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Simultaneous determination of rosuvastatin and fenofibric acid in human plasma by LC–MS/MS with electrospray ionization: Assay development, validation and application to a clinical study☆

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Abstract

A simple, sensitive and specific LC–MS/MS method for simultaneous determination of rosuvastatin (RST) and fenofibric acid (FFA) was developed and validated with 500 μL human plasma using carbamazepine as an internal standard (IS). The assay procedure involved a simple one-step liquid/liquid extraction of RST and FFA and IS from plasma into ethyl acetate. 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 X-Terra MS C-18 column (4.6 mm × 50 mm, 5.0 μm). Separation of RST, FFA and IS was achieved with a mobile phase consisting of 0.05 M formic acid:acetonitrile (45:55, v/v) at a flow rate of 0.40 ml/min. The API-3000 LC–MS/MS was operated under the multiple reaction-monitoring mode (MRM) using the electrospray ionization technique. Positive ion acquisition chromatographic run was used in the present method. Nominal retention times of RST, FFA and IS were 2.35, 4.70 and 2.32 min, respectively. Absolute recovery of RST, FFA and IS was 74, 61 and 69%, respectively. The lower limit of quantification (LLOQ) of RST and FFA was 1.00 ng/ml and 0.50 μg/ml, respectively. Response function was established for the range of concentrations 1.00–50.0 ng/ml and 0.50–20.0 μg/ml for RST and FFA, respectively, with a coefficient of determination (r^2) of 0.999 for both the compounds. The inter- and intra-day precision in the measurement of RST quality control (QC) samples 5, 15, 400 and 800 ng/ml, were in the range 8.93–9.37% relative standard deviation (R.S.D.) and 1.74–16.1% R.S.D., respectively. Similarly, the inter- and intra-day precision in the measurement of FFA quality control (QC) samples 0.5, 1.5, 8.0 and 15.0 μg/ml, were in the range 9.78–11.6% relative standard deviation (R.S.D.) and 0.22–17.4% R.S.D., respectively. Accuracy in the measurement of QC samples for RST and FFA were in the range 88.1–108 and 87–115%, respectively, of the nominal values. RST and FFA were stable in the battery of stability studies, viz., bench-top, auto-sampler and freeze/thaw cycles. Stability of RST and FFA was established for 1 month at −80 °C. The application of the assay to a clinical study confirmed the utility of the assay.

Keywords: Rosuvastatin; Fenofibric acid; Method validation; LC–MS/MS; Human plasma; Pharmacokinetics

1. Introduction

Rosuvastatin (Crestor®, Fig. 1), new member of a class of cholesterol-lowering drugs commonly referred to as "statins", was approved in the U.S. in August 2003 for the treatment of dyslipidemia [1–3]. 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. RST, a synthetic lipid-lowering agent, is a selective and competitive inhibitor of 3-hydroxy-3-methylglutaryl-coenzyne A (HMG-CoA) reductase, the key rate-limiting enzyme of cholesterol biosynthesis in liver. RST is used to reduce the amounts of LDL cholesterol, total cholesterol, triglycerides and apolipoprotein B in the blood. RST also modestly increases the level of HDL cholesterol in the blood. These actions are important in reducing the risk of atherosclerosis, which in turn can lead to several cardiovascular complications such as heart attack,

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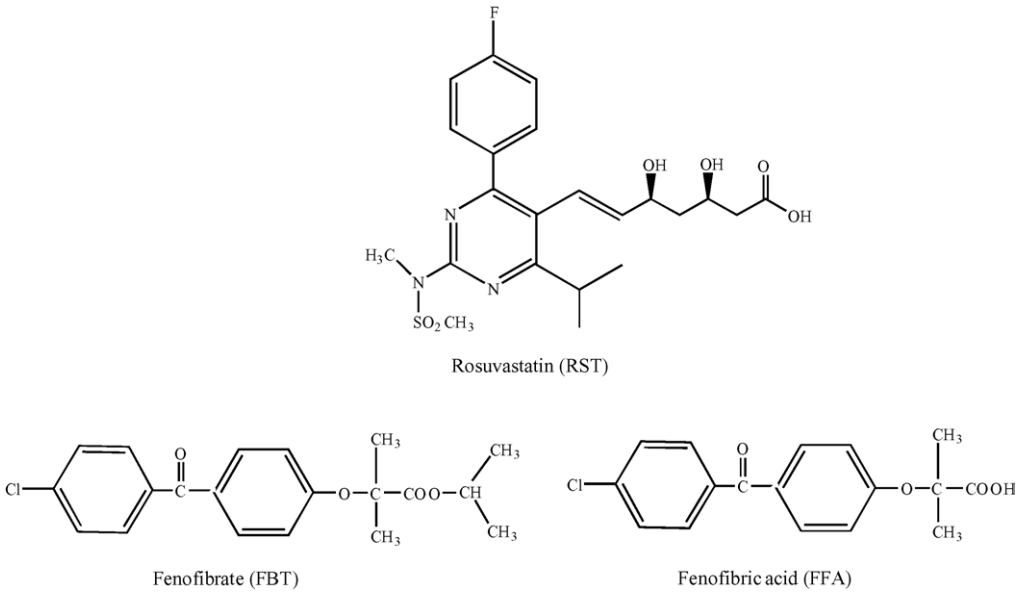

