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Determination of a neuroprotective agent (*S*)-(+)-BMS-204352 in human, rat and dog plasma by enantioselective liquid chromatography-tandem mass spectrometry

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Abstract

A sensitive liquid chromatography-electrospray ionization tandem mass spectrometry method (LC/ESI/MS/MS) for the enantioselective determination of (*S*)-(+)-BMS-204352, a potent and specific maxi-K channel opener, in human, rat and dog plasma was developed. (*S*)-(+)-BMS-204352, its enantiomer (*R*)-(−)-BMS-204353 and the internal standard (^{13}C-deuterated racemate of (*S*)-(+)-BMS-204352) were extracted from plasma using toluene. Chromatographic separation for the enantiomers was achieved on a Chiralcel OD-H analytical column with a run time of 8 min. An aqueous mobile phase modifier was added post column to enhance the mass spectrometer sensitivity. ESI mass spectra were acquired in the negative mode with selected reaction monitoring. The limit of quantitation (LLOQ) is 0.10 ng/mL for human plasma assay. Samples from a clinical study and two animal studies were processed using these procedures. Based on the in vivo data, lack of inversion of (*S*)-(+)-BMS-204352 to (*R*)-(−)-BMS-204353 was demonstrated in human, rat and dog after administration of the drug. A sensitive non-enantioselective LC/ESI/MS/MS assay has also been developed for (*S*)-(+)-BMS-204352 which uses a similar extraction procedure with a C18 column with a limit of quantitation at 0.05 ng/mL. Human study samples were analyzed by both methods and the correlation coefficient between both sets of data is greater than 0.99.

Keywords: (*S*)-(+)-BMS-204352; Enantioselective liquid chromatography; Tandem mass spectrometry

1. Introduction

Stroke is a major cause of death and long-term disability, annually affecting more than 700,000 people in the US alone [1]. During ischemic stroke, which represents 80% of all strokes, neurons at risk are exposed to pathologically high levels of intracellular calcium (Ca^{2+}), initiating a fatal biochemical cascade. To protect these neurons, the novel fluorooxindole, (*S*)-(+)-BMS-204352 (Fig. 1), has been developed as a potent, effective and unique opener of large-conductance, Ca^{2+}-actived (maxi-K or BK) potassium channels, thereby augmenting an endogenous mechanism for regulating Ca^{2+} entry and membrane potential [2]. Both enantiomers, (*S*)-(+)-BMS-204352 and (*R*)-(−)-BMS-204353 (Fig. 1), are very effective in increasing maxi-K current. Although not significantly different, (*S*)-(+)-BMS-204352 consistently produced a more robust increase in outward current than either the racemate or the (*R*)-(−)-BMS-204353 enantiomer [3]. To answer whether the enantiomers have different phamarcokinetic profiles and to demonstrate the lack of inversion of (*S*)-(+)-BMS-204352 to (*R*)-(−)-BMS-204353 in human, rat and dog after dosing, it was necessary to develop and validate a highly sensitive and enantioselective method for the enantiomers.

Liquid chromatography mass spectrometry (LC/ESI/MS) methods have already been reported for non-enantioselective

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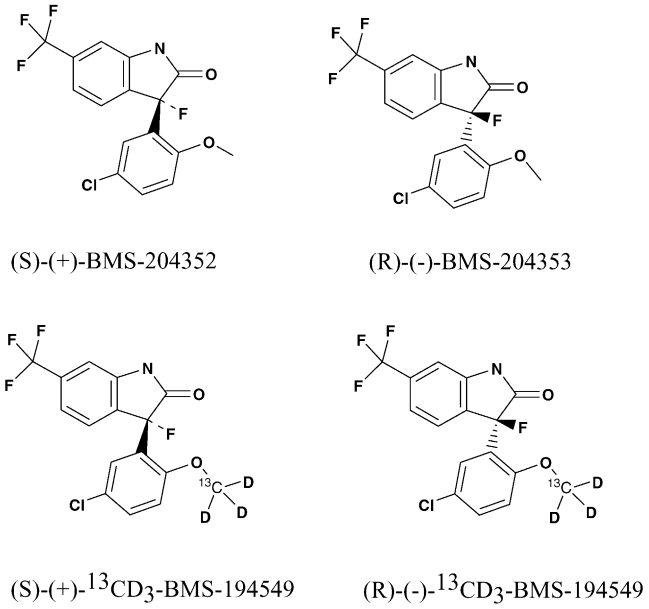

Fig. 1. Chemical structures of (*S*)-(+)-BMS-204352, its enantiomer, (*R*)-(−)-BMS-204353, and internal standard $^{13}CD_3$-BMS-194549.

determination of (*S*)-(+)-BMS-204352 in dog and rat plasma and rat brain [4–6]. The methods used conventional C18 column and have quantitation limits at 0.5–2.5 ng/mL. The sensitivity of the methods was limited by single quadrupole mass spectrometer used. Large sample volumes were used to further increase the sensitivity [4,6]. To date, no enantioselective method has been developed and reported for the quantitative determination of (*S*)-(+)- BMS-204352 in biological matrices.

