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Separation and quantification of two diastereomers of a Drug Candidate in rat plasma by ultra-high pressure liquid chromatography/mass spectrometry

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Abstract

Ultra-high pressure liquid chromatography (UHPLC) is a relatively new technology which utilizes chromatographic media with a 1.7 μm particle size. This technology has the potential to offer significant advantages in resolution, speed, and sensitivity for analytical determinations, particularly when coupled with mass spectrometric detection. Drug Candidate A, under development at Merck Research Laboratories, contains two chiral centers which have the absolute configuration *R*, *S*. Under *in vivo* and *ex vivo* conditions, one of the chiral centers readily epimerizes to produce the *R*, *R* diastereomer. Initially, a traditional high performance liquid chromatography/tandem mass spectrometry (HPLC–MS/MS) method was developed to separate and quantify these two diastereomers in rat plasma. The lower limit of quantification (LOQ) of the two analytes was 2 ng/mL, and a chromatographic run time of approximately 11 min was needed to separate R, S-(A) and R, R-(A). In this study, we explored a simple and robust UHPLC–MS/MS method in order to increase sample throughput and productivity. We were able to achieve a two-fold reduction in the lower limit of quantification and a three-fold reduction in retention time utilizing the UHPLC method, while keeping the same sample extraction procedure and similar MS/MS methodology. The new method exhibited good intra- and inter-day accuracy and precision, and was linear over a dynamic range of 1–500 ng/mL for each diastereomer. The method was successfully applied for the determination of R, S-(A) and R, R-(A) concentrations for *in vitro* and *in vivo* studies of epimerization of A in Sprague-Dawley rats.
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Keywords: Diastereomers; UHPLC–MS/MS; Quantitation

1. Introduction

The recently commercialized technology, *ultra-high pressure liquid chromatography* (*UHPLC*), has been made possible by the availability of reversed-phase chromatographic media with a 1.7 μm particle size. Based on the van Deemter equation, the use of smaller particles can significantly reduce the height equivalent of a theoretical plate (HETP) generated in a separation by improved mass transfer [1]. The separation efficiency is three times greater with 1.7 μm versus 5 μm particles, and two times greater compared to 3.5 μm particles. Furthermore, the resolution is 70% higher than with 3.5 μm particles. Typically, shorter retention times can be achieved using 1.7 μm particles compared to 5 μm particles with the same efficiency, and the flow rate can be three times higher, again with little loss of efficiency. An additional benefit of UHPLC is the enhanced sharpness of chromatographic peaks, which should, in concept, lower the limit of detection by at least a factor of two-fold relative to separations conducted with HPLC [1–5]. Thus, this technology could offer significant advantages in resolution, speed, and sensitivity for analytical determinations, particularly when coupled with mass spectrometric (MS) detection. Applications of UHPLC coupled with MS detection have been reported recently, such as the use of UHPLC–MS/MS for priority pesticides in baby food [6] and ground water [7], forensic and toxicological analysis [8], quantitative bio-analysis of drugs [9–12], and investigating metabolites using UHPLC–qTOF [13]. Another interesting

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study on the diastereomers *ephedrine* and *pseudoephedrine* demonstrated that utilizing a UHPLC–MS method significantly improved the sensitivity and reduced the analysis time compared to a well-optimized HPLC–MS method [14].

Drug Candidate A, under development at Merck Research Laboratories, contains two chiral centers which have the absolute configuration *R*, *S*. Under *in vivo* and *ex vivo* conditions, one of the chiral centers can readily epimerize to produce the *R*, *R* diastereomer. Since chiral compounds can have distinct pharmacokinetic, pharmacological and toxicological properties, they must be characterized individually if racemates are administered or if inter-conversion of stereoisomers is possible [15–18]. In order to address stereo-chemical concerns for developing new drugs under current FDA guidelines [19], a rapid and sensitive assay for Drug A was required to quantify the two diastereomers for *in vitro* and *in vivo* studies. Initially, a traditional high performance liquid chromatography/tandem mass spectrometry (HPLC–MS/MS) method was developed. With this assay, the limit of quantification (LOQ) of the two analytes in rat plasma was 2 ng/mL, and a chromatographic run time of approximately 11 min was needed to separate R, S-(A) and R, R-(A).

In this study, we developed a simple and robust UHPLC–MS/MS method in order to increase sample throughput and productivity using a reversed-phase UHPLC column. We were able to achieve a two-fold reduction in the lower limit of quantification and a three-fold reduction in retention time utilizing the UHPLC method while keeping the same extraction procedure and similar MS/MS methodology. This method was successfully validated by evaluating the extraction recovery, stability, sensitivity, linearity, precision and accuracy for these two diastereomers. Sample handling conditions were optimized in order to minimize the *ex vivo* inter-conversion of these two diastereomers. This new method was successfully used to determine the R, S-(A)/R, R-(A) concentration ratios in plasma from Sprague-Dawley rats both *in vitro* as well as *in vivo* following oral administration of A.