Fig. 1. Structural representation of rosuvastatin, fenofibrate and fenofibric acid.

stroke and peripheral vascular disease. RST peak plasma concentrations were reached by 3–5 h following oral administration in humans [4]. Pharmacokinetic studies in humans using oral doses (5–80 mg) showed that maximum plasma concentrations and areas under the concentration–time curve were approximately linear with dose [5]. The absolute oral bioavailability of RST is around 20% [6]. The elimination half-life was found to be approximately 19 h and steady-state concentration was reached within 4–5 days after dosing. Repeated dosing of RST was to have little or no effect on accumulation of drug in plasma [4,7]. Serum protein binding of RST was around 88% [6]. RST is not extensively metabolized in humans. In spite of the metabolism of RST not being extensive, McCormick et al. [8] have identified *N*-desmethyl rosuvastatin, as the primary metabolite. This metabolite was formed primarily by CYP 2C9 with lesser contributions coming from CYP 2C19 and 3A4 isozymes [8]. Additionally, it was found to be less potent than RST against HMG-CoA reductase inhibitory activity [9]. In a human mass balance study, the recovery of RST was primarily via the fecal route of elimination (90%) while renal excretion was a minor route of elimination (10%). Interestingly, RST pharmacokinetic profile is not affected by food ingestion or the time of dosing [4]. The traditional age and gender pharmacokinetics study revealed that RST (at a dose 40 mg) produced no clinically significant differences between elderly or young subjects, or between males and females were observed [7]. The pharmacokinetics of RST for patients with mild-to-moderate hepatic impairment and healthy subjects were not greatly different [10].

Fenofibrate (Tricor®, Fig. 1) has been widely used drug in the treatment of dyslipidaemia. Fenofibrate (FBT) originally launched in 1975 as a standard formulation is now marketed in over 85 countries. The current formulation of FBT shown an improved bioavailability due to the incorporation of a micronized process in product development [11,12]. Chemically, FBT is 2-[4-(4-chlorobenzoyl) phenoxy]-2-methyl-propanoic acid, 1-methylethyl ester. Fenofibric acid (FFA, Fig. 1), the active metabolite of FBT, contributes for the reductions in total cholesterol, LDL cholesterol, apolipoprotien B, total triglycerides and triglyceride rich lipoprotein [13,14]. In addition, treatment with FBT also resulted in elevation of high-density lipoprotein (HDL) and apoproteins, viz., apoAI and apoAII. The effects of FFA on lipid metabolism are mainly mediated through activation of peroxisome proliferator-activated receptor α [15]. Following oral administration FBT was well absorbed from gastrointestinal tract, and rapidly hydrolyzed by esterases to the active metabolite, FFA. The maximum plasma concentrations of FFA were achieved within 4–5 h following oral dosing of FBT. No unchanged FBT was detected in plasma after oral doing. Serum protein binding of FFA was reported to be around 99% and showed no concentration dependency over the concentrations expected in the therapeutic dose range [16]. The elimination half-life of FFA was around 20 h [17]. FBT is metabolized by CYP 3A4 isozyme to its active metabolite [18]. FFA was reported to be primarily conjugated with glucuronic acid and then excreted in urine [19]. To a small extent FFA was also reduced to a benzhydrol metabolite, which in turn, conjugated with glucuronic acid and was excreted in urine [19]. It has been reported following repeated administration no unexpected accumulation of FFA during pharmacokinetic studies [11].

