. Representative MRM chromatograms of human plasma samples from clinical subjects (A) pre-dose; (B) subject #20, 48-h post-dose (**1a** at 18.49 nM and **1c** at 1.71 nM) after administration of 500 mg **1a**; (C) plasma standard contains 200 nM of **1a–d** (left panel: isomers, right panel: I.S.).

2.8. Extraction recovery and matrix effect

SPE extraction efficiency was evaluated at the nominal concentrations of 0.5, 50 and 500 nM for each isomer ($n = 5$ at each analyte concentration) and 20 nM for **I.S.** ($n = 15$, five replicates at each of the three **1a** concentrations). Recovery of the extraction was determined by comparing the absolute peak areas of the pre-spiked analyte with those of the post-spiked analyte. The pre-spiked analyte was prepared by the extraction procedure described in Section 2.6. The post-spiked samples were prepared by extracting drug-free control plasma and then spiking with working stocks containing isomers and I.S. The matrix enhancement/suppression of ionization was evaluated by comparing the absolute peak areas of the post-spiked standards with those of the neat standards in reconstitution solvent.

3. Results and discussion

During the clinical development of **1a**, it was required to develop a sensitive and specific chiral assay to address the concern of chiral inversion. The reversed-phase chiral chromatography was estab-

lished to separate the four stereoisomers of **1a**. Mass spectrometry was used to provide the sensitivity needed to support *in vivo* studies.

3.1. Reversed-phase chiral column selection

Chiral separation can be achieved under normal-phase or reversed-phase chiral HPLC conditions. Reversed-phase chiral HPLC can be classified as direct or indirect (derivatization of enantiomers to form a pair of diastereomers) method. In this study, a direct reversed-phase separation on chiral stationary phases (CSPs) was chosen for the LC–MS analysis of stereoisomers **1a–d** due to its compatibility with the LC–MS. Among hundreds of available chiral stationary phases, columns from two categories (A) the macrocyclic glycopeptide-based Chirobiotic V (vancomycin) and Chirobiotic T (teicoplanin), and (B) polysaccharide-based Chiralpak AD-RH, Chiralcel OD-RH and Chiralcel OJ-RH have been reported to allow the separation of many drug enantiomers [13–16], and therefore, they were selected for evaluation of the chiral separation of **1a–d**, using polar organic mode (for category A columns) and reversed-phase mode (for category B columns).

Table 2
Intraday precision and accuracy for the determination of four stereoisomers of **1a** in five lots of human control plasma

Nominal concentration (nM)	Mean measured concentration (nM) ($n = 5$)	Accuracy[a] (%)	Precision[b] (%CV)
1a (3R, 6S)			
0.5	0.50	100.6	8.19
1	0.98	98.4	3.09
5	5.10	102.0	3.02
50	49.81	99.6	2.63
200	200.4	100.2	1.10
500	490.9	98.2	1.10
1000	999.6	100.0	3.04
5000	4949.8	99.0	1.96
Linear regression	$R = 0.9992$	Slope = 0.0495	Intercept = 0.00246
1b (3S, 6R)			
0.5	0.49	97.3	6.75
1	1.05	105.4	3.58
5	5.04	100.8	2.74
50	49.4	98.8	2.64
200	199.3	99.6	1.37
500	490.8	98.2	1.77
Linear regression	$R = 0.9987$	Slope = 0.0456	Intercept = 0.00131
1c (3S, 6S)			
0.5	0.49	98.4	12.4
1	1.02	102.4	8.75
5	5.22	104.5	6.46
50	48.5	97.1	4.16
200	202.8	101.4	6.94
500	481.3	96.3	7.60
Linear regression	$R = 0.9954$	Slope = 0.0447	Intercept = 0.00099
1d (3R, 6R)			
0.5	0.49	98.0	7.53
1	1.04	104.4	5.90
5	4.90	98.0	2.08
50	50.2	100.4	2.54
200	200.8	100.4	1.51
500	493.7	98.7	1.29
Linear regression	$R = 0.9985$	Slope = 0.0547	Intercept = 0.00307

[a] Expressed as ((mean measured concentration/nominal concentration) × 100) (%).
[b] Expressed as coefficient of variation (%CV).