Mass spectrometry as a sensitive detector combined with enantioselective HPLC applications has been increasingly used in pharmacokinetic studies for drug candidates where two enantiomers may have different pharmacological properties [7–18]. Many enantioselective chromatography procedures use normal phase condition and several technique have been published to solve the problem of compatibility of normal phase chromatography and mass spectrometry. To increase the ionization efficiency of the electrospray, it is common to add small amount of water or salt in the eluent before MS inlet through mobile phase or post column addition [15]. When a small amount of methanol or water exist in mobile phase, direct connection of HPLC and MS is possible [13,14], but the normal phase column may have shorter life time and chromatographic resolution may be affected. When large volumes of post column reagents, such as 2-propanol, ethanol, formic acid or ammonium acetate buffer, are added into the column eluent, splitting the flow into the mass spectrometer was needed [16–18]. We present here a method based on normal-phase Chiralcel OD-H chromatography using a tandem mass spectrometer as a detector, enabling the determination of (*S*)-(+)-BMS-204352 and its enantiomer, (*R*)-(−)-BMS-204353 in human, rat and dog plasma. The method relies on the use of a low flow rate addition of a post-column reagent. No water was needed in the mobile phase to enhance ionization nor was splitting of the column effluents required before the mass spectrometer.

A conventional non-enantioselective LC/ESI/MS/MS method for (*S*)-(+)-BMS-204352 in human plasma is also presented here with a limit of quantitation (LLOQ) at 0.05 ng/mL. Comparable analytical results from study samples were obtained from both methods.

2. Experimental for enantioselective LC/ESI/MS/MS method

2.1. Materials and reagents

(*S*)-(+)-BMS-204352, (chemical purity 99.8%), (*R*)-(−)-BMS-204353, (chemical purity 99.8%) and internal standard (IS) $^{13}CD_3$-BMS-194549, ^{13}C-deuterated racemate of (*S*)-(+)-BMS-204352, (chemical purity 99%), were obtained from Bristol-Myers Squibb (Princeton, NJ, USA). Methanol, ethanol, hexane, isopropanol and toluene, of HPLC grade, were purchased from EM Science (Gibbstown, NJ, USA). HPLC grade water was obtained from a Millipore Milli-Q Plus Water Purification System (Bedford, MA, USA). Ammonium acetate, an HPLC reagent, was purchased from J.T. Baker (Phillisburg, NJ, USA). Formic acid, an analytical reagent, was purchased from J.T. Baker (Phillisburg, NJ, USA). Control human, rat and dog EDTA plasma was purchased from Bioreclamation Inc. (Hicksville, NY, USA). All other chemicals, solvents and reagents were of highest chemical purity and were used without further purification.

2.2. LC/ESI/MS/MS conditions

The LC/ESI/MS/MS system consisted of Shimadzu LC-10AD pumps (Columbia, MD, USA), a Hewlett Packard HP1090 HPLC system (Wilmington, DE) used as the post-column reagent delivery pump, a Perkin-Elmer Series 200 LC autosampler (Norwalk, CT, USA) and a Micromass Quattro LC mass spectrometry (Beverly, MA, USA) connected with a Chiralcel OD-H column (5 μm; 150 mm × 2.1 mm i.d.) from Chiral Technologies Inc. (Exton, PA, USA). The injection volume was 10 μL and the run time was 8 min. A mixture of isopropanol and 0.1% formic acid in hexane (10/90, v/v) was used isocratically as the mobile phase at a flow rate of 0.2 mL/min at ambient temperature. An aqueous post column modifier (5 mM ammonium acetate in methanol/water (81/19, v/v)), which was not soluble in the mobile phase, was added into the liquid chromatography mobile phase stream prior to the mass spectrometer by a mixing tee at a flow rate of 0.1 mL/min. The total flow into the mass spectrometer was 0.3 mL/min.

Detection was performed by selected reaction monitoring (SRM) from the deprotonated molecular ion ($[M - H]^-$) of the analyte to its product ion using negative ion electrospray tandem mass spectrometry. The product ion efficiency of

the $[M-H]^-$ was optimized by varying capillary, cone and collision energies to their final settings of 2.60 kV, 37 V and 19 eV, respectively. The flow rate of nebuliser and drying gas (N_2; 99.999% purity) were approximately 80 and 800 L/h, respectively. The source block temperature and desolvation temperature were 120 and 300 °C, respectively. The collision-induced dissociation of the $[M-H]^-$ of the analyte was performed at quadrupole 2 using argon at a pressure of 8.0e–4 mBar. The monitoring ions for SRM analysis were set to *m/z* 358 to 338 for (*S*)-(+)-BMS-204352 and (*R*)-(−)-BMS-204353 and *m/z* 362 to 342 for the IS. The scan dwell time was set to 0.7 s. Originally, the mass spectrometer was tuned with the reverse phase non-enantioselective LC/ESI/MS/MS method's mobile phase which contained 5 mM ammonium acetate in methanol/water [5]. When this reverse phase mobile phase was used as the post column addition for the enantioselective method, it was found that the same mass spectrometer parameters used for that method gave the best sensitivity for the normal phase enantioselective method.

2.3. Preparation of standard and quality control (QC) samples in the human plasma

Two sets of primary stock solutions of (*S*)-(+)-BMS-204352 and (*R*)-(−)-BMS-204353 for standard and quality control samples were prepared from separated weighing by dissolving the analytes into ethanol. A standard working stock solution containing 5000 ng/mL each of (*S*)-(+)-BMS-204352 and (*R*)-(−)-BMS-204353 was prepared by appropriate dilution of the 1 mg/mL stock solutions with ethanol. A 40 μL portion of the 5000 ng/mL stock solution was diluted to 4 mL with control human EDTA plasma to yield a combined stock solution of 50.0 ng/mL for each analyte. This plasma pool was diluted to obtain the final concentrations in plasma standards of 50.0, 40.0, 25.0, 10.0, 5.00, 1.00, 0.50, 0.25 and 0.10 ng/mL for each analyte. Standard curves were prepared fresh daily. Similar dilutions were used to prepare four levels of QC samples in human plasma at 0.30, 15.0, 40.0 and 100 ng/mL, which were aliquoted and stored frozen at −30 °C. Additional QCs, containing only one of the enantiomers, were prepared to monitor the inversion of the analytes during storage and analysis. Other QCs were prepared at 1:100 and 100:1 ratio of (*S*)-(+)-BMS-204352 and (*R*)-(−)-BMS-204353 to demonstrate the ability to measure 1% of an enantiomer in the presence of an abundance of the other enantiomer.

