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Determination of RSD921 in human plasma by high-performance liquid chromatography–tandem mass spectrometry using tri-deuterated RSD921 as internal standard: application to a phase I clinical trial

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A fast, sensitive and specific method is presented for the quantification of RSD921 in human plasma by liquid chromatography coupled with tandem mass spectrometry using tri-deuterated RSD921 (3d-RSD921) as an internal standard. A single-step liquid/liquid extraction was performed with diethyl ether/hexane (80 : 20, v/v) using 0.5 ml of plasma. The plasma calibration curves were linear from 0.1 to 20 ng ml^{-1} ($r > 0.999$). Between-run precision, based on the percent relative deviation for replicate ($n = 40$) quality controls, was $\leq$7.27% (0.5 ng ml^{-1}), $\leq$7.39% (5.0 ng ml^{-1}), and $\leq$5.06% (20.0 ng ml^{-1}). Between-run accuracies, based on the relative error, were $\pm$2.59%, $\pm$1.23% and $\pm$1.64% respectively. The method was developed to evaluate the pharmacokinetic profile after 15 min of intravenous stepwise-ascending infusion dose of RSD921 in 18 healthy volunteers. A dissociation study of protonated RSD921 and 3d-RSD921 by collision-induced dissociation using in-source fragmentation and tandem mass spectrometry is also presented.

KEYWORDS: arylacetamide; antiarrhythmic; LC–MS/MS; quantification; fragmentation

INTRODUCTION

The compound (1*R*,2*R*)-(+)-*N*-methyl-*N*-[2-(1-pyrrolidinyl) cyclohexyl]benzo[*b*] thiophene-4-acetamide, RSD921 (Fig. 1), has local anaesthetic and antiarrhythmic actions both *in vivo* and *in vitro*[1–3] and has been presented as a potential alternative to lidocaine. Studies relate that RSD921 is a potent sodium channel-blocking agent.[4]

Only one quantitative method is presently available to measure RSD921 in biological matrices.[5] It employs reversed-phase high-performance liquid chromatography (RP-HPLC) coupled with ultra-violet (UV) detection to quantify RSD921 in rat whole blood and tissues. This method, however, shows low sensitivity for blood (140 ng ml^{-1}) and requires long run times (12 min). Many methods employing liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) have been described to determine drugs in biological matrices; hence LC–MS/MS plays a major role in pharmacokinetic investigations.[6]

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Herein we describe a rapid, specific and automated LC–MS/MS method to quantify RSD921 in human plasma using tri-deuterated RSD921 (3d-RSD921, Fig. 1) as the internal standard (IS). The method was employed in a phase I clinical trial.

A dissociation study by collision-induced dissociation (CID) was also performed by in-source fragmentation (ISF) and tandem mass spectrometry (MS/MS). The ISF was achieved by increasing the cone voltage during electrospray ionization (ESI) and occurred owing to collisional activation of the ions. When combined with MS/MS this two-step fragmentation proved to be useful for structure elucidation, and the mechanistic aspects and applications of ISF have been reviewed by Niessen.[7] Knowledge of the fragmentation pathway of a drug is important for screening metabolites and their structure elucidation. Today, LC–MS and LC–MS/MS are major techniques in the analysis of drug metabolism.[8]

EXPERIMENTAL

Materials

RSD921 and 3d-RSD921 were supplied by Rhythm Search Developments Ltd (Vancouver, Canada). The following analytical or HPLC-grade reagents were used: diethyl ether

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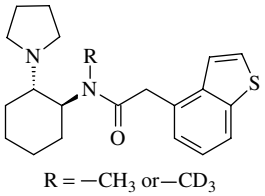

Figure 1. Structures of RSD921 and 3d-RSD921.

from Nuclear (São Paulo, SP, Brazil), heptane, ethylacetate, dichloromethane, formic acid, acetonitrile, hexane, sodium carbonate and sodium bicarbonate from Mallinckrodt (Paris, KY, USA) and Sigmacote siliconizing fluid from Sigma (Saint Louis, MO, USA).

Preparation of standard solutions and reagents

Stock standards of RSD921 and IS were prepared to provide solutions of 1.0 mg ml^{-1}. RSD921 working solutions were prepared by dilution from stock solution (1 mg ml^{-1}) in methanol : water (50 : 50 v/v) to provide concentrations of 10, 0.2, 0.1, 0.05 and 0.002 μg ml^{-1}. The IS solution was prepared by dilution from stock solution (1 mg ml^{-1}) in methanol : water (50 : 50 v/v) to give 10 μg ml^{-1}. A carbonate–bicarbonate solution was prepared by mixing one part of sodium carbonate and four parts of sodium bicarbonate (1 M each) followed by dilution with water to obtain a solution of 0.25 M (pH 9.4). The diethyl ether : hexane solution (80 : 20 v/v) was prepared freshly each day.