First, the macrocyclic glycopeptide based chiral columns, Chirobiotic V2 (250 mm × 2.1 mm, 5 μm) and ChiroBiotic T (250 mm × 2.1 mm, 5 μm), were evaluated because of their potential to be used in the polar organic mode (close to 100% organic modifier, such as methanol, with small amount of additives in the mobile phase), which might provide more sensitivity using MS detection than the reversed-phase mode. The test was conducted with various percentages of methanol in the mobile phase at the

Table 3
QC precision and accuracy for the four stereoisomers of **1a** in human plasma

Nominal concentration (nM)	Initial mean measured concentration (nM) ($n = 5$)	Accuracy[a] (%)	Precision[b] (%CV)	F/T stability[c] (%) ($n = 3$)
1a (3R, 6S)				
1.5	1.36	90.6	6.38	108.8
20	18.45	92.3	2.08	105.6
400	370.50	92.6	1.49	105.1
4000	4090.46	102.3	2.72	–[d]
1b (3S, 6R)				
1.5	1.46	97.7	1.87	106.8
20	18.90	94.5	1.54	108.8
400	387.56	96.9	1.30	106.2
1c (3S, 6S)				
1.5	1.37	91.7	2.62	93.7
20	20.28	101.4	4.22	90.6
400	401.18	100.3	3.55	95.5
1d (3R, 6R)				
1.5	1.45	96.5	3.86	103.4
20	19.18	95.9	1.52	106.4
400	383.70	95.9	1.63	108.8

[a] Expressed as ((mean measured concentration/nominal concentration) × 100) (%).
[b] Expressed as coefficient of variation (%CV).
[c] Stability after three cycles of freeze and thaw, expressed as ((mean measured concentration after freeze and thaw/initial mean measured concentration) × 100) (%).
[d] Not determined.

flow rate of 0.1 mL/min. ChiroBiotic T failed to demonstrate the required resolving power for the four isomers under all the tested conditions, while ChiroBiotic V2 provided a baseline separation (resolution Rs ≈ 1.5) between the enantiomers **1a** and **1b** or **1c** and **1d**, at 80% methanol. However, the diastereomers **1a** and **1d** overlapped at the retention time ∼9.4 min, while **1b** and **1c** overlapped at the retention time ∼10.5 min on ChiroBiotic V2. Although the diastereomer pairs can be separated on an achiral column, a chiral/achiral combination was not considered due to the favorable results obtained from the subsequent testing on polysaccharide chiral stationary phases.

Within the category of polysaccharide-based chiral selector, one amylose-based column (Chiralpak AD-RH, 150 mm × 4.6 mm, 5 μm) and two cellulose-based columns (Chiralcel OD-RH, 150 mm × 4.6 mm, 5 μm and Chiralcel OJ-RH, 150 mm × 4.6 mm, 5 μm) were evaluated as a single-column configuration with a variety of reversed-phase mobile phase combinations. Among the three tested columns, Chiralcel OJ-RH exhibited the best separation for all four isomers **1a–d** using a mobile phase of acetonitrile–water (35/63, v/v) at a flow rate of 0.5 mL/min (Figs. 2 and 3Figure 2B and Figure 3C). Retention times were 9.8, 10.9, 12.3 and 16.6 min, and the corresponding capacity factors (k') were 1.4, 1.7, 2.0 and 3.1.for **1c**, **1d**, **1a** and **1b**, respectively. The resolution between each adjacent bands **1c**/**1d**, **1d**/**1a** and **1a**/**1b** was 1.52, 1.56 and 2.79, respectively. The baseline separation of all four stereoisomers was achieved within 20 min. Evaluation of the effect of column temperature on the chiral separation showed a slightly longer retention, better separation, better peak shape (especially for the last peak, **1b**) and higher sensitivity at 40 °C comparing to those at room temperature. Introducing acetic acid or triethylamine at different concentration ratios to the mobile phase, in attempt to provide different mobile phase pH, caused about a 50-fold ion suppression. The analyte retention was not affected by changing mobile phase pH, which is consistent with the fact that the analytes studied here are neutral (pK_a = 2.20 and 9.53 from photometric titration) within the pH range restriction for chiral column operation (pH 2.0–8.0).