2.4. Sample processing procedure for human plasma

$^{13}CD_3$-BMS-194549, the ^{13}C-deuterated racemate of (*S*)-(+)-BMS-204352, was used as the internal standard. After the addition of 100 μL of the IS working solution at 50.0 ng/mL in ethanol/water (50/50, v/v), 0.5 mL of 5 mM ammonium acetate solution, which used as pH controller and diluter of the plasma, 4 mL of toluene were added to 0.5 mL of each calibration standard, QC sample and clinical sample. The concentration of each enantiomer of the IS was 5.00 ng/mL in plasma. The samples were shaken for 20 min, and then centrifuged to separate the liquid phases. The organic layer from each sample was transferred to a clean tube and evaporated to dryness. The dried extracts were re-dissolved in 100 μL of the reconstitution solution containing hexane/isopropanol (70/30, v/v) and transferred to injection vials. Ten microliters of the reconstitution solution was injected into the LC/ESI/MS/MS system.

2.5. Validation of the human plasma method

Calibration curve standards in plasma were prepared and analyzed in duplicate for three validation runs. Six replicates of each QC sample were assayed along with the standard curve. The enantiomeric stability QC samples were also analyzed in triplicate in all the validation runs and sample analysis runs. The specificity of the method were determined by extracting six lots of blank control human plasma both with and without the addition of (*S*)-(+)-BMS-204352 and (*R*)-(−)-BMS-204353 at the lower limit of quantitation level to detect any potential interference problems.

3. Experimental for non-enantioselective LC/ESI/MS/MS method

The material, reagents and instrumentation have been described in Sections 2.1–2.2. The same mass spectrometer conditions were used (Section 2.2). The HPLC column and mobile phase were changed. A YMC basic S5 column (5 μm; 50 mm × 2 mm i.d.) from Waters Corporation, (Milford, MA, USA) was used. The injection volume was 20 μL and the run time was 4 min. A mixture of 5 mM ammonium acetate in methanol/water (71.5/28.5, v/v) was isocratically used as the mobile phase at a flow rate of 0.2 mL/min at ambient temperature. The standard curve range was from 0.05 to 25.0 ng/mL and QCs were prepared at 50.0, 20.0, 10.0 and 0.15 ng/mL. The samples were processed following the enantioselective LC/ESI/MS/MS method procedure described in the Section 2.4 except 5 mM ammonium acetate in water/MeOH (40/60) was used as the reconstitution solution. Twenty microliters of the reconstituted sample was injected into the LC/ESI/MS/MS system. This method was used to evaluate the room temperature, 40 °C and freezer stability of (*S*)-(+)-BMS-204352 in human plasma. Long term storage stability of the compounds and processed sample stability were also evaluated.

4. Results and discussion

4.1. Chromatography and mass spectrometry

Under the LC/ESI/MS/MS conditions used for both the enantioselective and the non-enantioselective methods, (*S*)-

