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ORIGINAL RESEARCH

Determination of rosuvastatin in rat plasma by HPLC: validation and its application to pharmacokinetic studies[1]

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ABSTRACT: A specific, accurate, precise and reproducible high-performance liquid chromatography (HPLC) method was developed for the estimation of rosuvastatin (RST), a novel, synthetic and potent HMG-CoA inhibitor in rat plasma. The assay procedure involved simple liquid–liquid extraction of RST and internal standard (IS, ketoprofen) from a small plasma volume directly into acetonitrile. The organic layer was separated and evaporated under a gentle stream of nitrogen at 40°C. The residue was reconstituted in the mobile phase and injected onto a Kromasil KR 100-5C_{18} column (4.6 × 250 mm, 5 μm). Mobile phase consisting of 0.05 M formic acid and acetonitrile (55:45, v/v) was used at a flow rate of 1.0 mL/min for the effective separation of RST and IS. The detection of the analyte peak was achieved by monitoring the eluate using a UV detector set at 240 nm. The ratio of peak area of analyte to IS was used for quantification of plasma samples. Nominal retention times of RST and IS were 8.6 and 12.5 min, respectively. The standard curve for RST was linear ($r^2 > 0.999$) in the concentration range 0.02–10 μg/mL. Absolute recoveries of RST and IS were 85–110 and >100%, respectively, from rat plasma. The lower limit of quantification (LLOQ) of RST was 0.02 μg/mL. The inter- and intra-day precisions in the measurement of quality control (QC) samples, 0.02, 0.06, 1.6 and 8.0 μg/mL, were in the range 7.24–12.43% relative standard deviation (RSD) and 2.28–10.23% RSD, respectively. Accuracy in the measurement of QC samples was in the range 93.05–112.17% of the spiked nominal values. Both analyte and IS were stable in the battery of stability studies, viz. benchtop, autosampler and freeze–thaw cycles. RST was found to be stable for a period of 30 days on storage at –80°C. The application of the assay to determine the pharmacokinetic disposition after a single oral dose to rats is described. Copyright © 2006 John Wiley & Sons, Ltd.

KEYWORDS: statins; dyslipidemic disorders; oral dose; pharmacokinetic disposition in rats

INTRODUCTION

Rosuvastatin (Crestor®, Fig. 1) is the most recently approved drug in the statin family for treating various dyslipidemic disorders (Brown *et al.*, 2002; Olsson *et al.*, 2002; Jones *et al.*, 2003). The other approved drugs in this class include atorvastatin, simvastatin, prvastatin, fluvastatin, lovastatin. Rosuvastatin (RST) is chemically bis[(*E*)-7-[4-(4-fluorophenyl)-6-isopropyl-2-[methyl (methyl-sulfonyl)amino]pyrimidin-5-yl](3R,5S)-3,5-dihydroxyhept-6-enoicacid]calcium salt. Like other statins, RST selectively blocks the key rate-limiting enzyme responsible for cholesterol biosynthesis known as 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. The pharmacological action of RST includes significant lowering of blood circulating concentrations of lipids such as LDL, total cholesterol, triglycerides and apolipoprotein B. RST has been shown to increase HDL cholesterol levels in the blood to a modest extent. The clinical pharmacokinetic behavior of RST has been extensively studied and published (Martin *et al.*, 2002a,b). Across the wide range of doses administered orally (ranging from 5 to 80 mg), both peak plasma concentrations (C_{max}) and area under curve (AUC) showed approximate linear increase with the dose administered (Martin *et al.*, 2003a). The attainment of C_{max} in the circulation appeared to be consistent (3–5 h) following RST oral dosing (Martin *et al.*, 2002a). The steady state was achieved by the fifth day of dosing, which is in accordance the reported half-life value of 19 h (Martin *et al.*, 2002a). The oral bioavailability of RST was found to be 20% (Martin *et al.*, 2003b), which compared somewhat favorably with atorvastatin (12%), simvastatin (~5%) and pravastatin (18%) (Lennernas and Fager, 1997). Similar to other statins, serum protein binding of RST was not found to be very high (Martin *et al.*, 2003b). Work related to metabolism of RST has revealed the formation of *N*-desmethylrosuvastatin as a primary metabolite (McCormick *et al.*, 2000). Interestingly, these CYP isozymes (CYP 2C9, 3A4 and 2C19) were shown to have the potential to catalyze the formation of the