Preparation of calibration and quality-control samples

Calibration standards were prepared by spiking blank human plasma with standard solutions containing RSD921 to give concentrations of 0.1, 0.2, 0.5, 1, 2, 5, 10 and 20 ng ml^{-1}, and were prepared freshly in duplicate for each assay and extracted along with plasma samples and quality controls.

Quality control plasma samples were prepared by spiking pooled human plasma at final concentrations of 0.5, 5.0 and 20.0 ng per 1 ml of RSD921 followed by aliquoting and storing at −20 °C with the clinical samples until required.

Extraction of plasma samples

Calibration standards, quality controls and subject plasma samples (0.5 ml) were extracted after the addition of IS solution (20 ng ml^{-1}, final concentration) and carbonate buffer (0.5 ml). The tubes were vortex-mixed briefly and allowed to stand at room temperature for 5 min. Subsequently, 3 ml of diethyl ether : hexane (80 : 20 v/v) was added and the samples were again vortex-mixed for 30 s. The tubes were centrifuged at 2000 g for 10 min at 4 °C. The upper organic layer was carefully removed and transferred to siliconized tubes using siliconized Pasteur pipettes. The solvent was removed by a gentle stream of nitrogen in a dry bath at 37 °C. Mobile phase (200 μl, see chromatographic conditions) was added to the tubes followed by vortex-mixing for 15 s to reconstitute the residue. The solution was transferred to microvials that were then capped and placed in an HP 1100 autosampler rack.

LC–MS analysis

An HPLC 1100 system from Hewlett-Packard (Waldbronn, Germany) equipped with an analytical column (Genesis C18, 4 μm 150 × 4.6 mm i.d.) and guard column (Genesis C18, 4 μm 10 × 4 mm i.d.) from Jones Chromatography (Lakewood, CO, USA) were used. The column was operated at a temperature of 40 °C and a column switch was used to divert the column eluent to waste at appropriate times. The mobile phase was 50% acetonitrile containing 10 mM formate and was vacuum-filtered through a 0.45 μm filter; the final pH (3.2) was not adjusted. The mobile phase was pumped through the column at 1.0 ml min^{-1}. A split of approximately 1 : 15 was included so that only approximately 65 μl min^{-1} of the column eluent entered the mass spectrometer. The temperature of the autosampler was maintained at 8 °C, and 50 μl aliquots were injected automatically into the liquid chromatograph.

MS was performed using a Quattro II triple-stage quadrupole mass spectrometer from Micromass (Manchester, UK) equipped with an API electrospray source operating in the positive-ion mode using a crossflow counter electrode (a device that reduces source contamination). The mass spectrometer was calibrated with sodium iodide/cesium iodide solution in the range of m/z 20 to 1000, according to the instrument specifications. Multiple reaction monitoring (MRM) was used for the following reactions (m/z): 360.2 → 289.4 for 3d-RSD921 and 357.2 → 286.0 for RSD921. The dwell time, cone voltage, collision energy and gas pressure (argon) were 0.1 s, 25 V, 20 eV and 3.0×10^{-3} mbar, respectively. The data were processed with MassLynx (v 2.3 running on a Digital Celebris GL 6200 PC). Calibration curves were constructed using MassLynx from data generated with the calibration samples. The analyte to IS peak area ratios (PARs) were plotted against concentration using a weighted ($1/x$) least-squares linear regression. The PARs of unknown samples were then interpolated from the calibration curve to obtain the concentrations for RSD921.

Optimization of the mass spectrometric conditions for MRM and dissociation study (cone voltage and collision energy) was performed with infusion of standard solutions in mobile phase at a flow rate of 10 μl min^{-1} directly to the electrospray probe. The mass spectra (MS or MS/MS) reported represent background-subtracted sums of 13 scans in multi-channel acquisition (MCA) mode with a scan m/z range of 50–450, scan time of 2.1 s and interscan delay of 0.1 s.

RESULTS AND DISCUSSION

Method development

Full scan positive-ion mass spectra of RSD921 and 3d-RSD921 showed the protonated molecules, $[M + H]^+$, of m/z 357 and 360 respectively. Base peaks of m/z 286 and 289, for the non-deuterated and deuterated compounds respectively, were selected as product ions (Fig. 2) to quantify RSD921 and 3d-RSD921 in the MRM mode (m/z 357.2 → 286.0 and 360.2 → 289.4 respectively). To assess sample recovery, blank plasma was spiked at a final concentration of