2. Experimental

2.1. Chemicals, reagents and materials

Diastereomers (R, S-(A) and R, R-(A)) and the internal standard (IS) were synthesized at Merck Research Laboratories. HPLC-grade water and all analytical organic solvents were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Control rat plasma with sodium-EDTA anticoagulant was purchased from BioReclamation Inc. (Hicksville, NY, USA). Ammonium bicarbonate buffer was prepared by titrating 100 mM ammonium bicarbonate with ammonium hydroxide to pH 9.3 ± 0.05. Formate buffer was prepared by titrating 500 mM formic acid with sodium hydroxide to pH 3.0 ± 0.05. Deep 96-well collection plates were purchased from VWR Scientific Products (West Chester, PA, USA). Disposable glass centrifuge tubes (5 mL) were purchased from Kimble Corp. (Vineland, NJ, USA), and disposable culture tubes (13×100 mm) were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

2.2. Animal studies and sample collection

All studies were reviewed and approved by the Merck Research Laboratories Institutional Animal Care and Use Committee. Sprague-Dawley rats, 14–16 weeks old and weighing 300–400 g, were obtained from Charles River Laboratories (Wilmington, MA). The animals were fasted overnight before dosing and for 4 h post-dose, with water provided ad libitum. Pure R, S-(A) was administered orally by gastric gavage using a dosing vehicle composed of 0.5% aqueous methylcellulose containing 0.02% sodium lauryl sulfate at 5 mL/kg. Blood (~0.3 mL) was collected into sodium-EDTA-containing tubes at predetermined intervals (pre-dose, 0.25, 0.5, 1, 2, 4, 6, 8, 24, 48, and 72 h) from a previously implanted catheter in the femoral artery. Plasma samples were obtained after immediate centrifugation of blood at 4 °C and were stored at −70 °C until analyzed.

2.3. In vitro incubations

Pure R, S-(A) was dissolved in acetonitrile and added to fresh, EDTA-treated rat blood to produce a 10 μM final concentration. The total content of acetonitrile in blood was 0.5% (v/v). Incubations were carried out at 37 °C in a shaking water bath for predetermined intervals (0, 0.25, 0.5, 1, 2, 4 h). The incubations were terminated by placing the samples on ice. Plasma samples were obtained by centrifugation at 4 °C and then immediately extracted and analyzed by UHPLC–MS/MS.

2.4. Sample preparation for analysis

All sample preparations were performed in an ice–water bath, except as noted. The stock solutions of R, S-(A) and R, R-(A) (1 mg/mL) were prepared by dissolving the compounds separately in DMSO. An internal standard (IS) stock solution was prepared similarly. Two separate weighings for each diastereomer were used to prepare individual stock solutions for standard curve and quality control (QC) samples. All stock solutions were stored at −70 °C. Dilutions of the diastereomer and IS stock solutions were prepared in acetonitrile. Standard curve samples were prepared by spiking appropriate amounts of R, S-(A) and R, R-(A) separately into 100 μL of control rat plasma. The standard curves consisted of eight concentrations at 1.0, 2.5, 5.0, 12.5, 25.0, 125, 250, and 500 ng/mL in duplicate. The QC samples were prepared at four concentrations (1.0, 2.5, 100 and 400 ng/mL) by spiking appropriate amounts of R, S-(A) and R, R-(A) separately into 100 μL control rat plasma. The internal standard solution (20.0 μg/mL) was prepared in acetonitrile. A 20 μL aliquot of this solution was added to each sample in 5 mL glass centrifuge tubes. Then, 1 mL of an ethyl acetate/MTBE (methyl tert-butyl ether) (50/50, v/v) mixture was added, the tubes were capped and vortex-mixed for 5 min. Following centrifugation at 4 °C ($3000 \times g$ for 10 min), the aqueous layer was frozen in a dry-ice/acetone bath and the organic layer was transferred to clean disposable culture tubes. The culture tubes containing the organic layer were placed in a Zymark Turbo Vap LV (Hopkinton, MA, USA) and evaporated to dryness at

room temperature under a gentle stream of nitrogen gas. The samples were reconstituted in 150 μL of acetonitrile, and 5 μL aliquots were injected onto the UHPLC column for mass spectral analysis. In contrast, for the traditional HPLC method, 10 μL aliquots were injected to achieve the LOQ (2 ng/mL) for that method.

2.5. HPLC

The HPLC system consisted of a Leap HTS PAL autosampler coupled with two Perkin-Elmer series 200 micropumps. Chromatographic separation of the two diastereomers was achieved on a BetaBasic C-8 (2.1 mm ID × 150 mm, 5 μ particle size, Thermo Hypersil-Keystone Scientific) eluted at 0.5 mL/min. Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile/methanol (20/80). A gradient elution program was utilized where the solvent composition was held at 61% B for 2.5 min, changed linearly to 80% B over the next 5.5 min, and then rapidly changed to 90% B over the next 0.2 min followed by holding at 90% B for additional 2.5 min. The column was then re-equilibrated at the original solvent composition over 2.2 min. The total run time was 11 min.

2.6. UHPLC

UHPLC was conducted on an ACQUITY UPLC™ system, equipped with a binary solvent delivery system and an autosampler (Waters Corp., Milford MA, USA). The chromatography was performed on a 2.1 mm ID × 50 mm Waters Acquity UPLC™ BEH-C18 (1.7 μm particle size) column using a 0.5 mL/min flow rate. Mobile phase A was water with 0.1% formic acid, and mobile phase B was acetonitrile/methanol (20/80, v/v) with 0.1% formic acid. A gradient elution program was utilized where the initial solvent composition was held at 61% B for 0.5 min and then changed linearly to 75% B over the next 1.8 min. The proportion of solvent B was then changed linearly to 90% over the next 0.5 min and held for an additional 0.4 min. The solvent composition was then returned in a single step to the initial conditions for re-equilibration over 0.3 min. The total run time was 3.5 min.