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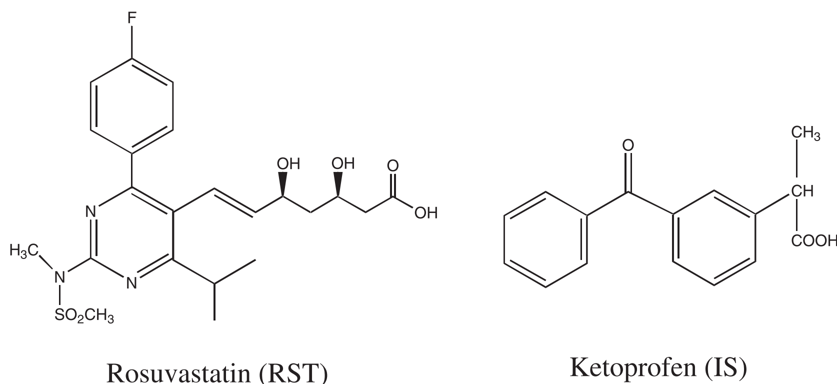

Figure 1. Structural representation of RST and ketoprofen (IS).

dealkylated metabolite of RST. However, it appeared that the contribution of CYP 2C9 was much greater than that of the other two isozymes. Since CYP 2C9 is a polymorphic isozyme, there may have been some concern if the *N*-desmethyl metabolite was very potent. However, the inhibition of HMG-CoA activity was found to be considerably lower compared with RST (Holdgate *et al.*, 2001).

Several drug–drug interaction studies have been carried out with RST, notably with agents that are co-administered with statins or have a narrow therapeutic window, or those that are known to inhibit CYP metabolism. Co-administration of ketoconazole (Cooper *et al.*, 2003a), fenofibrate (Martin *et al.*, 2003c) with RST resulted in no change in plasma concentrations of RST (Cooper *et al.*, 2003a; Martin *et al.*, 2003c). However, co-administration of itraconazole (Cooper *et al.*, 2003b), fluconazole (Cooper *et al.*, 2002), gemfibrozil (Schneck *et al.*, 2004) and cyclosporine (Simonson *et al.*, 2004a) with RST increased both AUC and C_{max} of RST. The increase in AUC and C_{max} with fluconazole and itraconazole was considered clinically insignificant (Cooper *et al.*, 2002; Cooper *et al.*, 2003b), on the other hand the increase in AUC and C_{max} observed with gemfibrozil and cyclosporine were considered clinically significant (Schneck *et al.*, 2004; Simonsom *et al.*, 2004a). While the co-administration of digoxin with RST resulted in no change in plasma concentrations of digoxin (Martin *et al.*, 2002c), the concomitant administration of oral contraceptives (Simonson *et al.*, 2004b) showed an increase in concentrations of oral contraceptives. Finally the co-administration of erythromycin along with RST resulted in clinically significant decrease in AUC and C_{max} (Cooper *et al.*, 2003c).

