ELSEVIER

Journal of Chromatography B, 759 (2001) 259–266

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Determination of an arylether antiarrhythmic and its N-dealkyl metabolite in rat plasma and hepatic microsomal incubates using liquid chromatography–tandem mass spectrometry

Vincent Tong[a], Tao Sheng[b], Michael J.A. Walker[c], Frank S. Abbott[a,*]

[a]*Division of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, The University of British Columbia, 2146 East Mall, Vancouver, B.C., V6T 1Z3, Canada*

[b]*Nortran Pharmaceuticals, 3650 Wesbrook Mall, Vancouver, B.C., V6S 2L2, Canada*

[c]*Department of Pharmacology and Therapeutics, Faculty of Medicine, The University of British Columbia, 2176 Health Sciences Mall, Vancouver, B.C., V6T 1Z3, Canada*

Received 22 November 2000; received in revised form 24 April 2001; accepted 24 April 2001

Abstract

A method was developed and validated for the quantification of (±)-*trans*-[2-morpholino-1-(1-naphthaleneethyloxy]cyclohexane monohydrochloride (RSD1070) and its N-dealkyl metabolite in rat plasma and hepatic microsomal incubates. Chromatographic separations were achieved using reversed-phase high-performance liquid chromatography coupled with positive ion electrospray ionization and detection by tandem mass spectrometry. The assay was linear from 2.5 to 100 ng/ml and this range was used for validation. Inter- and intra-assay variability (n=6), extraction recovery, and stability in plasma were assessed. The estimated limit of quantitation was in the range 2.5–3 ng/ml for both analytes in rat plasma. The analytical method was used in a pharmacokinetic study of RSD1070 in rats after a single i.v. bolus of 12 mg/kg.

Keywords: Arylether antiarrhythmic; (±)-*trans*-[2-Morpholino-1-(1-naphthaleneethyloxy]cyclohexane monohydrochloride

1. Introduction

RSD1070, (±)-*trans*-[2-morpholino-1-(1-naphthaleneethyloxy]cyclohexane monohydrochloride] (Fig. 1) is an experimental antiarrhythmic agent currently in preclinical stages of development [1]. However, information on the pharmacokinetics and metabolism of RSD1070 is lacking. RSD1070 is an analogue of a family of compounds developed to address the need for antiarrhythmics having enhanced potency but showing selectivity for patho-physiologically disturbed myocardial tissue (i.e. ischemia). Recently, an ester analogue of RSD1070 was developed that demonstrated increased antiarrhythmic potency under conditions of acidity and high potassium, conditions that mimic ischemia in vivo [2].

Previous analytical methods developed for this type of compound utilized reversed-phase HPLC–UV detection for the analysis of an arylacetamide analogue of RSD1070 in rat blood and other tissue [3]. Despite being a versatile and convenient method, the assay sensitivity of HPLC–UV was limited to a

*Corresponding author. Tel.: +1-604-822-2566; fax: +1-604-822-3035.

E-mail address: fabbott@interchange.ubc.ca (F.S. Abbott).

0378-4347/01/$ – see front matter
PII: S0378-4347(01)00230-4

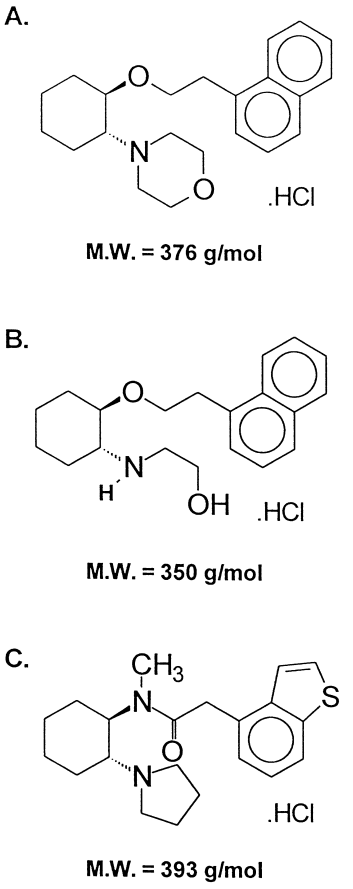

Fig. 1. Structures of (A) RSD1070, (B) its N-dealkyl metabolite and (C) the internal standard, RSD921.

concentration of approximately 0.1 μg/ml. Thus, there was a need for a more sensitive and selective assay to conduct pharmacokinetic studies of RSD1070 and to investigate its biotransformation.