2.7. MS/MS

The LC systems were alternately coupled to an MDS Sciex API 4000 triple quadrapole mass spectrometer (Toronto, Canada) equipped with an electrospray ionization (ESI) source. The instrument was operated in negative ionization mode using selected reaction monitoring (SRM). The ESI voltage was set to −4.5 kV and the auxiliary gas temperature was maintained at 400 °C. High purity nitrogen was used for GAS 1, GAS 2, curtain, and collision gases. The mass resolution was set to a peak width of 0.7 mass units at half-height for both Q1 and Q3. The electron multiplier was set at −2000 V. Declustering potential (−90 V), collision energy (−29 eV), entrance potential (−10 V), and collision cell exit potential (−20 V) were set as indicated. The R, S-(A) and R, R-(A) diastereomers and the internal standard were monitored using specific precursor ion → product ion transitions of *m/z* 442 → 399 and *m/z* 410 → 348, respectively. The dwell time of each SRM transition was 300 ms (UHPLC–MS/MS) or 1000 ms (HPLC–MS/MS), which was the sole change to the MS/MS conditions.

2.8. Data processing

MDS Sciex Analyst software (v. 1.4) was used for data acquisition and chromatographic peak integration. The peak area ratios of each diastereomer to the internal standard were plotted as a function of the nominal concentrations of the analytes. The standard calibration curves for each diastereomer were constructed using weighted ($1/x^2$) linear regression. A 3-point smoothing algorithm was applied to all ion chromatograms before integration. Intra- and inter-day assay relative standard deviations for QC samples were calculated using Watson™ LIMS software version. 6.4.0.04 (Thermo Scientific Inc. Woburn, MA).

2.9. Assay characterization and validation

The precision and accuracy of the method were evaluated using QC samples at four concentrations in three replicates on three separate days of analysis. The precision and accuracy were expressed as the coefficient of variation (CV) and percentage of bias from the nominal concentrations of the QC samples, respectively.

Extraction recovery for each diastereomer was evaluated under three pH conditions (acidic, neutral, and basic) at concentrations of 1.0, 100 and 400 ng/mL. The extraction recovery was determined in triplicate for each diastereomer by comparing the mean chromatographic peak area of spiked-before-extraction samples with the mean peak areas of the corresponding spiked-after-extraction standards. Control rat plasma was either acidified with 30% 0.5 M formate buffer (pH = 3), untreated (physiological pH), or adjusted to basic conditions with 30% 0.1 M ammonium bicarbonate buffer (pH = 9.3). The extent of *ex vivo* inter-conversion was also determined in triplicate at each of the three pH levels by comparing the concentration of R, S-(A) to that of R, R-(A) generated during sample preparation.

To determine the bench-top stability of each diastereomer, three sets of rat plasma QC samples from the low, mid and high range of the calibration curve were prepared in triplicate and left in an ice-water bath for 3 h. The samples were then processed and analyzed with a freshly prepared calibration curve. The concentrations determined for the QC samples were compared to their theoretical values. The freeze/thaw stability (1 cycle) was investigated in a similar manner. The freshly prepared QC samples were frozen overnight, thawed in an ice bath, and then analyzed with a freshly prepared calibration curve. The autosampler stability of the diastereomers in plasma sample extracts was also determined. The extracted QC samples were kept in the 96-well injection plate over a 24 h period at 5 °C and then analyzed with a freshly prepared calibration curve.

3. Results and discussion

3.1. UHPLC–MS/MS method development

During the tuning of the mass spectrometer by both infusion and flow injection, the ion response was found to be significantly more sensitive and reproducible using negative electrospray ionization (ESI) than with negative atmospheric pressure chemical ionization (APCI) mode. Even though using APCI would have the advantage of minimizing any matrix-induced suppression of ionization, the sensitivity was so poor using APCI that ESI mode had to be chosen as the ionization technique. The MS/MS conditions were optimized by tuning the instrument to obtain the maximum response with a stable product ion signal. The resulting product ion mass spectra for R, S-(A) and IS are shown in Fig. 1. The two diastereomers, R, S-(A) and R, R-(A), produced identical product ion mass spectra.

Reversed-phase separation was chosen since the polar mobile phase was readily compatible with ESI mode. In our original assay, the two diastereomers were separated under traditional HPLC–MS/MS conditions. A chromatographic run time of approximately 11 min was needed to achieve baseline separation using gradient elution conditions with a standard BetaBasic HPLC column. Fig. 2 shows a representative traditional HPLC–MS/MS ion chromatogram. Early validation experiments suggested a lower limit of quantification of 2 ng/mL was achievable under these conditions. The dynamic range for HPLC–MS/MS method was 2–500 ng/mL for each diastereomer of Drug Candidate A using linear regression curve, with correlation coefficients greater than 0.99 ($1/x^2$ weighting). Intra- and inter-day accuracy and precision for the two diastereomers were acceptable based on our acceptance criteria (data not shown). Subsequently, the method was transferred to the UHPLC in order to achieve higher sensitivity and greater sample throughput while keeping essentially the same sample handling procedure and similar MS/MS conditions.