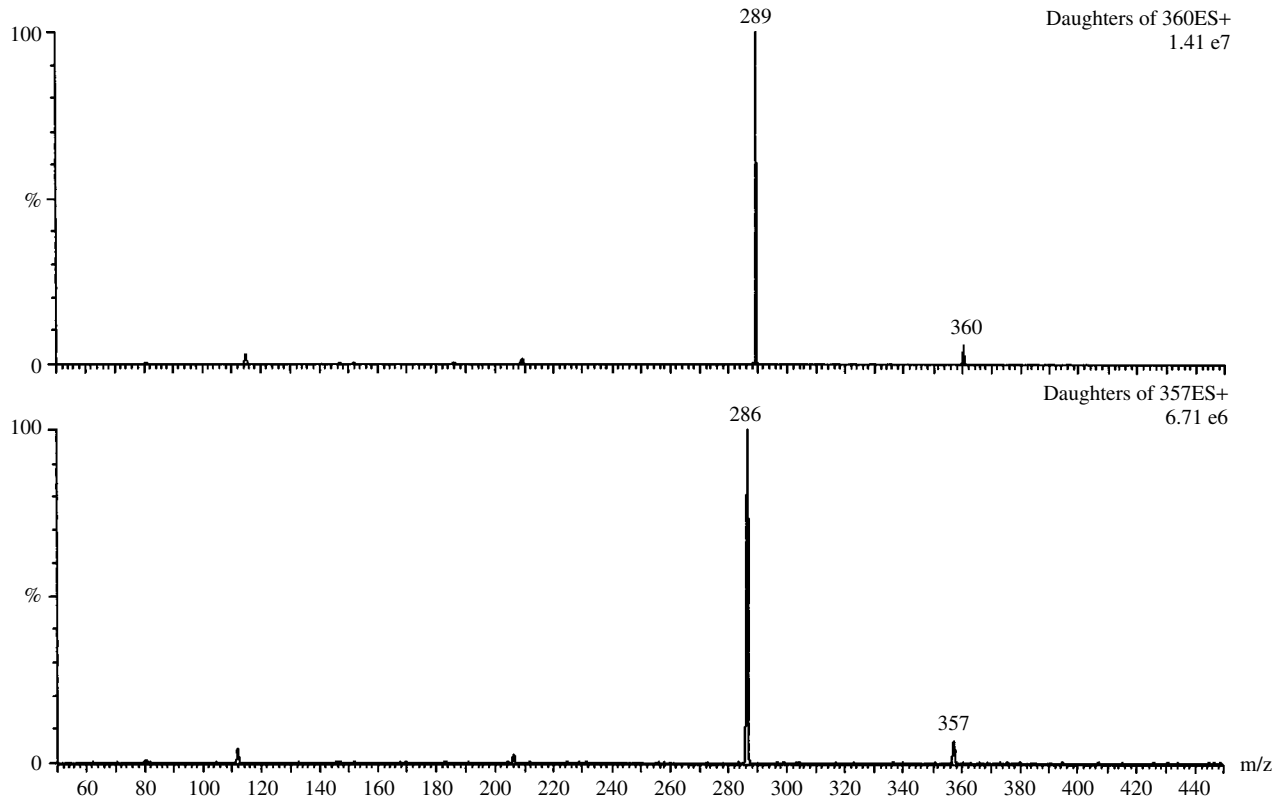

Figure 2. Atmospheric-pressure chemical ionization product ion spectra of $[RSD921 + H]^+$ (bottom) and $[3d\text{-}RSD921 + H]^+$ (top) at cone voltages of 25 V and collision energies of 20 eV.

20 ng ml^{-1} with RSD921 or 3d-RSD921 and extracted with different solvents. The resulting peak areas were compared with those of assayed peaks obtained with aqueous solutions at the same concentration and expressed as relative percent recovery and relative standard deviation (Table 1). The best results were obtained with diethyl ether : hexane (80 : 20, v:v). The lower signal from extracted samples of other solvents could partially be due to ESI suppression.

As Fig. 3a and b shows, no peak was observed in the mass chromatogram of human blank plasma under the LC–MS/MS conditions described above. A mass chromatogram at the lower limit of quantification (0.1 ng ml^{-1}) of RSD921 plus IS is shown in Fig. 3c and d. Retention time was 2.8 min for both RSD921 and IS.

Assay performance

Calibration curves were linear for plasma spiked with RSD921 (0.1 to 20 ng ml^{-1}) and a fixed amount of IS (20 ng ml^{-1}). The correlation coefficients r were typically $\geq$0.999 and the intercept values not significantly different from zero. Table 2 lists the slopes, intercepts, and correlation coefficients from different batches.