HPLC–MS–MS was the method of choice for this investigation for several reasons. Tandem mass spectrometry with the use of collision-induced dissociation (CID) of the parent compounds into characteristic daughter ions serves as a semidiagnostic tool that allows some inferences as to possible metabolite structures and molecular mass information [4,5]. The use of MS–MS for drug metabolism studies is based on the premise that metabolites retain substructures of the parent drug molecule and produce MS–MS product ions associated with those substructures. As a result, the application of HPLC–MS–MS for rapid structural identification of metabolites has been significant and has provided valuable insight into probable pathways of drug biotransformation [6,7]. For instance, recent applications of HPLC–MS–MS for the determination of related antiarrhythmic compounds and their metabolites have been described for lidocaine [8], propranolol [9], phenytoin [10], and verapamil [11]. Furthermore, the increased assay sensitivity and selectivity of HPLC–MS–MS allows for greater characterization of the terminal elimination phase of the plasma concentration versus time profile for pharmacokinetic studies of drug candidates. In order for the preclinical pharmacokinetic study to be reliably interpreted, the HPLC–MS–MS analytical method developed for the quantitative determination of RSD1070 and its metabolite in biological samples was characterized and validated. Although an enantioselelctive method would provide a more reliable interpretation of RSD1070 pharmacokinetics, this issue was not addressed at the preclinical stage.

2. Experimental

2.1. Instrumentation

HPLC–MS–MS detection and quantitation of RSD1070 was carried out using a Fisons VG Quattro tandem mass spectrometer (Micromass, Montreal, Canada) interfaced with a Hewlett-Packard (Avondale, PA, USA) 1090 II liquid chromatograph. Instrument operation and data acquisition were controlled by MASSLYNX® (v3.1, Micromass) software.

2.2. Reagents

RSD1070, its N-dealkyl metabolite and the internal standard (I.S.) RSD921 (Fig. 1) were supplied by Nortran (Vancouver, Canada). All the above analytes were greater than 99% pure as determined by GC–FID and NMR. Methyl *tert*-butyl ether (HPLC grade) was purchased from Fischer (Vancouver, Canada). Methanol (HPLC grade) was obtained from Caledon (Georgetown, Canada).

2.3. Stock standard solutions

Stock solutions of 1 mg/ml RSD1070 and N-dealkyl RSD1070 were prepared by dissolving the appropriate amount of each analyte in distilled water. The stock solutions were diluted with distilled water to give working solution mixtures of 10 μg/ml and 100 ng/ml. Aliquots of stock and working solutions were frozen at −20°C. A stock solution (1 mg/ml) and a working solution of I.S. (0.5 μg/ml) prepared in distilled water were also frozen at −20°C. Aliquots of all working solutions were thawed and used once for each assay.

2.4. Sample extraction

All samples, standards, and quality control samples were processed by a single step, liquid–liquid extraction procedure (pH 13). Appropriate volumes of biological fluids (up to 0.1 ml) were diluted with their respective blank matrix for analysis. Samples or the spiked standards were transferred into clean borosilicate glass tubes with PTFE-lined caps and deionized water was added to a final volume of 1 ml. In brief, 100 μl of I.S. (0.5 μg/ml) and 100 μl 2 *M* NaOH were added to the sample and vortex mixed. Methyl *tert*-butyl ether (5 ml) was added to each sample and the tubes were capped. The samples were vortex mixed for 15 s prior to and after placement in a Labquake® tube shaker (LabIndustries, Berkeley, CA, USA) for 30 min. The separation of the aqueous and organic layers was aided by centrifugation (2060 *g* for 10 min at room temperature). The organic layer was separated and transferred into a clean set of tubes and evaporated to dryness under a gentle stream of nitrogen (20 min at 30°C, 0.5 p.s.i.; 1 p.s.i.=6894.76 Pa) using a Zymark Turbo Vap® LV evaporator (Zymark, Hopkinton, MA, USA). All samples were reconstituted in 1 ml of mobile phase (MeOH–water, 40:60, v/v, with 0.2% formic acid).