The ACQUITY BEH column was selected for the UHPLC–MS/MS method since it is packed with a hybrid material in which the methyl groups are bridged for superior strength. This improved the column reliability and provided stability at high pH conditions [20]. Using the same mobile phase as the HPLC method, we found the gradient could be slightly modified under UHPLC conditions to reduce the run time approximately 3-fold to 3.5 min per sample while still maintaining baseline resolution of the diastereomers. A representative chromatogram obtained from the UHPLC method is shown in Fig. 3. The HPLC and UHPLC peak widths at baseline for the diastereomers were approximately 24 s and 12 s, respectively (Figs. 2 and 3). This sharper peak shape resulted in a two-fold gain in signal-to-noise with UHPLC versus HPLC conditions, which was significant considering the injection volume was decreased by 50% to 5 μL with UHPLC. In addition to the sensitivity and speed advantages of the UHPLC method, the solvent consumption was also decreased due to the reduced run time per sample. The backpressure generated by the UHPLC system under these conditions was approximately 7000 psi which is within the normal range encountered in UHPLC [2].

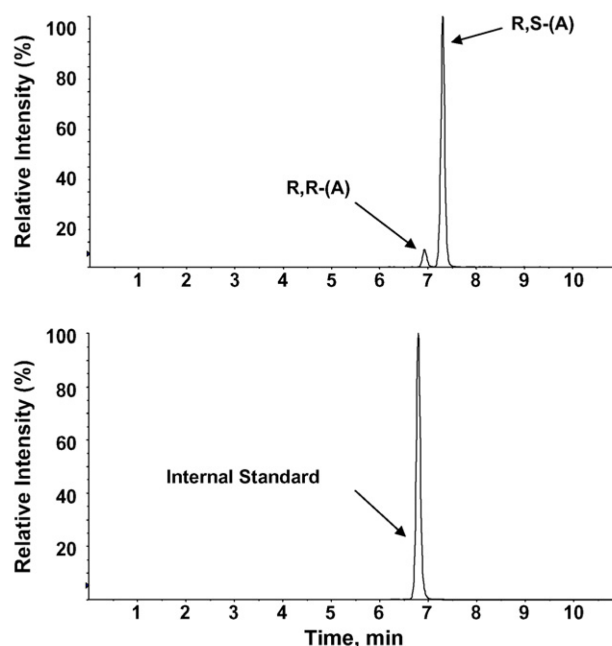

Fig. 2. Representative traditional HPLC–MS/MS ion chromatogram showing baseline resolution of the diastereomers (plasma extract).

3.2. Optimization of sample processing

As indicated earlier, Drug Candidate A contains two chiral centers, one of which can readily epimerize to produce

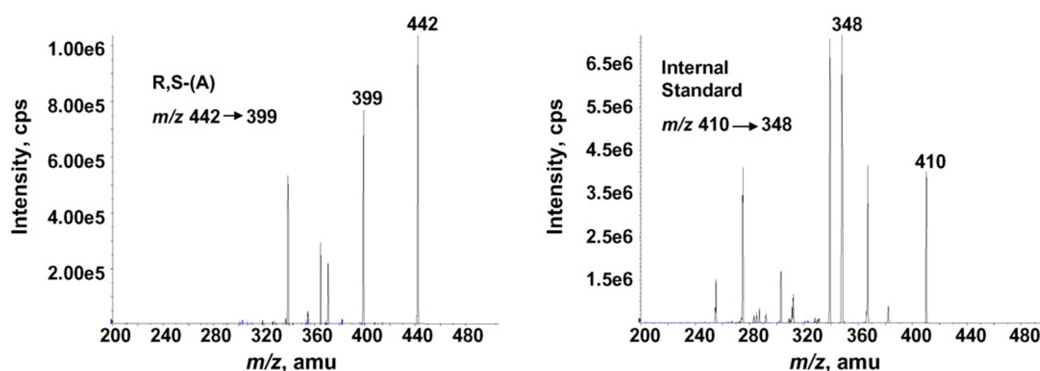

Fig. 1. MS/MS product ion mass spectra of R, S-(A) and the internal standard.