To date there are only two bioanalytical methods reported for the estimation of RST in human plasma using LC-MS/MS. The first LC-MS/MS method developed by Hull *et al.* (2002) utilizes automated solid-phase extraction, followed by high-performance liquid chromatography with positive ion TurboIonspray tandem mass spectrometry (Hull *et al.*, 2002). Recently, Trivedi *et al.* (2005) have developed an LC-MS/MS method for simultaneous estimation of RST and fenofibric acid from human plasma. This method involves simple liquid–liquid extraction of RST and fenofibric acid along with IS (carbamazepine) into ethyl acetate (Trivedi *et al.*, 2005). To the best of our knowledge there is no bioanalytical method employing a simple HPLC reported in literature for quantification of RST in plasma. However, the availability of HPLC methods is reported in the literature for the measurement of other statins like simvastatin (Carlcucci *et al.*, 1992; Ochiai *et al.*, 1997; Tan *et al.*, 2000) and pravastatin (Whigan *et al.*, 1989; Iacona *et al.*, 1994; Otter and Mignat, 1998; Sigurbjoernsson *et al.*, 1998; Li *et al.*, 2001; Siekmeier *et al.*, 2000; Bauer *et al.*, 2005). Potent statins have heightened awareness due to episodes of infrequently occurring muscle toxicity (rhabdomylosis). Additionally, the HPLC method can aid in the measurement of RST in routine monitoring, if necessary, in common laboratories. Herein we report an HPLC assay that is fully validated for the determination of RST in rat plasma. The assay was successfully applied to the analysis of RST in rat plasma samples obtained from pharmacokinetic studies, allowing the pharmacokinetics of the compound to be determined. The method offers the advantage of simplicity with adequate sensitivity, selectivity, precision and accuracy for the determination of RST. We believe that development of a method in rodent plasma would facilitate the ease of adaptability of RST assay in human plasma.

EXPERIMENTAL

Chemicals and reagents. RST and ketoprofen (IS, Fig. 1) were synthesized by the Medicinal Chemistry Group, Discovery Research, Dr Reddy's Laboratories Ltd (DRL), Hyderabad and were characterized using chromatographic and spectral techniques by Analytical Research Group,

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Discovery Research, DRL, Hyderabad. Purity was found to be more than 99% for both the compounds. Acetonitrile, methanol (HPLC-grade), and ethylene diamine tetra acetic acid disodium salt (EDTA) and formic acid (analytical reagent-grade) were purchased from Qualigens, Glaxo (India), Mumbai, India. All aqueous solutions including the buffer for the HPLC mobile phase was prepared with Milli Q (Millipore, USA) grade water. The control rat plasma was obtained from the Department of Pre-clinical Safety Evaluation, Discovery Research, DRL, Hyderabad, India.

Chromatography. The HPLC system consisted of a Shimadzu SCL-10A *VP* system controller (Kyoto, Japan), a Shimadzu LC-10AT *VP* pump (Kyoto, Japan), a Shimadzu SIL-10AD *VP* auto injector with sample cooler (Kyoto, Japan), a Shimadzu DGU-14A *VP* degasser (Kyoto, Japan) and a Shimadzu SPD-10A *VP* ultraviolet detector (Kyoto, Japan). The data were acquired and processed using Shimadzu VP software (version 5.03). The analytical column was a Kromasil KR100-5C_{18}-250 A, 4.6 × 250 mm, 5 μm particle size (Hichrom, UK). The isocratic mobile phase consisted of 0.05 M formic acid and acetonitrile mixture (55:45, v/v) was run at a flow rate of 1.0 mL/min. The eluate was monitored by an ultraviolet detector set at 240 nm, the maximal absorption for RST and the same wavelength was found adequate for monitoring the internal standard.

Standard solutions. Standards and QC stock solutions of RST and IS were prepared in methanol. Appropriate dilutions of RST were made in methanol to produce working stock solutions of 200, 100, 40, 20, 10, 4, 2, 1 and 0.4 μg/mL. Stock solutions were stored at approximately 5°C. Working stocks were used to prepare plasma calibration standards. A working IS solution (1 mg/mL) was prepared in methanol. Calibration samples were prepared by spiking with the appropriate amount of the analyte and IS on the day of analysis into 100 μL of control rat plasma. Samples for the determination of recovery, precision and accuracy were prepared by spiking control rat plasma in bulk at appropriate concentrations (0.02, 0.06, 1.6 and 8.0 μg/mL) and 100 μL volumes were aliquoted into different tubes and, depending on the nature of the experiments, were stored at –80°C until analysis.