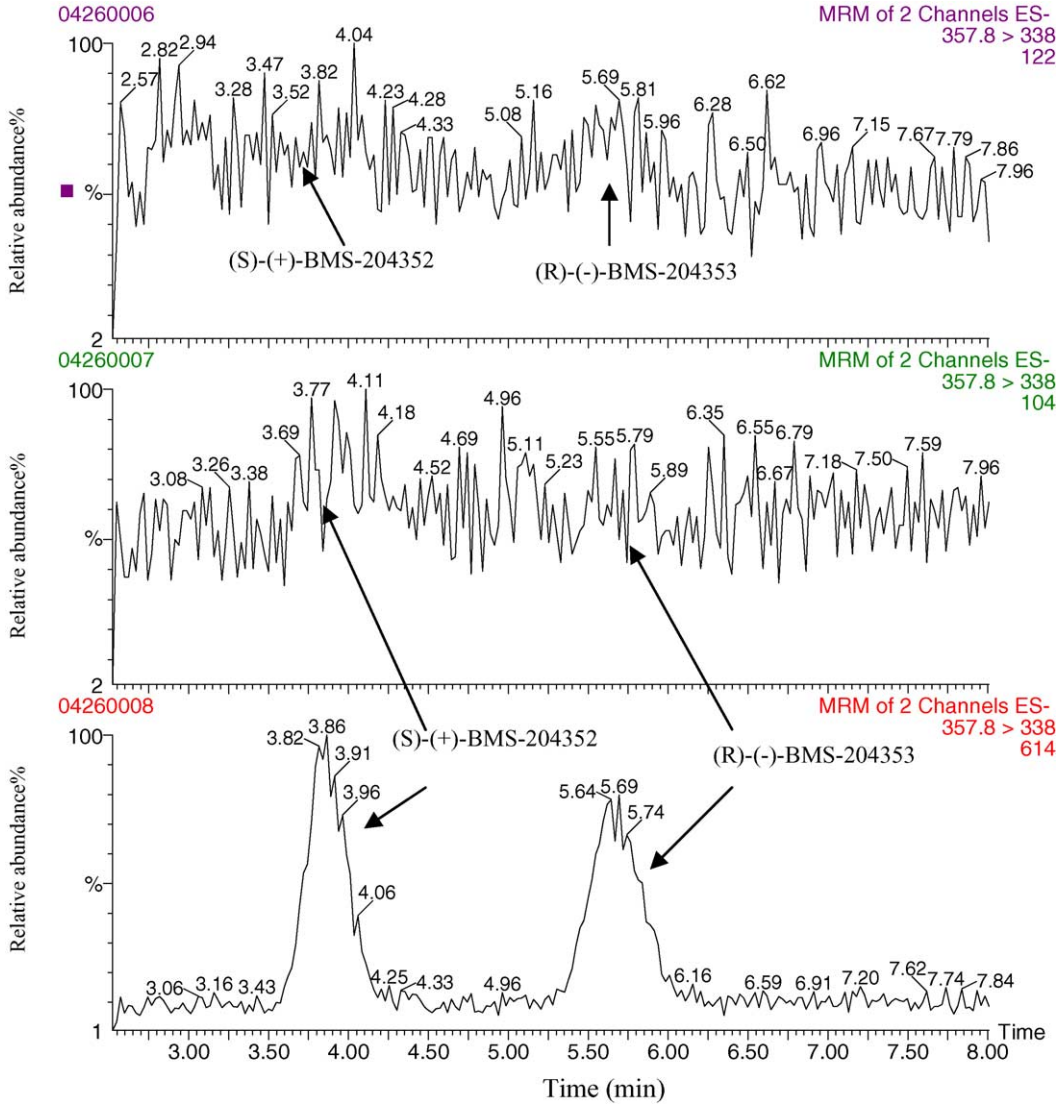

Fig. 2. Selected reaction monitoring chromatograms for (*S*)-(+)-BMS-204352 enantiomers obtained from: blank human plasma (top); human plasma containing only internal standard at 5.00 ng/mL (middle); human plasma containing (*S*)-(+)-BMS-204352 and (*R*)-(−)-BMS-204353 at lower limit of quantitation (0.10 ng/mL) and its internal standard at 5.00 ng/mL (bottom) in enantioselective method.

(+)-BMS-204352, (*R*)-(−)-BMS-204353 and their IS showed deprotonated ions $[M - H]^-$ at *m/z* 358 and 362 amu, respectively. Better signal intensity and lower background was obtained when negative ion electrospray was used. Depending on the collision energy used, either no fragmentation or too much fragmentation were observed for positive electrospray. For negative electrospray, fragmentation of the molecular ions using collision-induced dissociation resulted in dominant product ions, representing the lost of HF, for (*S*)-(+)-BMS-204352 and (*R*)-(−)-BMS-204353 (338 amu) and their IS (342 amu). Typical SRM mass chromatograms of blank human plasma, blank human plasma with IS and human plasma spiked with 0.10 ng/mL of (*S*)-(+)-BMS-204352 and (*R*)-(−)-BMS-204353 and 5.00 ng/mL of IS for the enantioselective assay are shown in Figs. 2 and 3 for both analyte and IS channels. No significant interfering peaks from the plasma were found at the retention time and in the ion channel of either of the analytes or the IS when six different lots of control human EDTA plasma blanks were analyzed.

Normal phase liquid chromatography, consisting of 90% hexane with 0.1% formic acid and 10% isopropanol, had to be used to achieve enantioselective separation for (*S*)-(+)-BMS-204352 and its enantiomer. Formic acid was used to reduce the peak tailing. Other enantioselective separation columns, such as cellulose-based Chiralcel OD-R column which can be used in reversed phase mode, were tried without success. Due to the lack of electrical conductivity of the organic solvents, there is almost no signal for the $[M - H]^-$ and its daughter ions when same tune parameters were used as the reverse phase method but no aqueous post column modifier was added into the normal phase infusion solution (Fig. 4). When the cone voltage was increased to 60 V and capillary voltage for mass spectrometry was increased to 5 kV, which was maximum voltage allowed for the instrument, the sen-