As RST and FBT act by unique and different mechanism of action in controlling elevated lipid levels in the body,

co-administration of these two drugs was tested in humans for their additive or synergistic benefit [20]. Martin et al. [20] found that there were minimal changes in RST and FFA exposure when RST and fenofibrate were co-administered in male volunteers and both the drugs were well tolerated when given alone or in combination. Till date, there is only one method reported for the estimation of RST in human plasma [21], which utilizes automated solid-phase extraction followed by high-performance liquid chromatography with positive ion Turboionspray tandem mass spectrometry. Several bioanalytical methods exist for quantitation of FFA [22–26]. Hitherto, no LC–MS/MS method is reported for the estimation of FFA. The necessity of developing an LC–MS/MS bioanalytical method did not arise because the therapeutic doses of FBT (160 mg) produced relatively high plasma levels of FFA permitting the employment of HPLC procedure. To the best of our knowledge, currently there is no bioanalytical method reported in literature describing the full details of a complete methodology and validation for simultaneously assaying RST and FFA using LC–MS/MS. The development of an assay for measurement of RST and FFA in plasma poses an inherent challenge of monitoring three-log differential therapeutic concentrations of two agents in a single analytical run. In this paper, we present a simple sensitive and reproducible triple quad mass spectrometric assay with commercially available IS for the simultaneous determination of RST and FFA in human plasma and its application to a clinical study.

2. Experimental

2.1. Chemicals and reagents

Rosuvastatin and fenofibric acid 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, Discovery Research, DRL, Hyderabad. Purity was found to be more than 99% for both the compounds. HPLC grades of acetonitrile, methanol, ethyl acetate and analytical grade of formic acid, ethylene diamine tetra acetic acid disodium salt (EDTA) and phosphoric acid (Analytical reagent grade) were purchased from Qualigens (Glaxo Mumbai, India). Carbamazepine (CAS No. 298-46-4) (IS) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All aqueous solutions including the buffer for the HPLC mobile phase were prepared with Milli Q (Millipore, Milford, MA, USA) grade water. The control human plasma was purchased from Cauvery Diagnostics and Blood Bank (Secunderabad, India).

2.2. Instrumentation and chromatographic conditions

An Agilent (Agilent Technologies, Waldbronn, Germany) 1100 series LC system equipped with degasser (G1322A), isopump (G1310A) and colcom (G1316A) along with Gilson 215 Model liquid handler (Gilson Inc., Middleton, WI, USA) was used to inject 40 μl aliquots of the processed samples on a X-Terra® MS C-18 column (4.6 mm × 50 mm, 5.0 μm) obtained from (Waters, UK), which was kept at ambient temperature. The isocratic mobile phase, a mixture of 0.05 M formic acid and acetonitrile mixture (45:55, v/v) was delivered at 0.4 ml/min into the mass spectrometer ionization chamber.

Quantitation was achieved by MS/MS detection in positive ion modes for analyte and IS, respectively, using a PE Sciex (Foster City, CA, USA) API 3000 mass spectrometer, equipped with a Turboionspray™ interface at 400 °C. The ion spray voltage was set at 5500 V. The common parameters, viz., nebulizer gas, curtain gas and collision gas were set at 7, 9 and 6, respectively. Detection of the ions was performed in the multiple reaction-monitoring (MRM) mode, monitoring the transition of the m/z 482.3 precursor ion to the m/z 258.2 product ion for RST, m/z 319.0 precursor ion to the m/z 233.1 product ion for FFA, m/z 237.1 precursor ion to the m/z 194.1 product ion for IS. Quadrupoles Q1 and Q3 were set on unit resolution. The analytical data were processed by Analyst software (Version 1.3).