2.5. High-performance liquid chromatography

The samples (10 μl) were chromatographed on a Hewlett-Packard Model 1090 II LC instrument using a Phenomenex Columbus C_{18} column (150×2 mm, 5 μm) (Torrance, CA, USA) and delivered at 0.2 ml/min at room temperature (23°C). The HPLC autoinjector syringe and sample loop volumes were 25 and 250 μl, respectively. The mobile phase consisted of a MeOH–water mixture with 0.2% formic acid (pH 2.5). Linear gradient conditions were as follows: 40–80% MeOH from 0 to 8 min and a return to 40% MeOH at 8.5 min. Total run time was 12 min.

2.6. Electrospray tandem mass spectrometry

The HPLC eluent was introduced to the stainless steel capillary probe held at 3 kV. Positive electrospray was used as the means of ionization and CID involved argon as the neutral target gas at a pressure of $\approx 3.5 \cdot 10^{-3}$ mbar and with collision energy of 40 eV. The cone voltage was set at 30 V with a source temperature of 140°C. The low-mass and high-mass resolutions were set at 12.5/12.5 for MS1 (mass peak width of 0.7 u at 50% peak height) and 5.0/5.0 for MS2 (mass peak width of 2.3 u at 50% peak height). Mass selective detection of RSD1070, N-dealkylated RSD1070 metabolite, and I.S. were performed by multiple reaction monitoring (MRM) with a dwell time of 0.3 s/channel and with an inter channel delay of 0.03 s. The ion transitions monitored were m/z 340→155 (RSD1070), m/z 314→142 (N-dealkyl RSD1070) and m/z 357→147 (RSD921, I.S.). These transitions were selected based on the predominant fragmentation pathways of the various compounds into their daughter ion spectra (Fig. 2).

2.7. Standard (calibration) curves

Calibration standards were run daily in triplicate and treated in the same manner as test samples. Working stock solutions of 100 ng/ml mixture of RSD1070 and N-dealkyl RSD1070 prepared in distilled water were used for all calibration curve standards. Calibration standards (at concentrations of 2.5, 5, 10, 25, 50 and 100 ng/ml for both RSD1070 and its metabolite) were prepared in triplicate by adding appropriate volumes of the working stock solution to 150 μl of blank plasma or boiled microsomal protein. The I.S. (50 ng of RSD921) was then added to each sample and the samples were processed and analyzed by HPLC–MS–MS as described above. Weighted linear regression (weighting

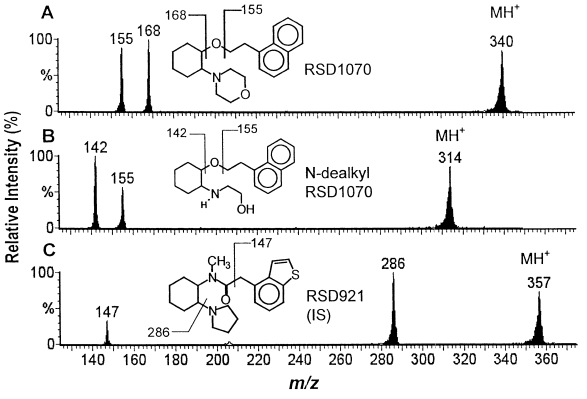

Fig. 2. Structures and electrospray MS–MS daughter ion spectra of (A) RSD1070 at m/z 340, (B) its N-dealkyl metabolite at m/z 314 and (C) internal standard, RSD921, at m/z 357. The structural diagrams show the product ions associated with specific substructures.

factor $= 1/y^2$) was performed between the peak area ratio of each analyte to that of the I.S. vs. the corresponding spiked concentration to reduce bias at the lower concentrations.