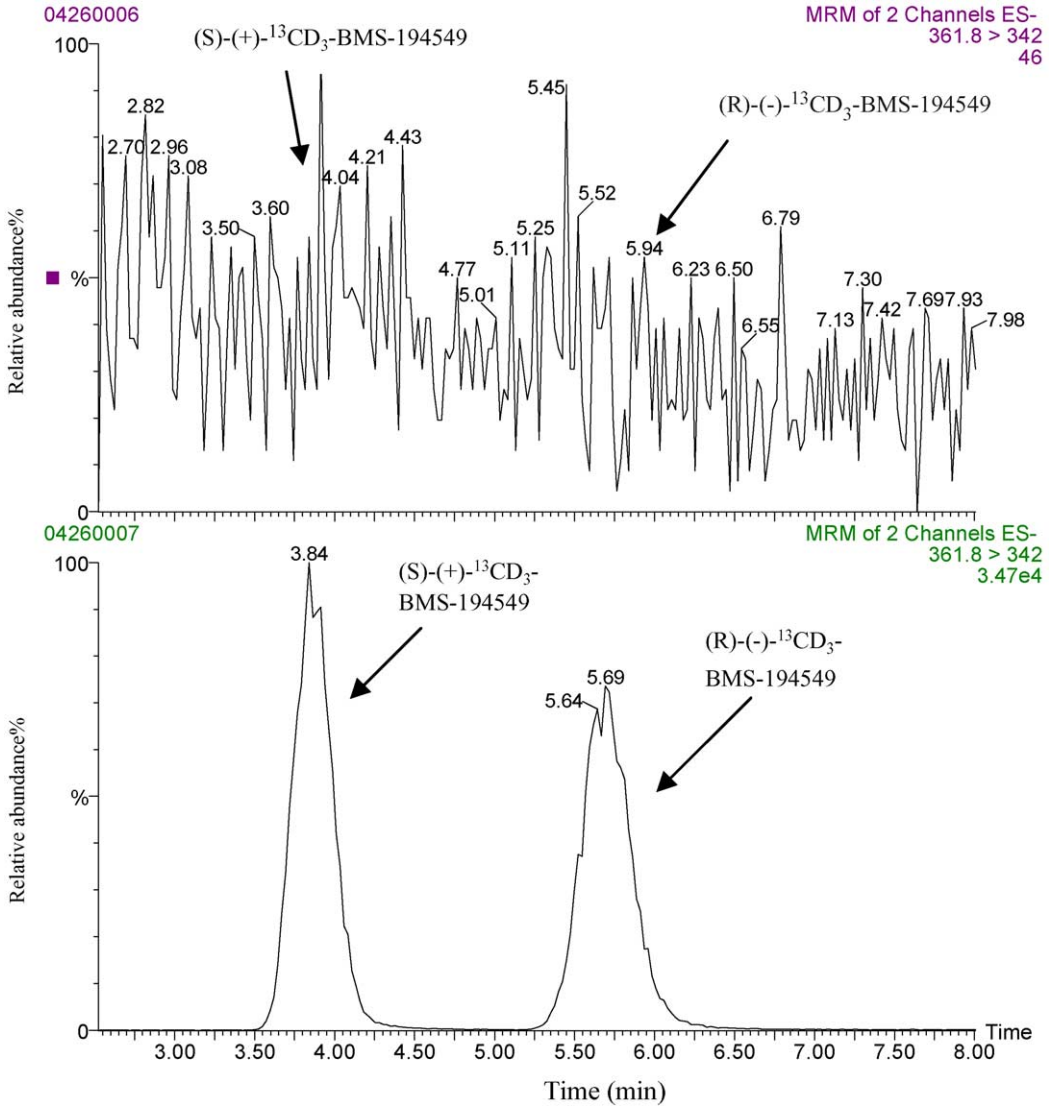

Fig. 3. Selected reaction monitoring chromatograms for the internal standard of (*S*)-(+)-BMS-204352 enantiomers obtained from: blank human plasma (top); human plasma containing only the internal standard at 5.00 ng/mL (bottom) in enantioselective method.

sitivity of the assay increase to about one-tenth that of the non-enantioselective reverse phase method. But the signal was very unstable and the sparking at the tip of the capillary caused charging and lower the sensitivity. The use of an aqueous post column modifier, which has the same components as the reverse phase assay's mobile phase with a slightly higher percentage of organic solvent, permitted lowering of the capillary voltage and increased the signal intensity about 700 times (Fig. 4). By controlling the flow rate of the mobile phase through the enantioselective column (0.2 mL/min) and post column modifier (0.1 mL/min), no split was necessary. This permitted the entire sample to be sent into the mass spectrometer. The post column modifier was conveniently added into the liquid chromatography system prior to the mass spectrometer by a mixing tee. Even though the modifier was not miscible with the mobile phase, its presence in the source was sufficient to significantly enhance the sensitivity. The signal to noise ratios at the limit of quantitation (0.10 ng/mL) is 10 when 10 μL of reconstitution solution was injected for the enantioselective assay and the signal to noise ratios at limit of quantitation (0.05 ng/mL) is 7.5 when 20 μL of reconstitution solution was injected for non-enantioselective assay (Figs. 2 and 5).

4.2. Accuracy and precision of calibration curve standards in human plasma

(*S*)-(+)-BMS-204352 enantiomers plasma concentrations were determined by inverse-prediction following $1/x^2$ weighted quadratic regression analysis of the standard curve data. A summary of the regression parameters (quadratic slope, slope, intercept and R^2) for the calibration curves in plasma for (*S*)-(+)-BMS-204352 and (*R*)-(−)-BMS-204353 is provided in Table 1. A quadratic response was observed

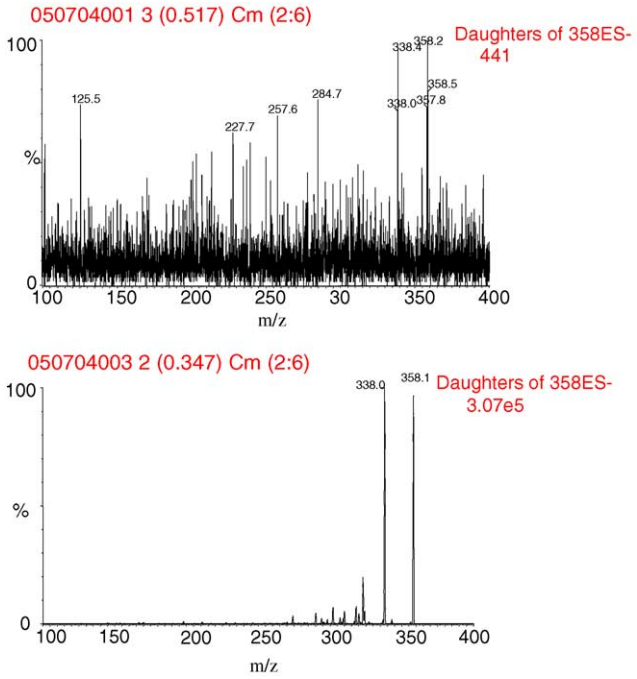

Fig. 4. Electrospray negative ion MS/MS product ion spectra of $[M - H]^-$ when infusing 1.0 μg/mL (*S*)-(+)-BMS-204352 in 0.1% formic acid in Hexane/IPA 90/10 without aqueous post column modifier (top); with 5 mM NH_4OAc in $MeOH/H_2O$ (70/30) as post column modifier (bottom).