2.3. Preparation of stock and standard solutions

Primary stock solutions of RST and FFA for preparation of standard and quality control (QC) samples were prepared from separate weighing. The primary stock solutions of the analytes and IS were prepared in methanol (1.0 mg/ml) and stored at −20 °C, which were found to be stable for one month. Appropriate dilutions were made in methanol for RST and FFA to produce working stock solutions of 1000, 400, 200, 100, 40, 20 ng/ml and 400, 200, 100, 40, 20, 10 μg/ml, respectively, on the day of analysis and these stocks were used to prepare calibration curve (CC). Another set of working stock solutions of RST and FFA were made in methanol (from primary stock) at 800, 300, 60, 20 ng/ml and 300, 160, 30, 10 μg/ml, respectively, for preparation of QC samples. Working stock solutions were stored at approximately 5 °C for a week (data not shown). Individually QC and CC two-in-one working stock solutions of RST and FFA were made before spiking into QC and CC samples accordingly. A working IS solution (2.00 μg/ml) was prepared in methanol. Calibration samples were prepared by spiking 475 μl of control human plasma with the appropriate amount of the analytes (25 μl containing both RST and FFA) and IS (10 μl) on the day of analysis. Samples for the determination of recovery, precision and accuracy were prepared by spiking control human plasma in bulk at appropriate concentrations [1.0 ng/ml (LLOQ), 3.0 ng/ml (QC low), 15.0 ng/ml (QC medium) and 40.0 ng/ml (QC high) for RST and 0.50 μg/ml (LLOQ), 1.50 μg/ml (QC low), 8.00 μg/ml (QC medium) and 15.00 μg/ml (QC high) for FFA] and 500 μl volumes were aliquoted into different tubes and depending on the nature of experiment samples were stored at −80 °C until analysis.

2.4. Sample preparation procedure

To 500 μl of plasma sample, IS solution (10 μl) equivalent to 40.00 ng was added and mixed for 15 s on a cyclomixer (Remi Instruments, Mumbai, India). After the addition of 3.00 ml of ethyl acetate, the mixture was vortexed for 3 min; followed by centrifugation for 5 min at 3200 rpm on a table-top centrifuge (Remi Instruments). The organic layer (2.7 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 40 μl was injected onto LC–MS/MS system.

2.5. Specificity and selectivity

The lack of chromatographic interference from endogenous plasma components was investigated using pooled blank samples and as well as samples obtained from individual volunteer.

2.6. Calibration curve

Calibration curves were acquired by plotting the peak area ratio of analyte (RST or FFA): IS against the nominal concentration of calibration standards. The concentrations used were 1.00, 2.00, 5.00, 10.00, 20.00 and 50.00 ng/ml for RST and 0.50, 1.00, 2.00, 5.00, 10.00 and 20.00 μg/ml for FFA. The results were fitted to linear regression analysis using $1/\chi^2$ as weighting factor. The calibration curve had to have a correlation coefficient (r^2) of 0.99 or better. The acceptance criterion for each back-calculated standard concentration was ±15% deviation from the nominal value except at LLOQ, which was set at ±20% [27,28].

2.7. Precision and accuracy

The intra-day assay precision and accuracy were estimated by analysing four replicates containing RST and FFA at four different QC levels, i.e., 1.00, 3.00, 15.00 and 40.00 ng/ml for RST and 0.50, 1.50, 8.00 and 15.00 μg/ml for FFA. The inter-assay precision was determined by analyzing the four levels QC samples on four different runs. The criteria for acceptability of the data included accuracy within ±15% deviation (DEV) from the nominal values and a precision of within ±15% relative standard deviation (R.S.D.), except for LLOQ, where it should not exceed 20% of CV [28,29].

2.8. Recovery

The recovery of RST, FFA and IS, through the liquid/liquid extraction procedure were determined by comparing the responses of the analytes extracted from replicate QC samples ($n = 4$) with the response of analytes from non-extracted standard solutions at equivalent concentrations [30]. Recoveries of RST and FFA were determined at low, middle and high concentrations (viz., 3.00, 15.00 and 40.00 ng/ml; 1.50, 8.00 and 15.00 μg/ml, respectively) and at the lower limit of quantification (LLOQ) (1.00 ng/ml and 0.5 μg/ml, for RST and FFA, respectively). The recovery of the IS was determined at a single concentration of 40 ng/ml.

2.9. Stability experiments

The stability of RST, FFA and IS in the injection solvent was determined periodically by injecting replicate preparations of processed samples up to 6 h (at ambient laboratory temperature) after the initial injection. The peak areas of the RST, FFA and IS obtained at initial cycle were used as the reference to determine the relative stability of the analytes at subsequent points. Stability of RST and FFA 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 and FFA in human plasma was assessed by analyzing the QC samples stored at −80 °C for at least 1 month. The stability of RST and FFA in human plasma following repeated freeze/thaw cycles was assessed using QC samples spiked with RST and FFA. The samples were stored at −80 °C between freeze/thaw cycles. The stability of RST and FFA 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% R.S.D.), except for LLOQ, where it should not exceed 20% of CV.