2.8. Validation

Method validation was performed by evaluating inter- and intra-assay accuracy (% bias) and precision (coefficient of variation, C.V.) of the low, mid and high QC concentrations. The QC standards of low (3 ng/ml), mid (15 ng/ml) and high (75 ng/ml) concentrations were prepared separately, frozen, and thawed for daily use. This was accomplished by analyzing six sets of calibration curves and QC samples on 6 separate days (inter-assay) and on the same day (intra-assay). Quantitation of QC samples was performed by analyzing the calibration curve standards and back calculating the concentration of each QC sample from the obtained slope, intercept and the peak area ratios.

The accuracy of the assay was assessed as the percentage bias of the nominal concentration observed for the spiked QCs and a bias of $< \pm 15\%$ at each concentration was considered to be acceptable accuracy. The precision of the assay (C.V.) was determined from the variance observed for the mean of replicate QCs of low, mid and high concentration and a precision of $<15\%$ C.V. at the mid and high QC concentrations and $<20\%$ at the low QC concentration was considered to be acceptable variability.

2.9. Extraction recovery

The relative percent recovery of RSD1070 and its N-dealkyl metabolite in rat plasma was determined at concentrations (2.5, 3, 5, 10, 15, 25, 50, 75 and 100 ng/ml) representing the entire range of the calibration curve. Two sets of samples, the control (non-extracted) group and the recovery (extracted) group, were prepared in triplicate. In the extracted samples, analytes were extracted as outlined above and the I.S. was added following the procedure. The non-extracted samples containing the I.S. were prepared in mobile phase and injected directly onto the LC. The extraction recoveries of the extracted group in rat plasma were determined as a percentage of the non-extracted samples at each concentration.

2.10. Analyte stability in rat plasma

Tests were carried out to establish the stability of the analytes in plasma under the routine sample handling in the laboratory. Bench-top stability was assessed with blank rat plasma spiked with analyte at concentrations representing the calibration curve (2.5–100 ng/ml). The samples were left on the bench-top overnight (24 h at room temperature) and processed the next day. Freeze–thaw stability was also assessed with spiked blank plasma at a concentration range of 2.5–100 ng/ml. The samples were subjected to three cycles of freezing (-20°C) and thawing at room temperature (22°C) before processing. The relative stability of analyte for each test was performed by comparing the PAR obtained from the stability testing to the PAR of a freshly prepared standard processed on the same day. The analytes were considered ‘stable’ if the measured PARs were within $\pm 10\%$ of the reference PARs.

2.11. Application of the method to a pharmacokinetic study of RSD1070 in rats

Male Sprague–Dawley rats (200–300 g, $n=3$) obtained from the University of British Columbia Animal Care Facility, were housed individually on a

bedding of wood shavings and fed with rat diet and water ad libitum. Animals were maintained in a holding room on a 12 h light–12 h dark cycle at constant temperature (22°C) and humidity. All animal experimentation was approved by the University of British Columbia Animal Care Committee (Vancouver, Canada). Rats were cannulated with PE-20 tubing at the abdominal aorta and the inferior vena cava under anesthesia (sodium pentobarbital, 65 mg/kg, i.p.). The cannulae were passed through a trocar and exteriorized by threading the trocar under the skin of the back and out through a small incision at the mid-scapular region. All incision sites were closed with silk sutures and the animal was housed individually. At least 24 h was allowed for recovery before commencing with the experiment. Rats were administered a single i.v. bolus antiarrhythmic ED_{90} dose of 12 mg/kg via the inferior vena cava cannula and housed in stainless steel metabolic cages. Blood (250 μl) was collected at various time points (2.5, 5, 10, 15, 20, 30, 45, 60, 120, 240, 360, 540 min postadministration) in Eppendorf® microcentrifuge tubes via the inferior vena cava cannula using heparinized syringes. All blood samples were immediately centrifuged (13 000 *g* for 5 min at room temperature) and the plasma removed and stored at −20°C until further analysis.