($R^2 \geq 0.99$) for all calibration curves. For all the runs, the deviations of the back-calculated concentrations from their nominal values were within the range from −15.4 to 15.5% for all the calibration standards for both (*S*)-(+)-BMS-204352 and (*R*)-(−)-BMS-204353. The mean concentration for six different lots of control human plasma spiked with (*S*)-(+)-BMS-204352 and (*R*)-(−)-BMS-204353 each at 0.100 ng/mL (LLOQ level) were 0.115 and 0.117 ng/mL, (ranging from 0.109 to 0.122 ng/mL for (*S*)-(+)-BMS-204352 and 0.112 to 0.121 for (*R*)-(−)-BMS-204353) with a mean deviation of 15.0 and 17.0%, respectively.

4.3. Accuracy and precision of quality control samples in human plasma

The accuracy and precision of the method was assessed by analyzing QC samples at concentrations within the lower, the second, and the upper quartile of the standard curve. To evaluate the dilution linearity and matrix effect, a fourth QC sample, with a concentration higher than the upper limit of the standard curve range, was also analyzed. This QC sample was diluted 1:20 with control human plasma and then the diluted sample was processed and analyzed. Six replicate samples at each concentration were analyzed in three separate runs. The accuracy was determined by calculating the deviations of the predicted concentrations from their nominal values. The intra- and inter-assay precision was determined by calculating the %R.S.D. values using a one-way ANOVA. The intra-assay precision was within 5.8% R.S.D. and the inter-assay precision was within 5.6% R.S.D for both (*S*)-(+)-BMS-204352 and (*R*)-(−)-BMS-204353, data was shown in Table 2. The assay accuracy was within ±10.6% of the nominal values for all the QC samples analyzed for both analytes.

4.4. Enantiomeric stability of the analytes in human plasma

The enantiomeric stability of (*S*)-(+)-BMS-204352 and (*R*)-(−)-BMS-204353 in human EDTA plasma during sample processing and analysis was evaluated using QCs with a large amount of one enantiomer and 1 or 0% of the other enantiomer. There is no observed inversion between (*S*)-(+)-BMS-204352 and (*R*)-(−)-BMS-204353 during the processing and analysis. The method was accurate and precise at low analyte concentrations and was demonstrated to be capable of quantifying either 1% of (*S*)-(+)-BMS-204352 in the presence of large amount of (*R*)-(−)-BMS-204353 or 1% of (*R*)-(−)-BMS-204353 in the presence of large amount of (*S*)-(+)-BMS-204352 to a limit of 0.10 ng/mL (LLOQ). The deviation at LLOQ level is less than 19% for both analytes.

4.5. Room temperature, 40 °C and freezer stability of (S)-(+)-BMS-204352 in human plasma

Since the assay was going to be used for analyzing study samples which have the potential for thawing and storage at elevated temperature, the stability of (*S*)-(+)-BMS-204352 in human plasma at room temperature and 40 °C was evaluated in triplicate using QC samples at 0.15 and 20.0 ng/mL with the non-enantioselective assay. The QC samples were kept

Table 1
Standard curve regression analysis results for (S)-(+)-BMS−204352 enantiomers in human EDTA plasma enantioselective method

(*S*)-(+)-BMS-204352					(*R*)-(−)-BMS-204353			
Run number	Quadratic slope ($\times 10^{-3}$)	Slope ($\times 10^{-1}$)	Intercept ($\times 10^{-3}$)	R^2	Quadratic slope ($\times 10^{-3}$)	Slope ($\times 10^{-1}$)	Intercept ($\times 10^{-3}$)	R^2
1	1.4025	1.891503	−1.1754	0.9940	1.4374	1.877940	−0.9972	0.9955
2	1.4890	1.784306	−4.2136	0.9919	1.4334	1.803547	−5.2005	0.9913
3	1.4237	1.853437	−2.5696	0.9919	1.3434	1.873736	−3.0496	0.9928
Mean	1.4384	1.843082	−2.6529		1.4047	1.851741	−3.0824	
S.D.	0.00451	0.054344	1.5208		0.00532	0.041790	2.1018	

Model: area ratio = $((\text{conc.})^2)$(quad slope) + (conc.)(slope) + intercept.