Table 1. Recoveries of RSD921 and 3d-RSD921 obtained from different organic solvents in human plasma by LC–MS/MS

	Recovery (%) Mean ± SD (n = 5)	
Solvent	3d-RSD921	RSD921
Dichloromethane	64.7 ± 9.2	62.0 ± 13.6
Hexane	4.25 ± 3.3	7.8 ± 3.6
Heptane/ethyl acetate	70.5 ± 22.8	67.7 ± 18.5
Ether/hexane	106.0 ± 18.1	92.0 ± 10.4

Both the precision and accuracy determine the error of an analytical measurement and are the primary criteria to evaluate an analytical method. Assay precision was calculated as percent relative standard deviation (%RSD) by the following formula: %RSD = (SD/M) × (100), in which M is the mean of the experimentally determined concentrations and SD is the standard deviation of M. Accuracy is defined as the percent relative error (%RE) and was calculated by the following formula: %RE = ($E - T$) × (100/T) in which E is the experimentally determined concentration and T is the theoretical concentration.[9,10]

Table 2 summarizes the between-run precision and accuracy for calibration standards. For the calibration curve, between-run precision was estimated from 1.07 to 4.90% over the calibration range, and accuracy was estimated from −3.33 to 2.16% over the calibration range.

A quality control sample (QC) was analyzed after a sequence of ten unknown samples. The within-run precision was ≤12.55% (QCA) at the lowest plasma quality control (0.5 ng ml^{-1}) and ≤13.91% (QCB) and ≤8.67% (QCC) for 5 ng ml^{-1} and 20 ng ml^{-1}, respectively. The within-run accuracy was ±7.00%, ±5.45% and ±5.59% for QCA, QCB and QCC respectively. The between-run precision was ≤7.27%, ≤7.39% and ≤5.06% for QCA, QCB and QCC respectively. The between-run accuracy was ±2.59%, ±1.23% and ±1.64% respectively.

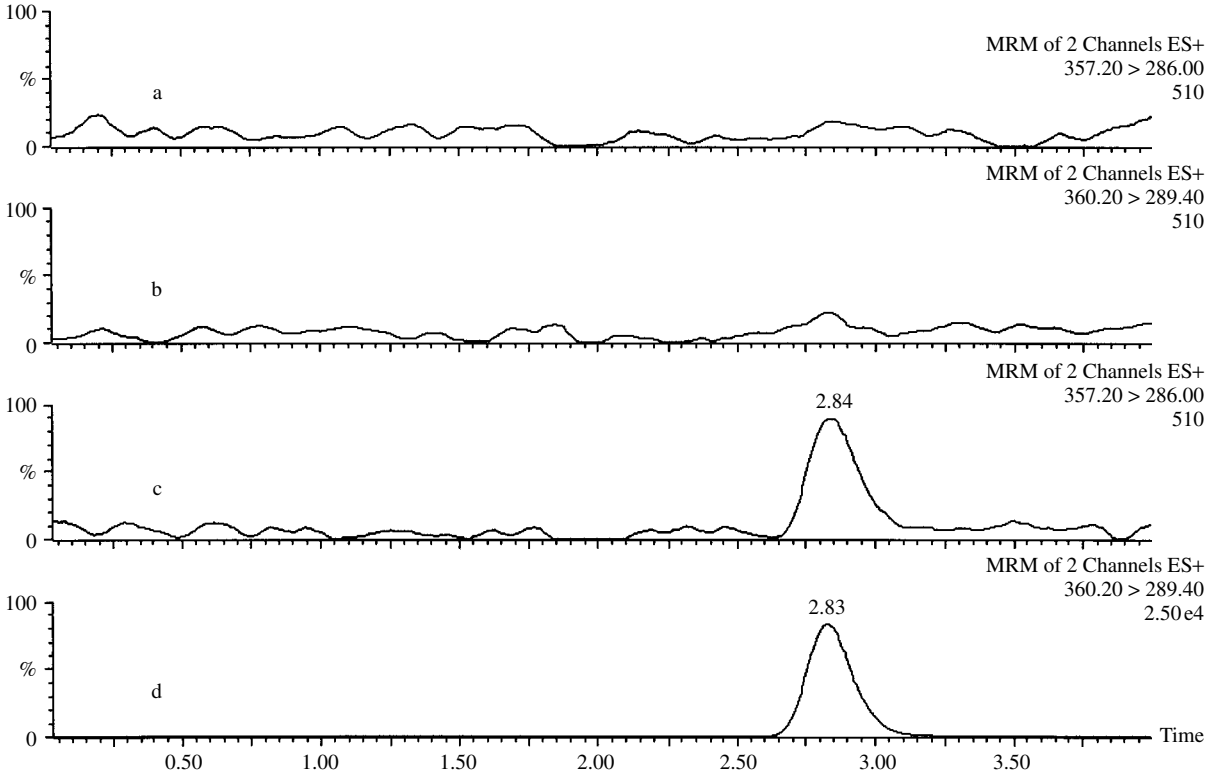

Figure 3. MRM chromatograms of blank human plasma: RSD921 (a) and 3d-RSD921 (b). MRM chromatograms of spiked blank human plasma at final concentration of 0.1 ng ml^{-1} plus internal standard: RSD921 (c) and 3d-RSD921 (d).