2.10. Clinical experiment

Blood samples were obtained from a male volunteer, who was co-administered with 40 mg of RST and 200 mg of FBT tablets. The ethics committee approved the protocol and the patient provided written informed consent. The samples were collected by vein puncture prior to dosage and at designated time points (1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 12 and 24 h) into polypropylene tubes containing EDTA solution as an anti-coagulant. Plasma was harvested by centrifuging the blood using Biofuge (Hereaus, Germany) at 1760 *g* for 5 min. Plasma (500 μl) samples were spiked with IS and processed as described above. Along with clinical samples, QC samples at a low, medium and high concentration were assayed in duplicate and were distributed among calibrators and unknown samples in the analytical run; no more than 33% of the QC samples were greater than ±15% of the nominal concentration. Plasma concentration–time data of RST and FFA were analyzed by non-compartmental methods using software package WinNonlin Version 4.0 (Pharsight Corporation, Mountain View, CA).

Table 1
Matrix effect data for RST in human plasma

Concentration (ng/ml)	Peak area of RST		Peak area of IS		Peak area ratio of RST/IS		Matrix effect (%)
	Set 1	Set 2	Set 1	Set 2	Set 1	Set 2	
1.00	5.92	6.32	170	176	0.04	0.04	103
2.00	13.6	13.4	185	187	0.07	0.07	97.5
5.00	32.3	31.5	208	197	0.16	0.16	103
10.0	56.9	36.6	204	201	0.28	0.32	113
20.0	97.2	103	149	164	0.65	0.63	96.3
50.0	232	251	141	166	1.64	1.511	91.9

Set 1: RST standard in methanol; set 2: RST spiked into post-extracted blank plasma; matrix effect expressed as the ratio of the mean peak area of RST spiked post-extraction (set 2) to the mean peak area of the RST standard (set 1) multiplied by 100.

3. Results and discussion

3.1. Method development, specificity and selectivity

Electrospray MS/MS in MRM mode was used for simultaneous estimation of RST and FFA, since it is a powerful analytical technique for pharmacokinetic studies and provides selectivity, sensitivity and specificity requirements for analytical methods. By the use of MRM, a key capability of tandem mass spectrometers, the selection and quantification of compound-specific ion-pairs enable a reduction in the interference by co-eluting substances and a considerable improvement in assay selectivity. Although, the detection in MS/MS technique is highly specific and sensitive, nevertheless, interference arising from endogenous substances can exist in much higher concentration than the analytes of interest and may co-elute with those affecting the ionization of the analytes leading to high imprecision and loss of sensitivity [31]. The matrix effect on the present method was evaluated by spiking blank plasma extracts across the linearity range and the results were compared with pure samples having similar concentrations (Tables 1 and 2) and found that there was no significant difference for peak responses between these samples.

Since the differential plasma concentrations of RST (1.8–30 ng/ml range) and FFA (5–15 μg/ml range) were almost three-log apart, it was an analytical challenge to manage the sensitivity and peak shape properties of the two analytes. However, detailed efforts in the evaluation of various mobile phases was undertaken to achieve a balanced results in terms of peak shape and sensitivity for the two analytes. The best signal and good ionization was achieved with 0.05 M formic acid:acetonitrile (45:55, v/v) at 0.4 ml/min flow rate. The nominal retention times of RST, FFA and IS under these conditions were 2.35, 4.70 and 2.32 min, respectively. While establishing the method extreme care was taken in optimizing declustering potential and collision energy so that the FFA signal (given the preponderance of its levels) would not artefactedily suppress RST signal. Therefore, adequate sensitivity of RST was achieved in the presence of higher levels of FFA in plasma. The MRM state file parameters were fully optimized for RST, FFA and IS, and are presented in Table 3. The optimized conditions enabled the establishment of LLOQ for RST at 1 ng/ml.

Fig. 2 left panels shows a typical overlaid chromatogram for the control human plasma (free of analyte and IS), human

Table 3
LC–MS/MS parameters for RST, FFA and IS

Parameters	RST	FFA	IS
Turbo gas (l/min)	3	3	3
Declustering potential (DP)	67	57	38
Focusing potential (FP)	180	155	145
Entrance potential (EP)	9	10	12
Collision energy (CE)	45	21	28
Collision cell exit potential (CXP)	14	15	11
Transition pair (Q1 → Q3)	482.3 → 258.2	319.0 → 233.1	237.1 → 194.1