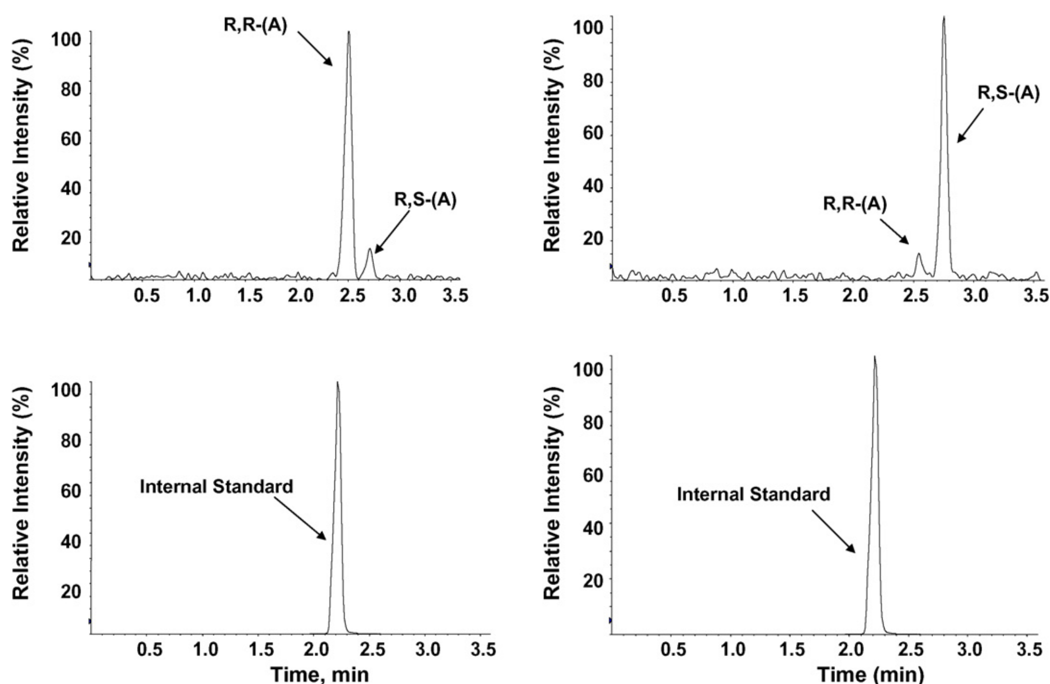

Fig. 3. Representative UHPLC–MS/MS ion chromatogram of the assay LLOQ spiked with 1 ng/mL R, R-(A) (Left) and R, S-(A) (Right) and 400 ng/mL internal standard.

a pair of diastereomers (R, S-(A) and R, R-(A)). Therefore, minimizing *ex vivo* inter-conversion of these two diastereomers during sample processing was one of our main goals. Based on our previous experience, and scientific literature with enantiomeric inter-conversion [17,18], we adapted the sample preparation procedure to utilize an ice–water bath, whenever possible. There are three basic plasma sample cleanup procedures: protein precipitation, solid phase extraction (SPE) and liquid–liquid extraction (LLE) to achieve a rugged assay for biological samples. Although protein precipitation is one of the simplest sample clean-up methods in bioanalysis [21], the significant amount of endogenous material remaining in the extracted samples ("dirty extracts") could cause rapid deterioration of chromatographic resolution and shorten the column lifetime. SPE offers much cleaner sample extracts, but there are technical challenges to perform SPE at reduced temperatures. Therefore, we developed a liquid–liquid extraction procedure for sample cleanup. The mean extraction recoveries for R, S-(A) and R, R-(A) at neutral and basic conditions were similar, ∼60%, while the recoveries for both diastereomers under acidic conditions were 3-fold lower. The extent of *ex vivo* inter-conversion during sample preparation and analysis was less than 10% under neutral conditions, while it exceeded 15% under basic conditions. Therefore, the sample extraction was performed under physiological pH conditions to maximize the recovery while minimizing the *ex vivo* inter-conversion of the diastereomers. Additionally, no column blockage or elevated backpressure was observed with our UHPLC column after more than 600 sample injections, which was at least in part due to the cleaner sample extracts from the liquid–liquid extraction procedure.

3.3. Method validation

In order to validate this UHPLC–MS/MS method for determining the plasma concentrations and diastereomeric ratio in rats, the following parameters were investigated: specificity, linear range of the calibration curve, accuracy, precision, and stability of the analytes.

3.3.1. Specificity and separation

Pooled, drug-free, control rat plasma samples were used to assess the specificity of the method. The described chromatographic conditions were found to be selective for both diastereomers and the internal standard. Samples of pooled blank rat plasma and rat plasma fortified with the IS were analyzed on each validation day. As shown in Fig. 4, there was no interference in the blank plasma chromatograms from endogenous substances or from the IS contributing to the Drug Candidate A SRM channel, confirming the selectivity of the method in rat plasma.

Figs. 3 and 5 illustrate representative ion chromatograms for the LLOQ (1 ng/mL) and ULOQ (500 ng/mL) of the calibration curve. With the resolving power of UHPLC, the diastereomers were completely separated to baseline, even at the ULOQ of the assay (Fig. 5). In addition, there was no analyte peak observed from an extracted drug-free plasma sample injected after the ULOQ, suggesting there was no significant carry-over under the described experimental conditions.