Table 2. Between-run plasma calibration quality report

	Concentration (ng ml^{-1})										
Standard Curve	0.10	0.20	0.50	1.00	2.00	5.00	10.0	20.0	Intercept	Slope	*r*
Mean of nine batches	0.10	0.21	0.50	1.00	2.01	5.08	9.78	20.1	0.010 58	0.998 39	0.999 80
SD	0.00	0.01	0.02	0.05	0.05	0.13	0.20	0.22	0.043 20	0.008 40	0.000 22
RSD (%)	3.37	4.84	3.77	4.90	2.37	2.52	2.01	1.07			
RE (%)	1.11	−3.33	0.67	0.44	−0.44	−1.69	2.16	−0.51			

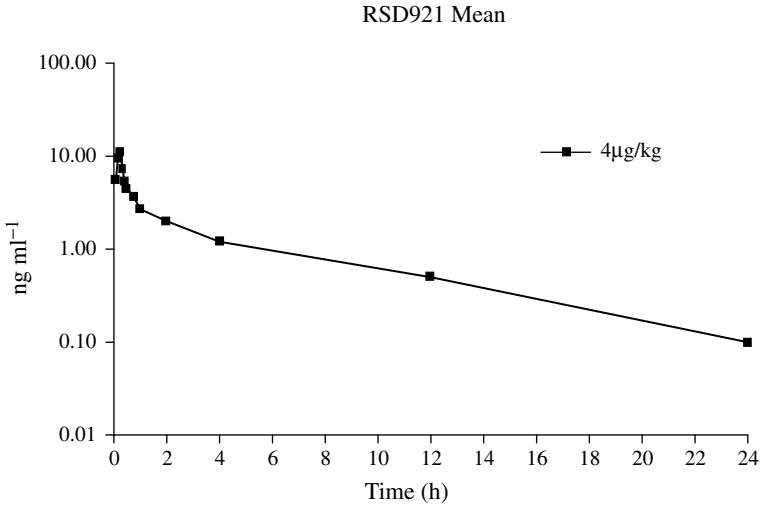

Figure 4. Mean plasma concentration of RSD921 in 18 healthy volunteers after a 15 min intravenous infusion administration dose of 4 µg kg^{-1}.

Figure 4 illustrates the mean plasma concentration curve of RSD921 after a 15 min intravenous administration of 4 μg kg^{-1} in 18 healthy volunteers.

Fragmentation pathway

Full-scan spectra (MS) at low cone voltages show primarily the protonated molecules: $[RSD921 + H]^+$ of m/z 357