Sample preparation. To 100 μL of plasma sample, methanolic solution of ketoprofen (IS) equivalent to 1 μg was added and mixed for 15 s on a cyclomixer (Remi Instruments, Mumbai, India). After the addition of 2 mL of acetonitrile, the mixture was vortexed for 2 min, followed by centrifugation for 5 min at 3200 rpm on a tabletop centrifuge (Remi Instruments, Mumbai, India). The organic layer (1.8 mL) was separated and evaporated to dryness at 40°C using a gentle stream of nitrogen (Zymark® Turbovap®, Kopkinton, MA, USA). The residue was reconstituted in 150 μL of the mobile phase and 100 μL were injected onto the HPLC column.

Calibration curves. Calibration curves were acquired by plotting the peak area ratio of RST:IS against the nominal concentration of calibration standards. The concentrations used were 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 μg/mL. The results were fitted to linear regression analysis using $1/X$ as weighting factor.

Precision and accuracy. The intra-assay precision and accuracy were estimated by analysing four replicates containing RST at four different QC levels, i.e. 0.02, 0.06 1.6 and 8.0 μg/mL. The inter-assay precision was determined by analyzing the four levels of QC samples on four different runs. The criteria for acceptability of the data included accuracy within ±15% deviation (DEV) from the nominal values and precision within 15% relative standard deviation (RSD) (*United States Pharmacopeia*, 1995; Shah *et al*., 1992).

Stability experiments. The stability of RST and IS in the injection solvent was determined periodically by injecting replicate preparations of processed samples for up to 24 h (in the auto sampler at 5°C) after the initial injection. The peak-areas of the analyte and IS obtained at initial cycle were used as the reference to determine the relative stability at subsequent points. Stability of RST in the biomatrix during 6 h (bench-top) was determined at ambient temperature (25 ± 3°C) at four concentrations in quadruplicates. Freezer stability of RST in rat plasma was assessed by analyzing the QC samples stored at –80°C for at least 1 month. The stability of RST in rat plasma following repeated freeze–thaw cycles was assessed using QC samples spiked with RST. The samples were stored at –80°C between freeze–thaw cycles. The samples were thawed by allowing them to stand at room temperature for approximately 2 h. After drawing out the required volume, the samples were then returned to the freezer. The stability of RST was assessed after three freeze–thaw cycles. The samples were processed using the same procedure as described in the sample preparation section. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (i.e. ±15% DEV) and precision (i.e. 15% RSD) except for LLOQ, where it should not exceed 20% of CV (*United States Pharmacopeia*, 1995; Shah *et al*., 1992).

Extraction recovery. Two sets of standards containing the analyte and IS at three different concentrations (0.06, 1.6 and 8.0 μg/mL) and at the lower limit of quantification (LLOQ) were prepared. One set was prepared in rat plasma and the other set was prepared in methanol (neat set). The recovery was determined by comparing peak areas of spiked plasma extracts with those of unextracted neat standards prepared in methanol. The recovery value was calculated at the various concentrations of RST. The recovery of the IS was determined at a single concentration of 10 μg/mL.

Animal study. The pharmacokinetic study was carried out in male Wistar rats. The animals were fasted overnight (~14 h) and had free access to water throughout the experimental period. RST was administered by oral gavage at a dose of 100 mg/kg, as a suspension in 0.25% sodium carboxymethylcellulose. Blood samples (0.25 mL) were collected from the retro-orbital plexus at designated time points (0.5, 1.5, 3, 5, 8, 10, 12 and 24 h) into microcentrifuge tubes containing 10 μL of EDTA. Plasma was harvested by centrifuging the blood using Biofuge (Hereaus, Germany) at 12,800 rpm for 5 min. Plasma (200 μL) samples were spiked with IS and processed as described above.

Pharmacokinetic analysis. Pharmacokinetic parameters were calculated by employing a non-compartmental analysis

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(Gibaldi and Perrier, 1982). The peak plasma concentration (C_{max}) and the corresponding time (T_{max}) were directly obtained from the raw data. The area under the plasma concentration vs time curve up to the last quantifiable time point, $AUC_{(0-t)}$, was obtained by the linear and log–linear trapezoidal summation. The $AUC_{(0-t)}$ was extrapolated to infinity (i.e. $AUC_{(0-\infty)}$) by adding the quotient of C_{last}/K_{el}, where C_{last} represents the last measurable time concentration and K_{el} represents the apparent terminal rate constant. K_{el} was calculated by the linear regression of the log-transformed concentrations of the drug in the terminal phase. The half-life ($t_{1/2}$) of the terminal elimination phase was obtained using the relationship $t_{1/2} = 0.693/K_{el}$.