As evident in the chromatograms, some inter-conversion of the diastereomers occurred (typically $<10\%$) despite our best efforts to control it. Therefore, separate calibration curves and QC samples were required for each day of analysis. Pooled

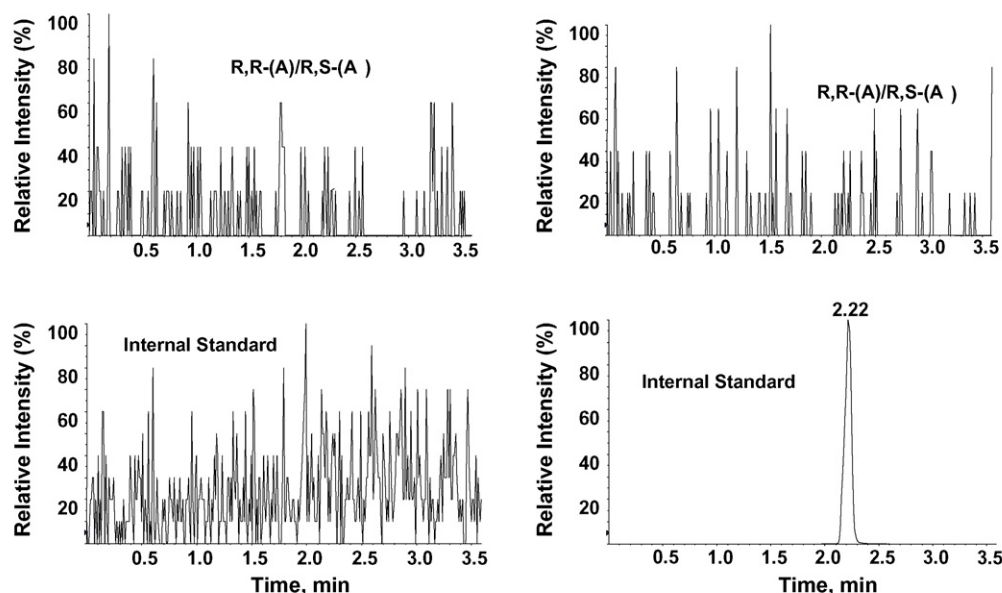

Fig. 4. Representative UHPLC–MS/MS ion chromatograms of a blank plasma sample and a blank plasma sample spiked with 400 ng/mL internal standard.

samples containing both diastereomers would have been ideal to increase productivity, but this was precluded by the stereochemical instability of the analytes.

3.3.2. Linearity, accuracy and precision

Calibration curves were found to be linear over the concentration range of 1–500 ng/mL for each diastereomer of Drug A, typically producing correlation coefficients greater than 0.99 with $1/x^2$ weighting using the UHPLC–MS/MS method.

Validation of the method included demonstration of its inter- and intra-day performance over three days of analysis. Calibration curves were prepared daily, in duplicate, for each diastereomer. In addition, three replicates of four QC concentrations within the dynamic range of the assay were analyzed on each validation day. Table 1 summarizes the validation data for accuracy and precision of each calibration standard concentration of R, S-(A) and R, R-(A). Table 2 presents the inter- and intra-assay precision for each of the QC samples. Our inter- and intra-day accuracy and precision (% CV) acceptance criteria for each QC and calibration standard was ≤15% (≤20% for the QC and standard at the LLOQ–1 ng/mL). The assay successfully met these criteria.

3.3.3. Stability

Preliminary experiments (data not shown) had determined that the bench-top stability of the diastereomers in rat plasma at room temperature was unacceptable due to rapid interconversion of the diastereomers. Indeed, sample preparation needed to be conducted at a reduced temperature (an ice–water bath) to minimize this effect. The bench-top stability was

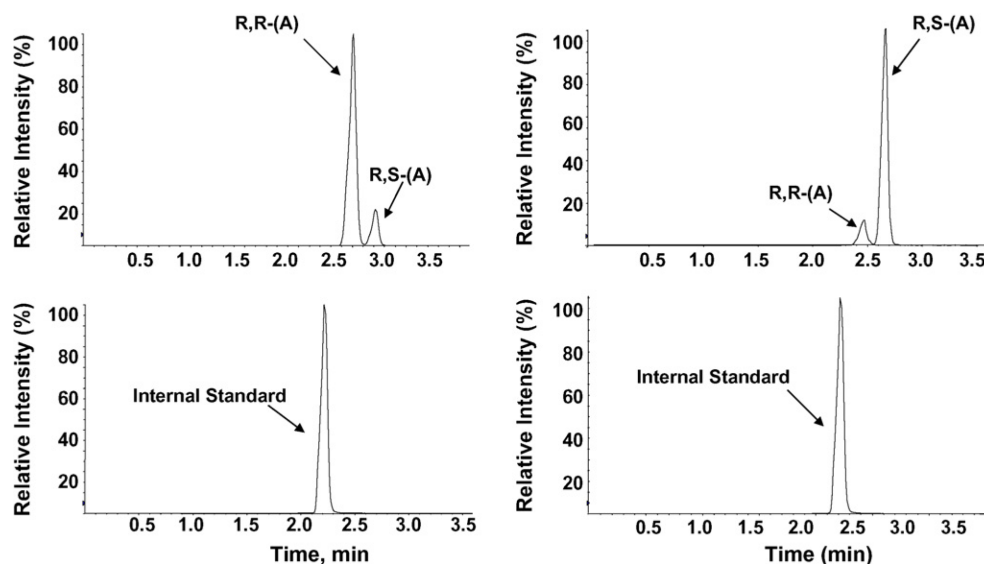

Fig. 5. Representative UHPLC–MS/MS ion chromatogram of the assay ULOQ spiked with 500 ng/mL R, S-(A) (left) and R, R-(A) (right) and 400 ng/mL internal standard.