3.2. Using appropriate mass-spectrometer-compatible additive to enhance detection sensitivity

Balancing between sensitivity and separation, 35% acetonitrile in water was chosen as a reversed-phase mobile phase, because any higher percentage of acetonitrile led to poor resolution, especially for the first eluting pair **1c**/**1d**. However, the water content in this 35% acetonitrile mobile phase was still so high that the ionization on the mass spectrometer (MS) was not efficient enough to provide the sensitivity required for quantitative analysis of the stereoisomers in clinical samples. Desai and Armstrong have reported that compounds with amine or amide functional groups could be effectively ionized in the presence of ammonium trifluoroacetate (NH₄TFA) [13]. In an attempt to increase the detection sensitivity, the addition of NH₄TFA to the mobile phase of 35% acetonitrile was evaluated. Over the range of 0.025–0.25% NH₄TFA, the use of 0.05% provided a maximum enhanced signal intensity—more than 30-fold higher than the signal obtained without NH₄TFA (Fig. 4). The salt additive allowed proper ionization of the analytes for MS detection without having a significant impact on the enantiomeric separation in terms of resolution and retention. The isocratic reversed-phase chiral method developed here provided a simple and MS compatible tool to allow simultaneous determination of four stereoisomers of **1a**.

3.3. Simultaneous quantification of the four stereoisomers of **1a**

After establishing the chiral separation conditions, a reversed-phase chiral LC–MS/MS assay for the simultaneous quantification
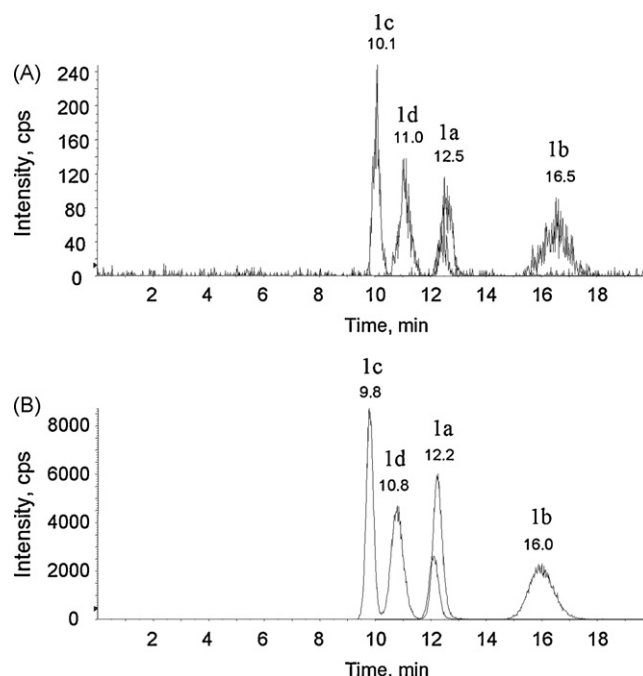

Fig. 4. Chromatograms of the chiral separation, showing a signal-intensity change, under mobile phases: (A) in the absence of 0.05% ammonium trifluoroacetate and (B) in the presence of 0.05% ammonium trifluoroacetate.

of the four stereoisomers of **1a** in human plasma was developed and validated over the ranges of 0.5–5000 nM for **1a** and 0.5–500 nM for its three isomers (**1b–d**) using compound **2**, a deuterium labeled **1a** (Fig. 1), as the internal standard. Since the concentration of **1a** were much higher than other isomers in the clinical samples, to avoid two-step analysis (i.e. measuring **1a** with dilution and others without dilution), the upper limit of quantification (ULOQ) for **1a** were designed to be higher than that for others. Due to the large dynamic range for **1a** analysis, quality control (QC) samples for **1a** were prepared and tested at four concentration levels, while three concentrations were used for its isomers (**1b–d**). Following the solid-phase extraction using Oasis μElution HLB 96-well plate, the analytes and I.S. were separated on a Chiralcel OJ-RH column (150 mm × 4.6 mm, 5 μm) under reversed-phase condition, and monitored by MS/MS under the positive-ion mode with a turbo ion-spray interface using multiple reaction monitoring (MRM) to obtain the optimal sensitivity and selectivity.