RESULTS AND DISCUSSION

Specificity and chromatography

In the chosen chromatographic conditions, specificity was indicated by the absence of any endogenous interference at retention times of peaks of interest as evaluated by chromatograms of blank rat plasma and plasma spiked with RST and IS. Both the analyte and IS were well separated with retention time of 8.6 and 12.5 min, respectively. System suitability parameters for the method were as follows: theoretical plates for RST > 8338 and for IS > 7312, asymmetry factor <1.14 and resolution between RST and IS >2.20. Figure 2 shows a typical overlaid chromatogram for the control rat plasma (free of analyte and IS), rat plasma spiked with RST at the LLOQ (0.02 µg/mL) and an *in vivo* plasma sample obtained after oral administration of RST at 100 mg/kg.

Calibration curve

Peak area ratios of RST to the IS were measured and acted as a surrogate for quantitation. A representative calibration graph of peak-area ratio (RST to IS) vs RST concentration in the range 0.02–10 µg/mL resulted in the regression equation $y = 0.118x + 0.0004$ ($R^2 > 0.999$). The standard curve had a reliable reproducibility over the standard concentrations of the analyte across the calibration range. The lowest concentration with the RSD < 20% was taken as LLOQ (Shah *et al.*, 1992) and was found to be 0.02 µg/mL. The RSD and signal-to-noise ratio at LLOQ were found to be 6.80% and 5.75, respectively.

Accuracy and precision

Accuracy and precision data for intra- and inter-day plasma test samples are presented in Table 1. The intra-day accuracy (%) ranged from 106.37 to 112.17 at 0.02 µg/mL, 94.54 to 100.49 at 0.06 µg/mL, 99.42 to 106.64 at 1.6 µg/mL and 93.05 to 101.45 at 8.0 µg/mL. The inter-day accuracy (%) was 109.65, 98.54, 103.56 and 97.07 at 0.02, 0.06, 1.6 and 8.0 µg/mL, respectively. The intra-day precision (% RSD) ranged from 4.10 to 8.54 at 0.02 µg/mL, 3.98 to 10.23 at 0.06 µg/mL, 2.28 to 8.13 at 1.6 µg/mL and 2.65 to 8.44 at 8.0 µg/mL. The