Table 2
Matrix effect data for FFA in human plasma

Concentration (μg/ml)	Peak area of FFA		Peak area of IS		Peak area ratio of FFA/IS		Matrix effect (%)
	Set 1	Set 2	Set 1	Set 2	Set 1	Set 2	
0.50	1.25	1.11	1.73	1.81	0.72	0.61	84.8
1.00	3.19	2.85	1.88	1.84	1.70	1.55	91.3
2.00	7.30	6.82	2.05	1.76	3.54	3.88	110
5.00	19.2	18.5	1.83	1.89	10.5	9.79	93.3
10.0	35.4	32.4	1.49	1.57	23.8	20.6	86.9
20.0	84.8	85.7	1.81	1.74	46.9	49.2	105

Set 1: FFA standard in methanol; set 2: FFA spiked into post-extracted blank plasma; effect expressed as the ratio of the mean peak area of FFA spiked post-extraction (set 2) to the mean peak area of the RST standard (set 1) multiplied by 100.

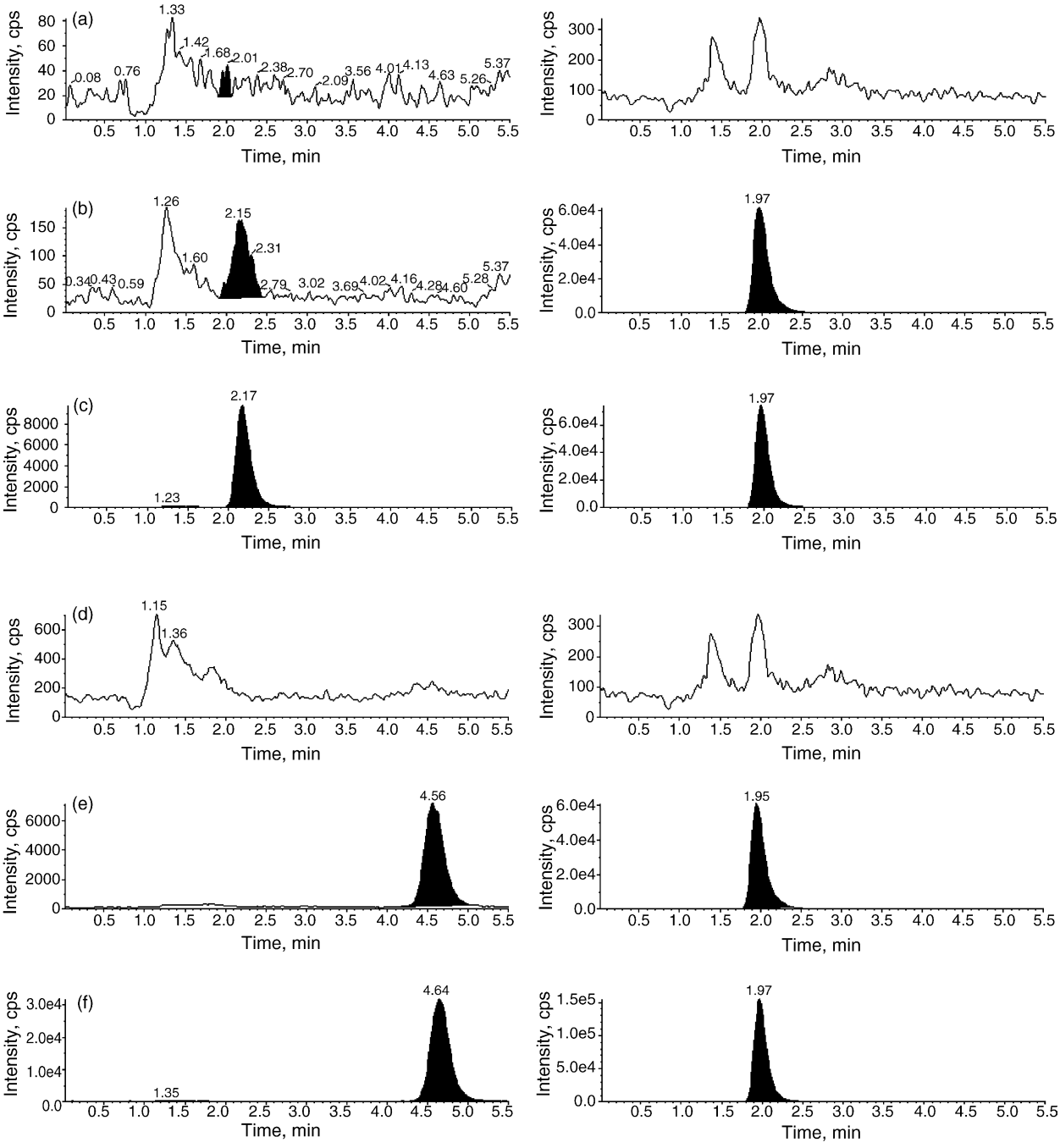

Fig. 2. Chromatograms showing the mass transition of RST (left panels, a–c), FFA (left panels, d–f) and IS (right panels) in (a and d) human blank plasma, (b) human plasma spiked with RST at LLOQ (1 ng/ml), (c) a 4.0 h in vivo plasma sample showing RST peak obtained following oral dose of RST (e) human plasma spiked with FFA at LLOQ (0.5 μg/ml) and (f) a 4.0 h in vivo plasma sample showing FFA peak (released from FBT) obtained following oral dose of RST.

plasma spiked with RST and FFA at their respective LLOQs and an in vivo plasma sample obtained at 4 h after oral co-administration of RST and FBT (FFA was quantified) tablets.

3.2. Calibration curve

The plasma calibration curve was constructed using six calibrators in duplicate (viz., 1.00–50.0 ng/ml for RST and 0.50–20.0 μg/ml for FFA). The standard curve had a reliable reproducibility over the standard concentrations for both the analytes across the calibration range. Calibration curves were prepared by determining the best fit of peak area ratios (peak area analyte/peak area IS) versus concentration, and fitted to the $y = mx + c$ using weighing factor ($1/\chi^2$). The average regression ($n = 4$) was 0.999 for both the analytes. The lowest concentration with the R.S.D. < 20% was taken as LLOQ [28]