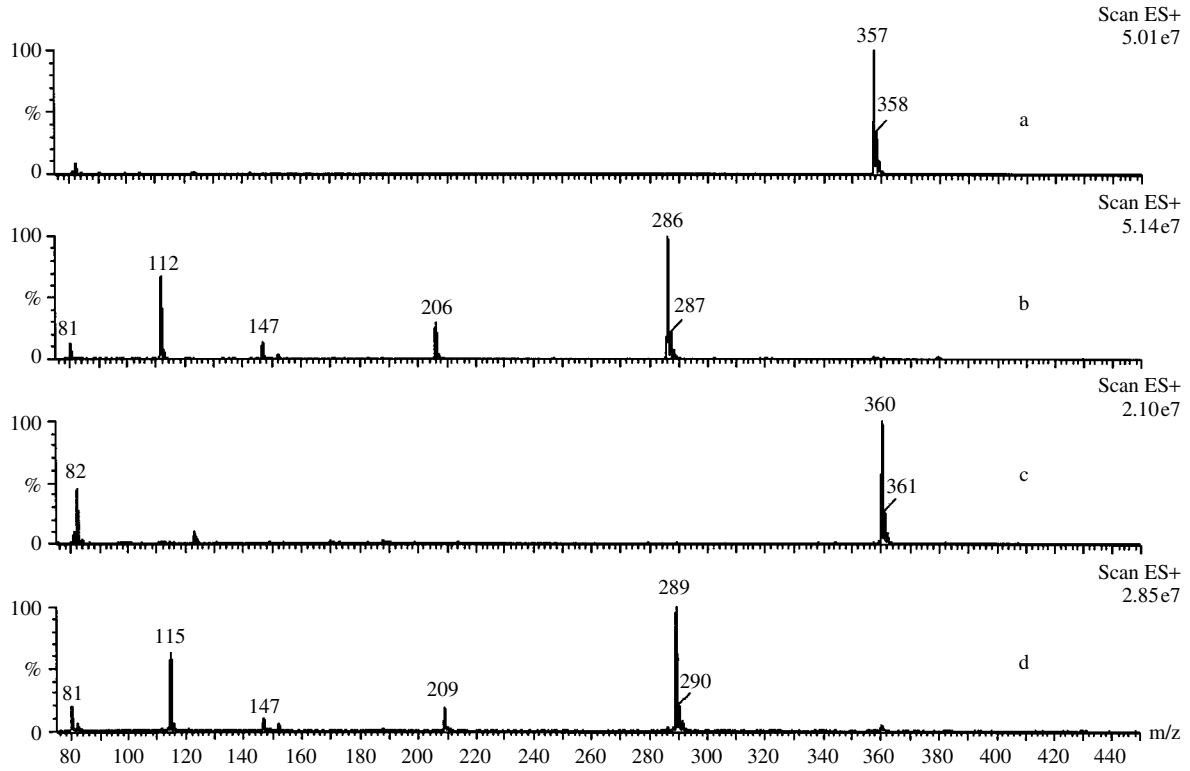

Figure 5. ESI-MS spectra of RSD921 (a) and 3d-RSD921 (c) at cone voltage of 20 V. ISF-MS spectra of in-source-formed fragments of $[RSD921 + H]^+$ (b) and $[3d\text{-}RSD921 + H]^+$ (d) at cone voltage of 60 V.

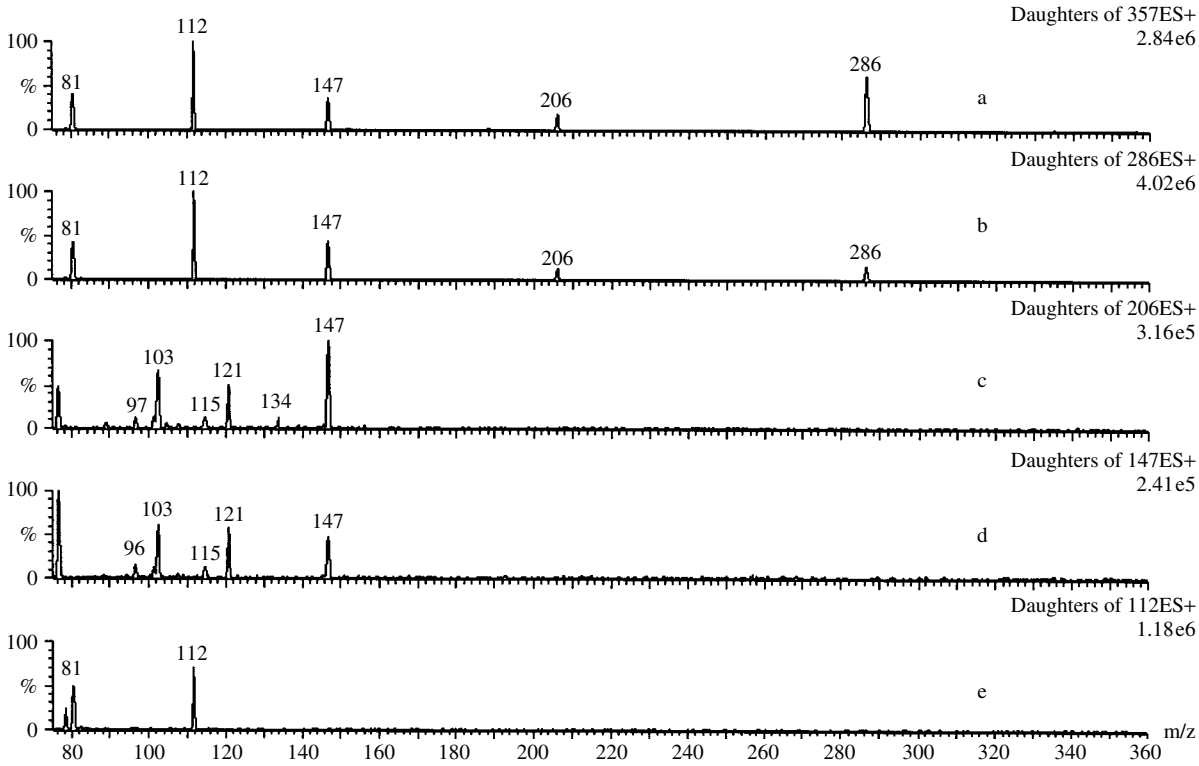

Figure 6. (a) Product ion mass spectra (MS/MS) of $[RSD921 + H]^+$ (m/z, cone voltage, collision energy: 357, 20 V, 30 eV). Product ion mass spectra of in-source-formed fragments (ISF-MS/MS) of RSD921 (m/z, cone voltage, collision energy): (b) 286, 40 V, 25 eV; (c) 206, 60 V, 45 eV; (d) 147, 60 V, 30 eV; (e) 112, 60 V, 20 eV.