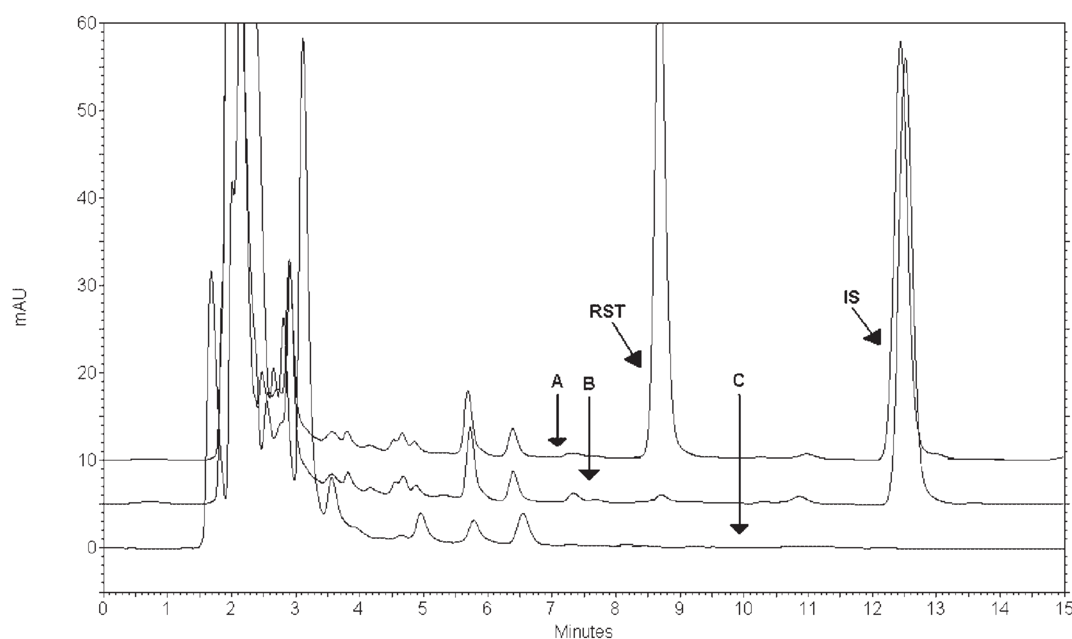

Figure 2. HPLC chromatograms of a 100 µL injection of (a) a 0.5 h *in vivo* plasma sample obtained from rat dosed with RST at 100 mg/kg, p.o., (b) blank plasma spiked with RST at LLOQ (0.02 µg/mL) and 1 µg/mL of IS and (c) rat blank plasma.

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Table 1. Intra and inter-day precision of determination of RST in rat plasma

Theoretical concentration (μg/mL)	Run	Measured concentration (μg/mL)			
		Mean	SD	RSD	Accuracy (%)
Intra-day variation (four replicates at each concentration)					
0.02	1	0.02	0.001	4.10	112.17
	2	0.02	0.001	6.18	106.37
	3	0.02	0.002	8.54	109.26
	4	0.02	0.001	6.98	107.06
0.06	1	0.06	0.005	8.16	99.70
	2	0.06	0.002	3.98	94.54
	3	0.06	0.006	10.23	99.43
	4	0.06	0.004	6.22	100.49
1.6	1	1.70	0.13	8.13	106.64
	2	1.67	0.03	2.28	104.34
	3	1.66	0.06	4.08	103.85
	4	1.59	0.10	6.72	99.42
8.0	1	7.86	0.32	4.15	98.37
	2	8.12	0.68	8.44	101.45
	3	7.64	0.25	3.36	95.44
	4	7.44	0.19	2.65	93.05
Inter-day variation (16 replicates at each concentration)					
0.02		0.02	0.002	7.36	109.65
0.06		0.05	0.007	12.43	98.54
1.6		1.63	0.15	9.22	103.56
8.0		7.60	0.55	7.24	97.07

RSD, relative standard deviation (SD × 100/mean).

inter-day precision (% RSD) was 7.36, 12.43, 9.22 and 7.24 at 0.02, 0.06, 1.6 and 8.0 μg/mL, respectively.

Stability

Autosampler and benchtop stability. Over a period of 24 h injection time in the auto-sampler at 5°C and over the bench-top for 6 h period, the predicted concentrations for RST at 0.02, 0.06, 1.6 and 8.0 μg/mL samples deviated within the nominal concentrations. The results were found to be within the assay variability limits (Table 2).

Freeze–thaw stability. Table 2 shows the results of the analyses of the QC samples following three freeze–thaw cycles. RST was shown to be stable in the frozen plasma at –80°C for at least three freeze–thaw cycles (Table 2).

Freezer stability. RST was found to be stable when stored at –80°C for at least one month. Both accuracy and precision of QC samples in this evaluation were within the assay variability of ±15% (Table 2).

Extraction recovery

The results of the comparison of neat standards vs plasma-extracted standards were estimated at 0.06, 1.6 and 8.0 μg/mL concentrations. The absolute recoveries ranged from 85 to 110% across the concentrations. The absolute recovery of internal standard at 10 μg/mL was >100%.