Assay specificity was accessed by testing six lots of control human plasma extracts. The lower limit of quantification (LLOQ) of the assay, defined as observing <20% precision (CV) and accuracy at the low end of each standard curve prepared from five different lots of human plasma (data in Table 2), was 0.5 nM on a Sciex API3000 for each of the four isomers when 0.2 mL of plasma sample was processed. Representative chromatograms of extracts of drug free human plasma and of a plasma standard containing 0.5 nM (LLOQ) of each isomer are shown in Fig. 2. No endogenous interference was found within the retention time windows of the four isomers and the internal standard.

The dynamic range for each isomer was determined from the intraday validation data that showed <12.4% precision (%CV) and <6.0% inaccuracy for each isomer from five calibration curves constructed with five different lots of human plasma (Table 2). Using a weighted ($1/x^2$) linear regression, correlation coefficients ranged from 0.995 to 0.999. QC samples, prepared and stored at −70 °C, gave accuracy and precision that ranged from 90.6 to 102.3% and 1.30–6.8%, respectively, for the four stereoisomers over

Table 4
Assay recovery and matrix effect of the four stereoisomers of **1a** from five lots of human control plasma

Nominal concentration (nM)	%CV of peak area, pre-spiked ($n = 5$)	Recovery[a] (%)	Matrix effect[b] (%)
1a (3*R*, 6*S*)			
0.5	8.1	94.2	99.6
50	4.3	83.1	97.9
500	2.8	81.5	95.7
1b (3*S*, 6*R*)			
0.5	4.3	84.2	96.4
50	4.7	81.6	97.6
500	3.9	81.0	97.7
1c (3*S*, 6*S*)			
0.5	11.3	72.1	91.8
50	7.6	67.6	99.3
500	9.3	68.1	96.7
1d (3*R*, 6*R*)			
0.5	8.1	92.8	98.4
50	3.3	85.8	98.7
500	1.9	84.7	96.5
I.S.			
20 ($n = 15$)	3.9	82.2	97.2

[a] Expressed as ((peak area of pre-spiked/peak area of post-spiked) × 100) (%).
[b] Expressed as ((peak area of post-spiked/peak area of neat solution) × 100) (%).

the tested QC concentrations (Table 3). The QCs were stable after three cycles of freeze/thaw (Table 3). The autosampler stability was evaluated using the extracted QC samples by comparing the QC accuracy/precision before and after an analytical run, and the results indicated that the four isomers in post-extracted samples were stable for at least 40 h in the autosampler. Carryover was minimized during method development and monitored by injecting a blank sample immediately after an extracted high standard during the study to ensure that the extent of carryover did not have a negative impact on assay performance.

The extraction recovery of each isomer from plasma was determined by comparing five replicates of plasma standards spiked before SPE extraction (pre-spiked) versus standards spiked after extraction of blank plasma (post-spiked). The matrix effect was assessed by taking the peak area ratio of post-spiked standards versus that of neat analyte solution at the same concentration. The results (Table 4) showed that the mean recoveries were above 65% at 0.5, 50 and 500 nM with limited variability (%CV < 11.3% from five lots of plasma), and no significant matrix effect was observed for the four analytes.

3.4. Assay application to evaluate the chiral integrity of post-dosed human plasma and pharmaceutical formulation

The validated method was applied to investigate the chiral integrity of **1a** in clinical plasma samples, where six subjects that received 500-mg oral dose of **1a** were selected. Typical chromatograms of pre-dose and post-dose plasma extract are given in Fig. 3. As illustrated in Fig. 3B, in comparison with 3C, the only detectable stereoisomer was **1c**—an inversion at 3-position, and no **1b** or **1d** was detected in all the tested subject samples under the current validated assay. The plasma concentrations of **1a** and **c** were determined, and their mean plasma profiles are presented in Fig. 5. The area-under-curve (AUC) of **1c** ranged from 2.4% to 5.2% (geometric mean is 3.5%, $n = 6$) of parent exposure. The terminal half-life of **1c** was longer than that of **1a**. This could lead to a more significant interference to the parent concentration at the later sample collection time points if the diastereomers were not separated chromatographically during clinical sample analysis.
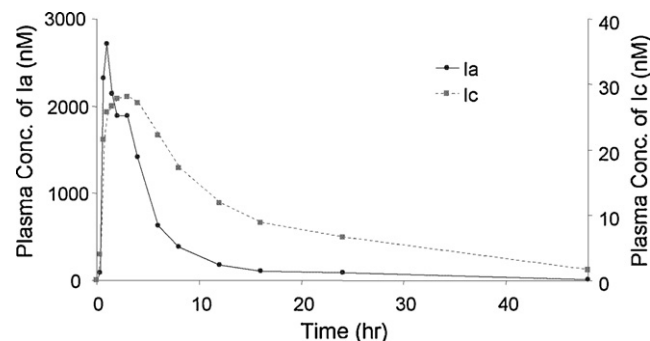
Fig. 5. Mean plasma concentration–time profile ($n = 6$) of **1a** and its diastereomer **1c** observed from human subjects receiving a single oral administration of **1a**.