3. Results and discussion

3.1. Chromatography and detection of RSD1070 and its N-dealkyl metabolite

HPLC–MS–MS was an effective method to obtain optimal selectivity and sensitivity for the assay of RSD1070 in all of the biological samples examined. Fig. 2 illustrates the daughter ion mass spectra of RSD1070, N-dealkylated RSD1070, and the I.S. The collision induced fragmentation pattern of each molecular ion precursor (MH^+) allowed for the selection of the desired product ions to be detected by MRM. RSD1070 (MH^+ m/z 340) fragmentation resulted in product ions m/z 168 and 155, corresponding to the cyclohexyl N-morpholino backbone and the 1-naphthyl side chain, respectively. N-dealkylated RSD1070 (MH^+ m/z 314) fragmentation resulted in product ions m/z 142 and 155, corresponding to the N- and O-dealkylated N-morpholino backbone and the 1-naphthyl side chain, respectively. Fragmentation of the I.S. (MH^+ m/z 357) resulted in product ions m/z 286 and 147. Ion m/z 147, which corresponds to the benzothiophene side chain, was monitored for quantitation purposes. Ion m/z 286, which corresponds to the molecular ion with the loss of the pyrrolidine moiety, produced an intense signal. However, ion m/z 286 was not monitored because this was a constituent species of the I.S. stock solutions, and is believed to be a starting material contaminant. Based on the daughter ion spectra, the following parent–daughter ion transitions were selected for detection: RSD1070 at m/z 340→155, its N-dealkylated metabolite at m/z 314→142, and I.S. at m/z 357→147. Sample LC–MS–MS chromatograms using MRM detection mode are illustrated in Fig. 3. The chromatographic conditions used in the assay provided the conditions necessary for adequate resolution of the product ions of interest and resulted in sharp symmetrical peaks.

3.2. Assay validation

RSD1070 and N-dealkylated RSD1070 calibration curves were prepared daily in triplicate based on PAR to the internal standard. Initially, the LOQ was estimated to be 2.5 ng/ml based on a signal-to-noise ratio of approximately 5 to 1. Calibration curves demonstrated linearity over the range 2.5–100 ng/ml based on having linear regression coefficients >0.999 (weighting factor $=1/y^2$). The mean slope and intercept values ($n=6$) for RSD1070 were 0.049 ± 0.011 and 0.028 ± 0.011, respectively. The mean slope and intercept values for N-dealkyl RSD1070 were 0.043 ± 0.014 and 0.020 ± 0.006, respectively. Based on the published guidelines of Shah et al. [12], the $1/y^2$ weighting function resulted in acceptable regression bias and precision at the lower, mid and upper range of the calibration curves (summarized in Table 1). The inter- and intra-assay variability and precision based on low, mid, and high quality control (QC) samples in microsomal matrix were $<15\%$ C.V. and $<\pm15\%$ bias for both RSD1070 and its N-dealkylated metabolite. QC samples prepared in rat plasma on six separate days demonstrated inter-assay variability of $<10\%$ C.V. at mid and high QC concentrations, however, 18.2%