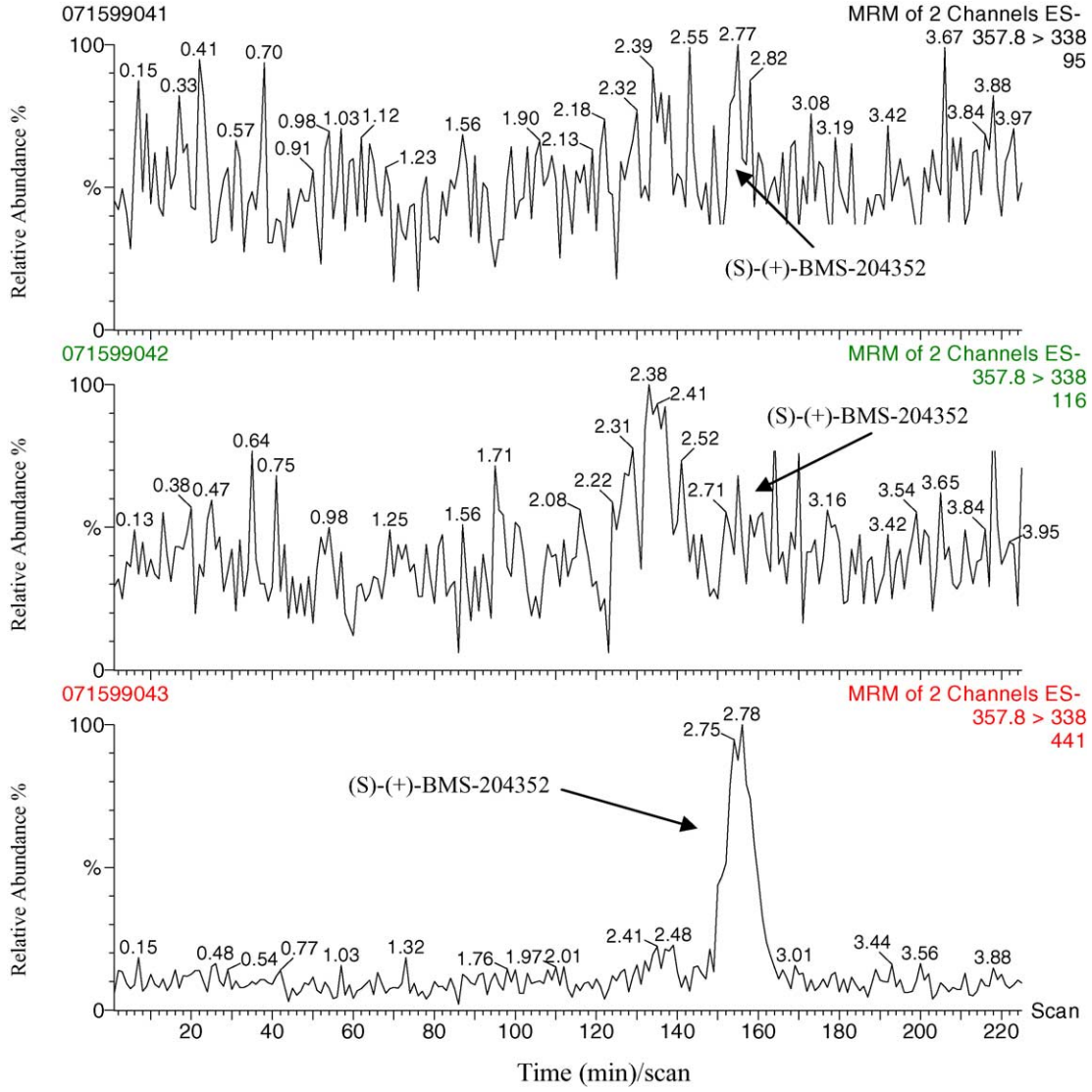

Fig. 5. Selected reaction monitoring chromatograms for (*S*)-(+)-BMS-204352 obtained from: blank human EDTA plasma (top); human EDTA plasma containing only internal standard at 2.00 ng/mL (middle); human EDTA plasma containing (*S*)-(+)-BMS-204352 at lower limit of quantitation (LLOQ) (0.05 ng/mL) and its internal standard at 2.00 ng/mL (bottom) in non-enantioselective method.

at room temperature and 40 °C for 1, 2, 5 and 7 days prior to analysis. The deviations of the mean predicted concentrations of the test QC samples from the nominal concentrations were used as an indicator of the room temperature and 40 °C stability of (*S*)-(+)-BMS-204352 in human plasma. (*S*)-(+)-BMS-204352 was stable for at least 7 days at room temperature with maximum deviation at −6.7%. (*S*)-(+)-BMS-204352 started to degrade when stored at 40 °C for 2 days or more. The deviation from nominal concentration at 2 and 5 days are −14.0 and −24.5%, respectively. The long term storage stability of (*S*)-(+)-BMS-204352 in human plasma at −30 °C was evaluated in triplicate using QC samples at 0.15, 10.0 and 20.0 ng/mL stored for a period of 35 weeks. The deviations of the mean predicted concentrations of the QC samples from

Table 2
Accuracy and precision for (*S*)-(+)-BMS-204352 enantiomers in human EDTA plasma enantioselective method, *n* = 18

	(*S*)-(+)-BMS-204352				(*R*)-(−)-BMS-204353			
Nominal concentration (ng/mL)	0.300	15.0	40.0	100[a]	0.300	15.0	40.0	100[a]
Mean observed Concentration (ng/mL)	0.281	16.5	40.5	110	0.293	16.6	41.1	110
Deviation (%)	−6.3	10.3	1.3	10.6	−2.3	10.4	2.7	10.5
Between run precision (%)	3.3	3.2	1.1	5.6	4.1	2.9	2.9	4.5
Within run precision (%)	5.1	3.1	4.5	4.1	5.8	2.4	4.9	3.8

[a] Dilution factor = 20.

the nominal concentrations were within 9.8%, indicating that (*S*)-(+)-BMS-204352 was stable for at least 35 weeks at −30 °C.