and was found to be 1 ng/ml and 0.5 μg/ml for RST and FFA, respectively. The R.S.D. at LLOQ for RST and FFA was found to be 8.95 and 11.1, respectively.

3.3. Accuracy and precision

Accuracy and precision data for intra- and inter-day plasma test samples are presented in Tables 4 and 5. The intra-day accuracy (%) for RST ranged from 88.1 to 110 at 1.00 ng/ml, 90.3 to 106 at 3.00 ng/ml, 93.7 to 109 at 15.0 ng/ml and 92.6 to 108 at 40.0 ng/ml; FFA ranged from 93.8 to 115 at 0.50 μg/ml, 87.2 to 109 at 1.50 μg/ml, 87 to 108 at 8.00 μg/ml and 87.3 to 109 at 15.0 μg/ml. The inter-day accuracy (%) for RST was 103, 102, 103 and 99.7 at 1.00, 3.00, 15.0 and 40.0 ng/ml; FFA was 108, 98.5, 99.9 and 99.1 at 0.50, 1.50, 8.00 and 15.0 μg/ml. The intra-day precision (% R.S.D.) for RST ranged from 1.74 to 7.89 at 1.00 ng/ml, 4.09 to 16.1 at 3.00 ng/ml, 5.23 to 13.2 at 15.0 ng/ml and 4.12 to 8.65 at 40.0 ng/ml; FFA ranged from 2.82 to 17.6 at 0.50 μg/ml, 1.15 to 13.4 at 1.50 μg/ml, 0.22 to 10.3 at 8.00 μg/ml and 1.12 to 9.30 at 15.0 μg/ml. The inter-day precision (% R.S.D.) for RST was 8.95, 9.51, 8.93 and 9.37 at 1.00, 3.00, 15.00 and 40.00 ng/ml; FFA was 11.1, 11.6, 10.6 and 9.78 at 0.50, 1.50, 8.00 and 15.00 μg/ml.

3.4. Recovery

The results of the comparison of neat standards versus plasma-extracted standards were estimated for RST and FFA at 3.00, 15.0, 40.0 ng/ml and 1.50, 8.00, 15.0 μg/ml, respectively. The absolute mean recoveries were 74.61 and 69% for RST and FFA, respectively, across the concentrations. The absolute recovery of IS at 2.0 μg/ml was about 69%.