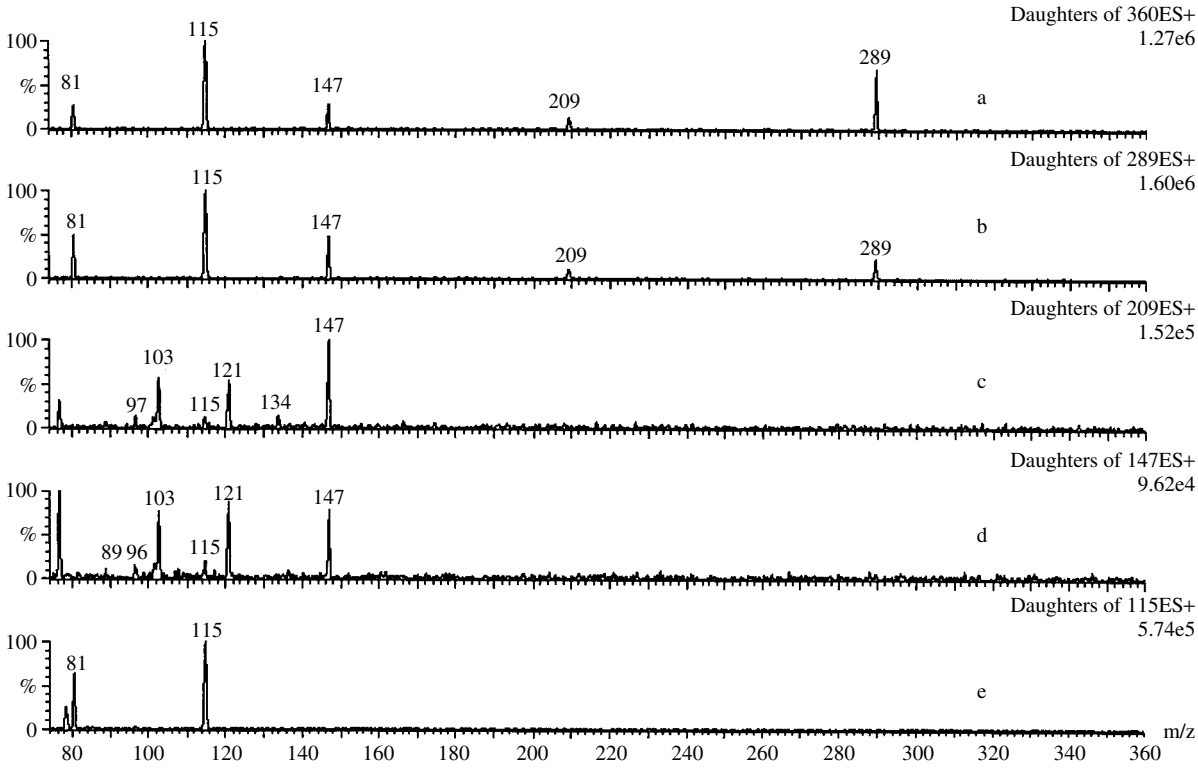

Figure 7. (a) Product ion mass spectra (MS/MS) of $[3d\text{-}RSD921 + H]^+$ (*m/z*, cone voltage, collision energy: 360, 20 V, 30 eV). Product ion mass spectra of in-source-formed fragments (ISF-MS/MS) of 3d-RSD921 (*m/z*, cone voltage, collision energy): (b) 289, 40 V, 25 eV; (c) 209, 60 V, 45 eV; (d) 147, 60 V, 30 eV; (e) 115, 60 V, 20 eV.

and $[3d\text{-}RSD921 + H]^+$ of m/z 360 (Fig. 5a and c). Both ISFs (Fig. 5b and d) and MS/MS CID (Figs 6a and 7a) produce ions of m/z 286, 206, 147, 112 and 81 for $[RSD921 + H]^+$ and of m/z 289, 209, 147, 115 and 81 for $[3d\text{-}RSD921 + H]^+$. Since ISF-MS and MS/MS produce nearly the same spectra, each one of these ISF-formed ions was mass-selected and subjected to MS/MS (ISF-MS/MS) so as to study the mechanism of dissociation by CID. ISF-MS/MS of the ions of m/z 286 and 289, for $[RSD921 + H]^+$ and $[3d\text{-}RSD921 + H]^+$ respectively, produce the product ions of m/z 206, 147, 112 and 81 for $[RSD921 + H]^+$ and of m/z 209, 147, 115 and 81 for $[3d\text{-}RSD921 + H]^+$ (Figs 6b and 7b). These spectra reveal that all other species (m/z 206/209, 147, 112/115, and 81) were directly or indirectly derived from m/z 286 and 289. ISF-MS/MS of the ions of m/z 206 and 209, from $[RSD921 + H]^+$ and $[3d\text{-}RSD921 + H]^+$ respectively, produce major product ions of m/z 147, 121, 103 and 77 (Figs 6c and 7c). These spectra reveal that only the ion of m/z 147 is derived from m/z 206/209. When the ISF-formed ions of m/z 147 (for both $[RSD921 + H]^+$ and $3d\text{-}RSD921 + H]^+$) were subjected to MS/MS their major product ions were those of m/z 121, 103 and 77 (Figs 6d and 7d). These spectra reinforce that m/z 112 and 115 are derived from m/z 286 and 289 respectively. ISF-MS/MS of the ions of m/z 112 and 115, from $[RSD921 + H]^+$ and $[3d\text{-}RSD921 + H]^+$ respectively, produce major product ions of m/z 81 for both RSD921 and 3d-RSD921 (Figs 6e and 7e). These spectra reveal that m/z 81 is derived from m/z 112 and 115. Based on these data, a dissociation route for $[RSD921 + H]^+$ and $[3d\text{-}RSD921 + H]^+$ by CID (ISF and MS/MS) is proposed (Scheme 1).