4.6. Enantioselective LC/ESI/MS/MS methods for rat and dog plasma

The enantioselective LC/ESI/MS/MS method has also been validated for the quantitation of (*S*)-(+)-BMS-204352 and its enantiomer in 0.5 mL of dog EDTA plasma and 0.05 mL of rat plasma. The method utilized the same HPLC and MS conditions described for the human method. Similar extraction procedures were followed. The validation consisted of a one run for the determination of accuracy, precision, lower limit of quantitation and specificity. The standard curves ranged from 0.10 to 50.0 ng/mL for the dog plasma assay and 0.50 to 500 ng/mL for rat. For dog plasma assay, the intra-assay precision was within 7.1% CV and the assay accuracy was within ±7.5% of the nominal values. At the LLOQ of 0.10 ng/mL, the deviations of the predicted concentrations from the nominal value for the six LLOQ samples were within ±12.0% for both analytes. For the rat plasma assay, the intra-assay precision was within 2.1% CV and the assay accuracy was within ±9.9% of the nominal values. At the LLOQ of 0.50 ng/mL, the deviations of the predicted concentrations from the nominal value for five of the six LLOQ samples were within ±17.8% for both analytes. No significant interfering peaks from both rat or dog plasma were found at the retention time and in the ion channel of either the analytes or the IS when six different lots of control rat and dog EDTA plasma blanks were analyzed. The recovery of the method is 77% in rat plasma.

4.7. Non-enantioselective LC/ESI/MS/MS method validation for human plasma

The enantioselective LC/ESI/MS/MS method used same mass spectrometer conditions and a C18 column. The mobile phase used was similar to the post column modifier of the enantioselective method. The standard curve, which ranged from 0.050 to 25.0 ng/mL, was fitted to a $1/x$ weighted quadratic regression model. The intra-assay precision was within 3.8% R.S.D. and inter-assay precision was within 2.3% R.S.D. The assay accuracy was within ±6.7% of the nominal values. At the lower limit of quantitation of 0.050 ng/mL, the deviations of the predicted concentrations from the nominal value for six of the seven LLOQ samples were within 16.8%. The processed samples were stable for 48 h at room temperature.

5. Assay feasibility

Both the enantioselective and non-enantioselective LC/ESI/MS/MS methods were applied to evaluate the pharmacokinetics of (*S*)-(+)-BMS-204352 in human subjects who received a single 10 mg intravenous dose of the drug. (*S*)-(+)-BMS-204352 was infused over a 5 min period. Serial blood samples were collected for up to 148 h to characterize the pharmacokinetics of (*S*)-(+)-BMS-204352. Fig. 6 shows the (*S*)-(+)-BMS-204352 plasma concentration versus time profile in a typical subject following the administration of a single intravenous dose of 10 mg of (*S*)-(+)-BMS-204352 using the enantioselective and non-enantioselective assays. The correlation coefficient determined when comparing the subject's samples (*S*)-(+)-BMS-204352 concentrations from the enantioselective and non-enantioselective methods was greater than 0.99, demonstrating the two assays can be interchangeably used for the analysis of (*S*)-(+)-BMS-204352 in clinical studies. There was no inversion of (*S*)-(+)-BMS-204352 to (*R*)-(−)-BMS-204353 observed in human plasma samples. The enantioselective assay was used for the analysis of several hundred clinical samples and non-enantioselective assay was used for more than several thousand samples. For the enantioselective assay, up to 124 samples were injected in a single run including all the standards, QCs and clinical samples.

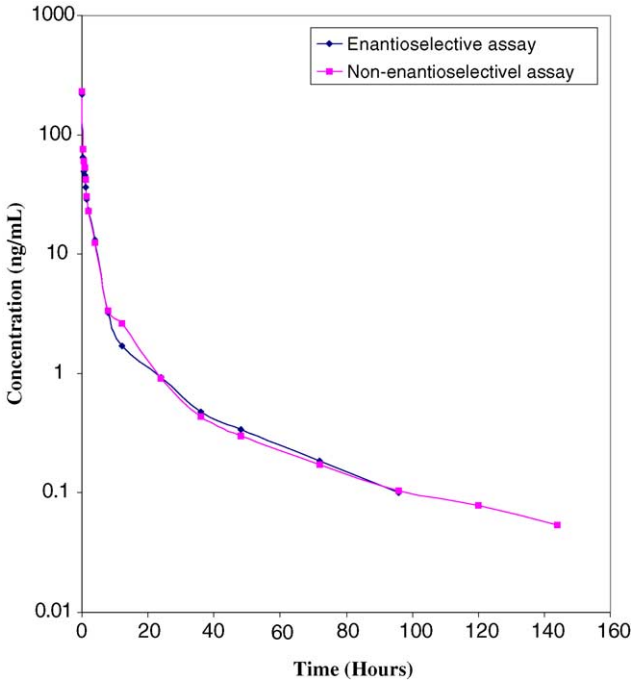

Fig. 6. The plasma concentration vs. time profile in a typical subject following the administration of a single intravenous dose of 10 mg of (*S*)-(+)-BMS-204352 using the enantioselective and non-enantioselective assays to analyze the plasma concentration.

Animal studies also demonstrated that there was no inversion of (*S*)-(+)-BMS-204352 to (*R*)-(−)-BMS-204353 after administration of a single intravenous dose of 2 mg/kg of (*S*)-(+)-BMS-204352 in dog and 6 mg/kg in rat, respectively. In these studies, serial blood samples were collected up to 8 h for dog and 24 h for rat. Plasma samples were analyzed with the rat and dog enantioselective assays.

6. Conclusions

The validated enantioselective and non-enantioselective LC/ESI/MS/MS methods in the three species demonstrated high sensitivity, enantio-selectivity, accuracy, precision and ruggedness. Plasma concentration results from human clinical study samples showed excellent correlation between the enantioselective and non-enantioselective assays. Based on the in vivo data, lack of inversion of (*S*)-(+)-BMS-204352 to (*R*)-(−)-BMS-204353 was demonstrated in all three species. The developed assays are suitable for the determination of the pharmacokinetics of (*S*)-(+)-BMS-204352 and (*R*)-(−)-BMS-204353 in animals and humans.

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