Due to the observation of **1c** in post-dose samples [5], further investigation was conducted in attempt to rule out the possibility of *in vitro* inversion during blood collection and plasma sample storage. First of all, the analyte stability in blood was tested following a similar procedure used for clinical sample processing at the clinical site. The human blood QCs, 4000 nM **1a** in blood, were incubated at 4 °C or 35 °C for 1 h, which covered the time frame and temperature range that blood samples were exposed to after collection. The blood QCs were centrifuged at 3000 rpm at 4 °C for 10 min at the end of incubation. Plasma was transferred from the blood QCs and tested along with plasma standards. The result showed that no inversion was detected (Fig. 6A) and **1a** was stable in blood after incubation at 4 °C or 35 °C for up to 1 h. Secondly,
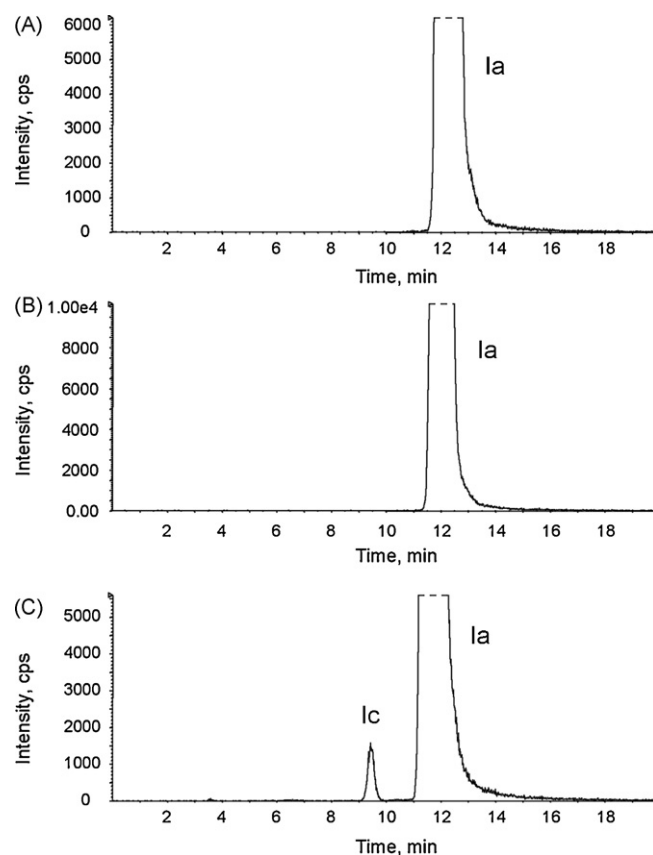
Fig. 6. Representative MRM chromatograms for (A) human blood QC containing 4000 nM of **1a** after incubation at 35 °C for 1 h; (B) human plasma QC containing 4000 nM of **1a** after storage at −70 °C for 5 months; (C) the capsule formulation used in the clinical studies.

the stability of **1a** in control human plasma at 4000 nM after storage at −70 °C for 5 months was tested. The result indicated that **1a** in plasma was stable during storage and sample preparation in the analytical laboratory (Fig. 6B). Thirdly, the capsule formulation (100 mg active ingredient/capsule) used in the clinical study was tested and the amount of **1c** observed in this formulation was about 0.12% of **1a** (Fig. 6C). The above results confirmed that **1c** observed in the clinical sample was not generated during sample collection and storage. This led to a possibility that a small percent of formulation impurity (**1c**) could accumulate up to 2.4–5.2% of parent exposure in humans due to the different tissue distribution between **1a** and **1c**. Since the exposure of **1c** was observed in animal species with the same **1a** formulation (data not show) and the exposure of **1c** in human was less than 10% of parent drug systemic exposure at steady state, safety should not be a concern based on the FDA guidance for industry safety testing of drug metabolites [17].