CONCLUSIONS

Phase I therapeutic drug monitoring and pharmacokinetic studies require highly selective and sensitive analytical methods to quantify the drug accurately. Data from calibration curves and quality-control samples revealed excellent sensitivity, accuracy and precision. Thus, the LC–MS/MS method developed to analyze RSD921 with 3d-RSD921 as the IS is suitable for support routine analysis of RDS921 in human plasma. In addition, the combined two-step dissociation ISF-MS/MS was successfully employed to elucidate the fragmentation pathway of protonated RSD921 and 3d-RSD921. This fragmentation pathway information will be employed in future metabolism studies.

Acknowledgements

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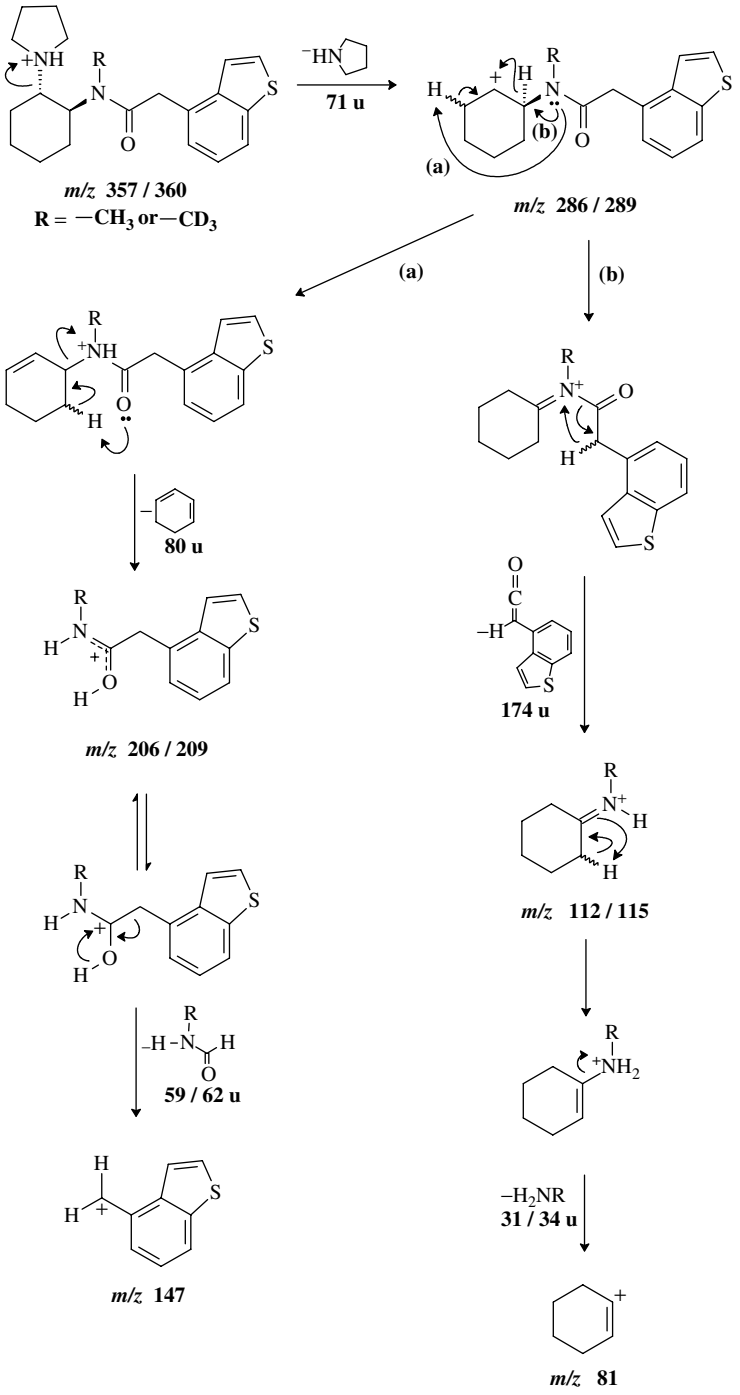

Scheme 1

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