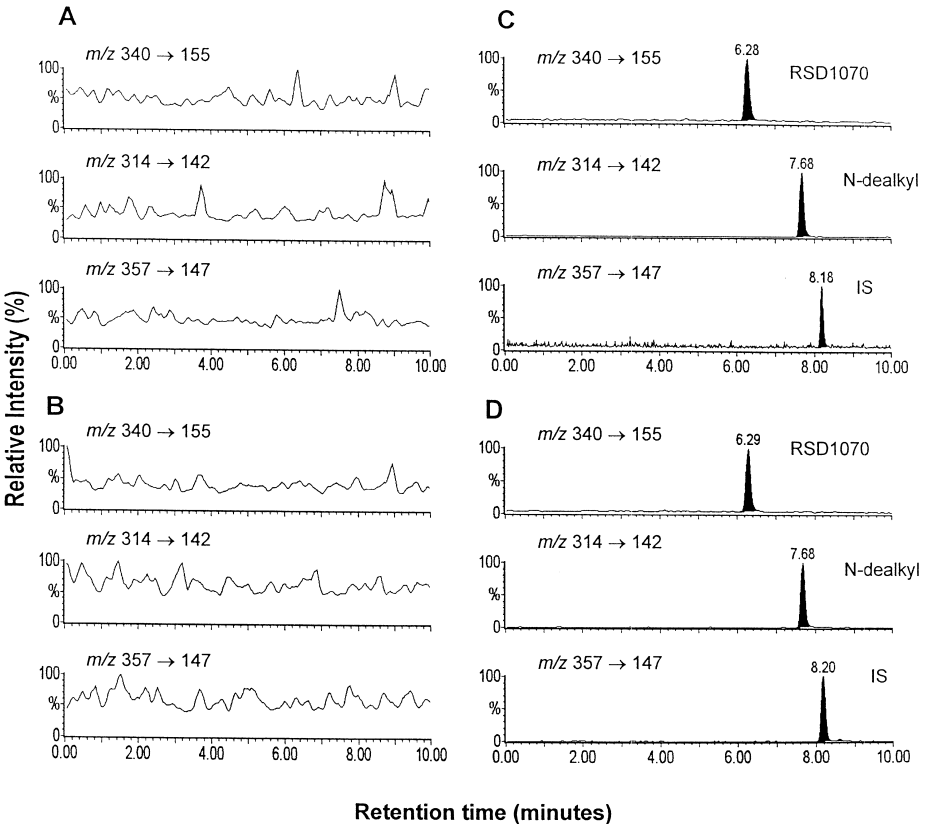

Fig. 3. LC–MS–MS chromatograms obtained by MRM of ion transitions m/z 340>155 (RSD1070), m/z 314>142 (N-dealkylated RSD1070), and m/z 357>147 (IS). (A) Blank rat plasma, (B) blank microsomal matrix, (C and D) rat plasma and microsomal matrix spiked with reference standards (50 ng/ml of each analyte). The y-axis scales in (A) and (B) have been magnified to show the baselines at different MRM ion transitions. The LC and MS–MS conditions and specifications are described in the text.

Table 1
Inter-assay and intra-assay variability and bias of the method in rat plasma and liver microsome incubates

Analyte (matrix)	Parameter	Inter-assay ($n=6$)			Intra-assay ($n=6$)		
		QC low	QC mid	QC high	QC low	QC mid	QC high
Nominal conc. (ng/ml)		3	15	75	3	15	75
RSD1070	C.V. (%)	18.2	8.1	7.3	6.4	3.8	7.3
(rat plasma)	Bias (%)	2.2	12.1	4.0	−4.2	0.4	5.2
RSD1070	C.V. (%)	12.1	5.2	3.3	5.1	6.0	8.9
(rat liver microsome)	Bias (%)	−3.1	9.1	−1.2	−1.1	0.3	−1.6
N-dealkyl RSD1070	C.V. (%)	19.1	9.0	4.2	10.8	4.4	6.1
(rat plasma)	Bias (%)	3.9	15.6	−6.3	1.1	7.0	0.5
N-dealkyl RSD1070	C.V. (%)	8.4	5.0	4.6	11.6	5.4	6.0
(rat liver microsome)	Bias (%)	11.2	10.6	−0.3	5.4	5.4	−3.7

C.V. was reported with the low QC. The accuracy (% bias) of all QC samples prepared in rat plasma for both inter- and intra-assay validations was within 84.4–106.3% of the expected nominal concentrations. The LOQ for both RSD1070 and N-dealkylated RSD1070 was estimated to be in the range 2.5–3 ng/ml, based on the validation results and in accordance of having a C.V. of $<20\%$ and a signal-to-noise ratio of 5:1.

3.3. Extraction recovery and stability

Methyl *tert*-butyl ether was previously used as the organic solvent for the extraction of the I.S. RSD921 [3] and also provided maximal recovery of RSD1070 and its N-dealkyl metabolite in our method. The mean analytical recovery for the studied analytes in rat plasma over the concentration range 2.5–100 ng/ml was determined to be $>95\%$. Studies were conducted to evaluate the stability of the analytes in rat plasma under conditions to mimic routine sample handling in the laboratory. RSD1070 and its metabolite were stable in plasma for 24 h at room temperature (22°C) and after three cycles of freezing and thawing.