4. Conclusions

A polysaccharide-type chiral stationary phase (Chiralcel OJ-RH) has been demonstrated to be an appropriate sorbent to carry out chiral HPLC separation of the four stereoisomers of **1a** under the reversed-phase condition without derivatization. The quality of the separation and peak shape allowed simultaneous determination of the four stereoisomers, **1a**–**d**, in human plasma with high sensitivity, specificity and reliability; and the *direct* reversed-phase chiral separation on LC–MS/MS provided an additional advantage compared to the normal-phase chiral assay and indirect (derivatization of enantiomers) RP-HPLC, which require much more restricted instrument operation and complicated sample preparation. Since the organic content in the RP *chiral* assay is generally lower than the conventional non-chiral RP-HPLC, the assay sensitivity could be an issue for chiral RP-LC/MS detection. Addition of ammonium trifluoroacetate into mobile phase significantly enhanced ionization for the tested analytes, and provided a useful alternative to resolve the low sensitivity issue. The investigation on the chiral integrity of **1a** in clinical samples indicated that the diastereomer **1c**, inversion at azepinone-3 carbon, was the only isomer observed in the tested post-dose samples, and accounted for 2.4–5.2% of parent exposure

in human circulating system. The possibility of inversion during blood collection, plasma storage or sample preparation was ruled out, while inversion during clinical formulation (100 mg **1a** in capsule) was observed accounting for ∼0.12% of **1a**. The application of this reported chiral assay to investigate the chiral integrity of **1a** and source of inversion demonstrated a pathway on how to address the issue, interpret the result and evaluate the impact of the observation on drug development process following the FDA guidance.

References

[1] A. Richards, R. McCague, Chem. Ind. (1997).
[2] C. Mislanova, M. Hutta, J. Chromtogr. B 797 (2003) 91.
[3] C.S. Burgey, Z.J. Deng, D.N. Ngugen, D.V. Paone, A.W. Shaw, T.M. Williams, The United State Patent and Trademark Office (USPTO) October 11, US Patent number 6,953,790 (2005).
[4] T.W. Ho, L.K. Mannix, X. Fan, C. Assaid, C. Furtek, C.J. Jones, C.R. Lines, A.M. Papoport, Neurology (2007) (Epub ahead of print).
[5] Y. Xu, K.J. Willson, D.G. Musson, J. Chromatogr. B 863 (2008) 64.
[6] C. Mislanova, M. Hutta, J. Chromatogr. B 797 (2003) 91.
[7] M. Maftouh, C. Granier-Loyaux, E. Chavana, J. Marini, A. Pradines, Y.V. Heyden, C. Picard, J. Chromatogr. A 1088 (2005) 67.
[8] C. Perrin, N. Matthijs, D. Mangelings, C. Granier-Loyaux, M. Maftouh, D.L. Massart, Y.V. Heyden, J. Chromatogr. A 966 (2002) 119.
[9] R. Cirilli, A. Simonelli, R. Ferretti, A. Bolasco, P. Chimenti, D. Secci, E. Maccioni, F. La Torre, J. Chromatogr. A 1101 (2006) 198.
[10] J. Zagrobelny, B.K. Matuszewski, W.F. Kline, S.H. Vincent, J. Pharm. Biomed. Anal. 17 (1998) 1057.
[11] E. Lipka, A. Selouane, D. Postel, C. Len, M.P. Vaccher, J.-P. Bonte, C. Vaccher, J. Chromatogr. A 1034 (2004) 161.
[12] M. Lipka, A. Guelzim, S. Yous, J.-P. Bonte, C. Vaccher, J. Biochem. Biophys. Methods 64 (2005) 46.
[13] M.J. Desai, D.W. Armstrong, J. Chromatogr. A 1035 (2004) 203.
[14] K. Petritis, A. Valleix, C. Elfakir, M. Dreux, J. Chromatogr. A 913 (2001) 331.
[15] Z. Basakova, I. Klouckova, E. Tesarova, J. Chromatogr. B 770 (2002) 63.
[16] E. Yashima, J. Chromatogr. A 906 (2001) 105.
[17] Food and Drug Administration, US Department of Health and Human Services, Guidance for Industry: Safety Testing of Drug Metabolites, February 2008.