Table 1
Accuracy and precision of calibration standards for R, S-(A) and R, R-(A) in rat plasma

	Nominal concentration (ng/mL)	Mean determined concentration (ng/mL, $n = 6$)	Accuracy (%)	CV (%)
R, S-(A)	1.00	1.01	101	8.93
	2.50	2.44	97.6	3.91
	5.00	4.86	97.2	4.07
	12.5	12.3	98.4	6.04
	25.0	26.0	104	9.57
	125	131	105	4.71
	250	247	98.8	3.86
	500	488	97.6	3.16
R, R-(A)	1.00	0.989	98.9	5.98
	2.50	2.56	102	8.47
	5.00	5.03	101	6.09
	12.5	12.4	98.9	6.09
	25.0	25.4	101	8.35
	125	129	103	7.71
	250	252	101	2.95
	500	473	94.7	10.1

Table 2
Intra- and inter-day accuracy and precision of QC samples of R, S-(A) and R, R-(A) in rat plasma

	Norminal concentration (ng/mL)	Mean determined concentration (ng/mL)	Accuracy (%)	CV (%)
Intra-day ($n = 3$)				
R, S-(A)	1.00	1.03	103	9.35
	2.50	2.63	105	6.19
	100	102	102	8.32
	400	381	95.3	3.82
R, R-(A)	1.00	1.05	105	8.35
	2.50	2.31	92.3	10.9
	100	104	104	5.94
	400	393	98.3	8.08
Inter-day ($n = 9$)				
R, S-(A)	1.00	1.03	103	9.35
	2.50	2.71	109	7.90
	100	103	103	13.7
	400	374	93.6	10.1
R, R-(A)	1.00	1.05	105	8.35
	2.50	2.47	98.8	7.39
	100	106	106	6.86
	400	400	100	8.81

investigated to determine whether the samples were adequately stable under these conditions over a sufficient period of time to cover the sample preparation process. The resulting data (listed in Table 3) indicated that both diastereomers were stable.

Freeze–thaw stability was evaluated after one cycle of freezing and thawing three sets of rat plasma QC samples at low, mid and high concentrations. The data listed in Table 3 indicates that both diastereomers were stable after one freeze-thaw cycle.

The stability of both diastereomers in the final injection solvent was also evaluated. The results indicated that they were stable in the autosampler for at least 24 h at 5 °C (Table 3).

3.4. *In vitro R, S-(A)/R, R-(A) inter-conversion in rat blood and plasma*

Results from experiments to investigate the *in vitro* rate of inter-conversion from R, S,-(A) to R, R-(A) at 37 °C in rat blood are shown in Table 4 and graphically in Fig. 6. The values for the R, S,-(A)/R, R-(A) concentration ratio plotted in the figure were determined by quantifying the concentration of each diastereomer in plasma obtained after incubating R, S,-(A) with fresh EDTA-treated pooled rat blood. Based on previous work on chiral compound inter-conversion [17,18], an excessive amount of organic solvent may affect the epimerization of a compound, possibly by minimizing the differential effect of plasma protein binding of the individual diastereomers. Therefore, in this study, the total content of acetonitrile was kept at 0.5% (v/v) when spiking the drug into fresh blood.

As evident from the data in Table 4 and Fig. 6, there was rapid inter-conversion of stereoisomers in rat blood following incubation at 37 °C, resulting in the achievement of an R, S-(A)/R, R-(A) ratio of 1.0 within 1 h. The R, S-(A)/R, R-(A) ratio decreased slightly to 0.7 at 4 h (Fig. 6). Similar results were obtained by incubating R, S-(A) at two other concentrations, 1 μM and 50 μM, which suggested that the R, S-(A)/R, R-(A) ratio was not concentration dependent (data not shown). Interestingly, similar results were obtained by incubating R, S,-(A)

Table 3
Assessment of stability of R, S-(A) and R, R-(A)

Nominal concentration (ng/mL)	Bench-top 4 °C (ice bath) (3 h)		Freeze/thaw (1 cycle)		Autosampler 5 °C (24 h)	
	Mean found concentration (ng/mL, $n = 3$)	Accuracy (%)	Mean found concentration (ng/mL, $n = 3$)	Accuracy (%)	Mean found concentration (ng/mL, $n = 3$)	Accuracy (%)
R, S-(A)						
2.50	2.52	101	2.61	105	2.26	90.3
100	82.2	82.2	88.6	88.6	85.2	85.2
400	364	90.9	406	102	458	114
R, R-(A)						
2.50	2.60	104	2.78	111	2.27	90.9
100	107	107	111	111	106	106
400	394	98.4	428	107	390	97.5

Table 4
R, S-(A)/R, R-(A) ratio in fresh rat blood incubated with R, S-(A)[a]

Concentration (μM)	Time (h)	Rat blood	Rat blood	Rat blood	Mean	S.D.[b]
10	0	20.9	21.1	20.7	20.9	0.205
	0.17	4.53	4.50	4.69	4.58	0.0991
	0.5	2.00	2.05	2.03	2.03	0.0216
	1	1.14	1.17	1.20	1.17	0.0269
	2	0.816	0.829	0.826	0.824	0.00666
	4	0.697	0.669	0.678	0.681	0.0144

[a] R, S,-(A) was incubated in fresh rat blood at 37 °C with organic solvent at 0.5% (v/v).
[b] S.D. = Standard deviation.

with fresh rat plasma (data not shown). Taken together, these data demonstrate the need for immediate cooling of blood samples for *in vivo* studies, immediate freezing of the harvested plasma, and careful attention to the effects of temperature during sample processing. Also demonstrated is the value of higher speed analysis using UHPLC. The higher throughput enabled us to run more samples during the period of time that the diastereomers of Drug Candidate A were stable in the autosampler.