Application of the method

After a single oral administration of 100 mg/kg RST to male Wistar rats, the plasma concentrations of RST were determined by the described method. The mean plasma concentration vs time profiles for RST are depicted in Fig. 3. Inspection of Fig. 3 reveals that the newly developed analytical method has the required sensitivity to characterize the absorption, distribution and elimination phases of RST following oral dosing. The pharmacokinetic parameters were calculated using a non-compartmental analysis. Maximum concentration in plasma (C_{max} 1.98 ± 1.08 μg/mL) was achieved at 0.50 ± 0.00 h (T_{max}). The half-life ($t_{1/2}$) of RST was 11.72 ± 3.27 h, while the $AUC_{(0-\infty)}$ was 12.98 ± 2.78.

CONCLUSION

The assay developed is specific, accurate, precise and reproducible for the analysis of RST in rat plasma. The use of the method can easily enable the characterization of RST pharmacokinetics after single oral dose. The assay can be easily extended to quantitate RST in plasma for routine monitoring of levels of RST in laboratories.

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Table 2. Stability data of RST quality controls in rat plasma

QC (spiked) concentration (μg/mL)	Stability	Mean ± SD[a] (μg/mL), $n = 4$	Accuracy (%)[b]	Precision (%CV)
0.02	0 h (for all)	0.020 ± 0.001		
	3 F/T	0.023 ± 0.001	102.36	5.39
	6 h (BT)	0.020 ± 0.001	91.27	7.33
	6 h (in injector)	0.020 ± 0.001	118	11.0
	30 days at −80°C	0.024 ± 0.001	105.30	2.56
0.06	0 h (for all)	0.060 ± 0.005		
	3 F/T	0.058 ± 0.001	97.15	2.35
	6 h (BT)	0.060 ± 0.005	99.69	7.83
	6 h (in injector)	0.060 ± 0.002	111	4.23
	30 days at −80°C	0.060 ± 0.005	100.44	7.79
1.6	0 h (for all)	1.70 ± 0.13		
	3 F/T	1.67 ± 0.01	98.01	1.07
	6 h (BT)	1.56 ± 0.04	91.56	2.86
	6 h (in injector)	1.72 ± 0.22	107	7.17
	30 days at −80°C	1.60 ± 0.03	94.05	1.96
8.0	0 h (for all)	7.86 ± 0.32		
	3 F/T	7.63 ± 0.04	97.05	0.63
	6 h (BT)	7.70 ± 0.12	97.87	1.67
	6 h (in injector)	7.78 ± 0.46	102	8.19
	30 days at −80°C	7.34 ± 0.45	93.41	6.12

QC, quality control; %CV, coefficient of variation; F/T, freeze–thaw; BT, benchtop.
[a] Back-calculated plasma concentrations.
[b] (Mean assayed concentration/mean assayed concentration at 0 h) × 100.

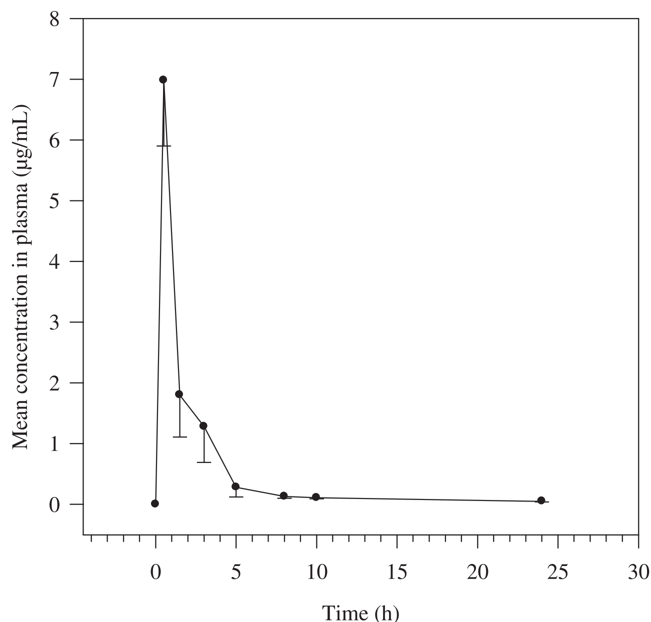

Figure 3. Plasma concentration vs time profiles of RST after single dose oral administration of 100 mg/kg in male Wistar rats. The data points are means and standard deviation bars of four observations.

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