3.4. Pharmacokinetics of RSD1070 in rat

The applicability of the developed method was demonstrated in a pharmacokinetic study in rat, designed to study the disposition of RSD1070 and the formation its N-dealkyl metabolite, the suspected major RSD1070 metabolite. Fig. 4 illustrates the mean plasma profiles of RSD1070 and N-dealkyl RSD1070 following a single iv bolus administration of an antiarrhythmic (ED_{90}) dose of 12 mg/kg in rats ($n=3$). The data shows that RSD1070 plasma concentration declined rapidly displaying three-compartment elimination kinetics after drug administration. At 360 min post-administration, RSD1070 plasma concentrations were below the LOQ. All plasma samples analyzed had plasma concentrations that exceeded 10 ng/ml, and thus were greater than the LOQ concentration of 3 ng/ml. The plasma formation of N-dealkyl RSD1070 was maximal (0.27 μg/ml) at 3 min and its elimination profile was parallel to the elimination of RSD1070. Whether or not N-dealkyl RSD1070 is a major metabolite is yet to be determined and further investigation involving urinary mass balance data will be required.

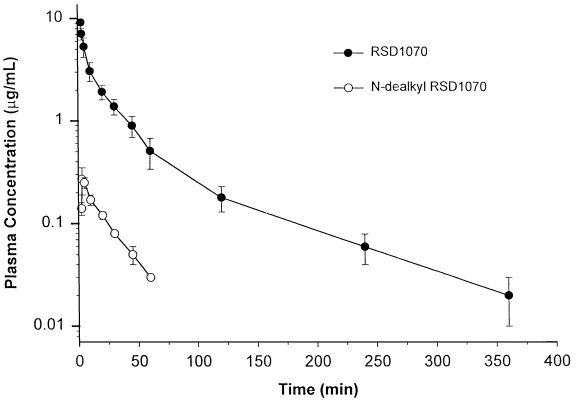

Fig. 4. Plasma concentration vs. time profiles of RSD1070 (●) and N-dealkyl RSD1070 (○) in rat ($n=3$) after single i.v. bolus dose of 12 mg/kg RSD1070 via the inferior vena cava.

Acknowledgements

The authors thank Nortran Pharmaceuticals for the provision of RSD1070 and the internal standard; Dr. Lillian Clohs and Mr. Mark Buss (Nortran Pharmaceuticals) for discussion of analytical method development, and Mr. Harvey Wong (Faculty of Pharmaceutical Sciences, UBC) for assistance in the pharmacokinetic studies. Technical assistance from Mr. Roland Burton (Faculty of Pharmaceutical Sciences, UBC) in the use of HPLC–MS–MS is greatly appreciated. Funding to Vincent Tong from Nortran Pharmaceuticals is acknowledged.

References

[1] G.N. Beatch, Nortran Pharmaceuticals, personal communications.
[2] S.L. Yong, R. Xu, G. Mclarnon, A.B. Zolotoy, G.N. Beatch, M.J.A. Walker, J. Pharmacol. Exp. Ther. 289 (1997) 236.
[3] M.L. Walker, R.A. Wall, M.J.A. Walker, J. Chromatogr. B 675 (1996) 257.
[4] R.J. Perchalski, R.A. Yost, B.J. Wilder, Anal. Chem. 54 (1982) 1466.
[5] T.R. Covey, E.D. Lee, J.D. Henion, Anal. Chem. 58 (1986) 2453.

[6] R.J. Perchalski, M.S. Lee, R.A. Yost, J. Clin. Pharmacol. 26 (1986) 435.
[7] M.S. Lee, E.H. Kerns, M.E. Hail, J. Liu, K.J. Volk, LC·GC 15 (1997) 542.
[8] M. Abdel-Rehim, M. Bielenstein, Y. Askemark, N. Tyrefors, T. Arvidsson, J. Chromatogr. B 741 (2000) 175.
[9] F. Beaudry, J.C. Yves Le Blanc, M. Coutu, I. Ramier, J.P. Moreau, N.K. Brown, Biomed. Chromatogr. 13 (1999) 363.
[10] S. Bardin, J.C. Ottinger, A.P. Breau, T.J. O'Shea, J. Pharm. Biomed. Anal. 23 (2000) 573.
[11] O. von Richter, M. Eichelbaum, F. Schonberger, U. Hofmann, J. Chromatogr. B 738 (2000) 137.
[12] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman, S. Spector, Pharm. Res. 9 (1992) 588.