3.5. *In vivo R, S-(A)/R, R-(A) inter-conversion in the rat*

The *in vivo* R, S-(A)/R, R-(A) ratio in rat plasma was determined after oral administration of R, S-(A) at 25 and 250 mg/kg in female and male rats. The ratio was determined at different time points between 15 min and 72 h (Fig. 7). Consistent with the results from the *in vitro* experiments, equilibration of the two diastereomers was rapid, with the R, S-(A)/R, R-(A) ratio achieving a constant value of 0.6–0.7 within 2 h after administration of R, S-(A). The ratio remained constant for up to 72 h. Moreover, there appeared to be no significant gender difference in the ratio, and it was not significantly affected by concentration, since the ratio remained similar in female versus male rats and at two doses differing by an order of magnitude. In addition, the short inter-conversion half-life determined from the *in vitro* and *in vivo* experiments, <1 h, and the *in vivo* elimination half-life of both diastereomers of 13–14 h in rats (based on pharmacokinetic calculations – data not shown) revealed that the epimerization of R, S-(A) was much faster than their elimination. Importantly, the data also demonstrated that rats dosed with R, S-(A) would also be exposed to approximately equivalent concentrations of R, R-(A) within a short period of time following administration.

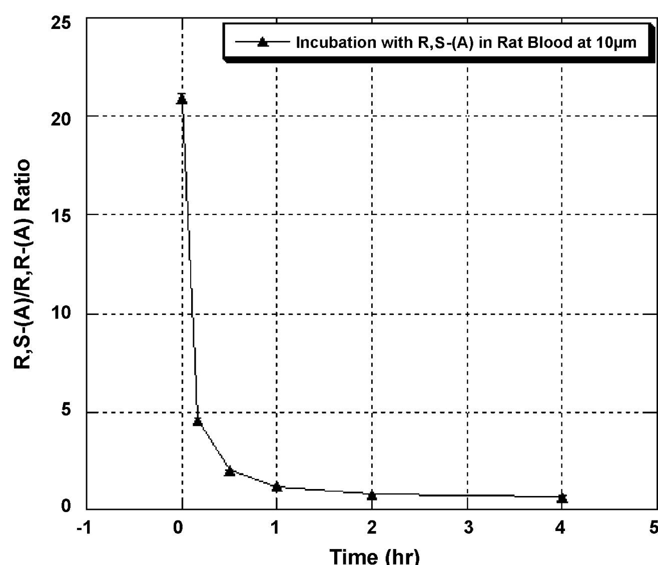

Fig. 6. R, S-(A)/R, R-(A) ratio *in vitro* in rats.

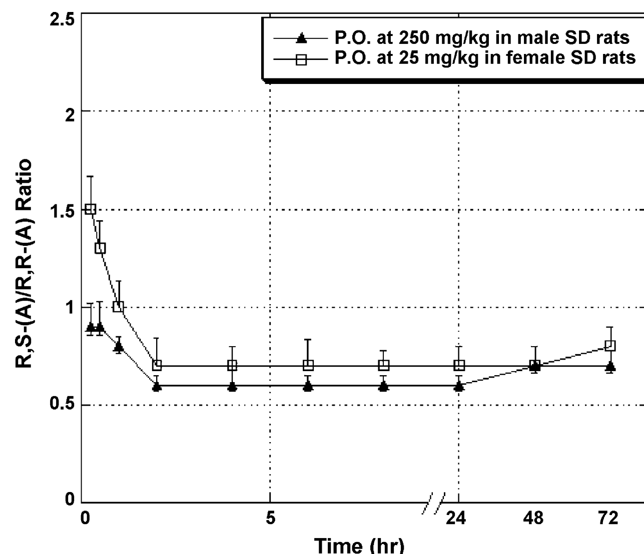

Fig. 7. R, S-(A)/R, R-(A) ratio *in vivo* in rats.

4. Conclusions

A reliable and high throughput UHPLC–MS/MS method for the independent measurement of two diastereomers, R, S-(A) and R, R-(A) in rat plasma was successfully developed and validated. Overall, the new UHPLC–MS/MS method resulted in significant improvements in speed (nearly three-fold) and sensitivity (two-fold) compared to our traditional HPLC–MS/MS method. The new method exhibited good intra- and inter-day accuracy and precision, and calibration curves were linear over a dynamic range of 1–500 ng/mL for both R, S-(A) and R, R-(A). This new method allowed us to expedite important experiments for the determination of *in vivo* and *in vitro* R, S-(A)/R, R-(A) ratios in rats, in which we confirmed rapid inter-conversion of the diastereomers both *in vitro* and *in vivo*. *Ex vivo* inter-conversion was also prevalent, but careful optimization of the sample handling and processing techniques was found

to effectively mitigate this potential problem. The higher speed analysis afforded by UHPLC–MS/MS was critical to this study.

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