Table 4
Intra- and inter-day precision of determination of RST in human plasma

Nominal concentration (ng/ml)	Run	Measured concentration (ng/ml)			
		Mean	S.D.	R.S.D.	Accuracy (%)
Intra-day variation (4 replicates in each run)					
1.00	1	0.93	0.02	1.74	92.6
	2	1.10	0.06	5.83	110
	3	1.08	0.05	4.34	108
	4	0.88	0.07	7.89	88.1
3.00	1	3.18	0.15	4.81	106
	2	2.97	0.48	16.1	99.1
	3	2.99	0.14	4.77	99.6
	4	2.71	0.11	4.09	90.3
15.0	1	16.3	1.19	7.27	109
	2	16.0	0.84	5.23	107
	3	14.0	0.90	6.38	93.7
	4	15.3	2.02	13.2	102
40.0	1	43.3	1.78	4.12	108
	2	37.1	3.06	8.26	92.6
	3	39.2	3.39	8.65	98.0
	4	39.3	1.72	4.38	98.2
Inter-day variation (16 replicates at each concentration)					
1.00		1.03	0.09	8.95	103
3.00		3.05	0.29	9.51	102
15.0		15.5	1.38	8.93	103
40.0		39.9	3.74	9.37	99.7

R.S.D.: relative standard deviation (S.D. × 100/mean).

Table 5
Intra-day and inter-day precision of determination of FFA in human plasma

Nominal concentration (μg/ml)	Run	Measured concentration (μg/ml)			
		Mean	S.D.	R.S.D.	Accuracy (%)
Intra-day variation (4 replicates in each run)					
0.50	1	0.57	0.04	7.61	115
	2	0.55	0.10	17.6	111
	3	0.56	0.02	2.82	113
	4	0.47	0.02	3.37	93.8
1.50	1	1.42	0.12	8.14	94.9
	2	1.63	0.10	5.88	109
	3	1.54	0.21	13.4	102
	4	1.31	0.01	1.15	87.2
8.00	1	8.39	0.87	10.3	105
	2	8.66	0.59	6.85	108
	3	7.70	0.55	7.17	96.3
	4	6.96	0.02	0.22	87.0
15.0	1	16.30	0.18	1.12	109
	2	14.30	1.08	7.55	95.3
	3	15.33	1.42	9.30	102
	4	13.10	0.30	2.29	87.3
Inter-day variation (16 replicates at each concentration)					
0.50		0.54	0.06	11.1	108
1.50		1.48	0.17	11.6	98.5
8.00		7.99	0.85	10.6	99.9
15.0		14.8	1.45	9.78	99.1

R.S.D.: relative standard deviation (S.D. × 100/Mean).

3.5. Stability

3.5.1. Auto-sampler and bench-top stability

Over a period of 6 h injection time in the auto-sampler at ambient temperature and over the bench-top for 6 h period, the predicted concentrations for RST and FFA at 1.0, 3.00, 15.0, 40.0 ng/ml and 0.50, 1.50, 8.00, 15.0 μg/ml, respectively, samples deviated within the nominal concentrations. The results were found to be within the assay variability limits (Tables 6 and 7).

3.5.2. Freeze/thaw stability

Tables 6 and 7 shows the results of the analyses of the QC samples following repeated three freeze/thaw cycles. Both RST and FFA were shown to be stable in the frozen plasma at −80 °C for at least three freeze/thaw cycles.

Table 6
Stability data of RST quality controls in human plasma

QC (spiked) concentration (ng/ml)	Stability	Mean ± S.D.[a], $n = 4$ (ng/ml)	Accuracy (%)[b]	Precision (%CV)
1.00	0 h (for all)	0.93 ± 0.02		
	3 F/T	0.89 ± 0.08	95.7	9.35
	6 h (B.T.)	1.00 ± 0.06	108	5.96
	6 h (in-injector)	1.07 ± 0.05	115	5.00
	30 day at −80 °C	0.88 ± 0.07	94.6	7.89
3.00	0 h (for all)	3.18 ± 0.15		
	3 F/T	2.74 ± 0.16	86.2	5.85
	6 h (B.T.)	2.86 ± 0.23	89.9	8.21
	6 h (in-injector)	3.14 ± 0.24	98.7	7.59
	30 day at −80 °C	2.71 ± 0.11	85.2	4.09
15.0	0 h (for all)	16.33 ± 1.99		
	3 F/T	14.33 ± 0.31	87.8	2.29
	6 h (B.T.)	16.01 ± 1.51	98.6	9.41
	6 h (in-injector)	14.15 ± 0.06	86.7	4.26
	30 day at −80 °C	15.30 ± 2.02	93.7	13.2
40.0	0 h (for all)	43.33 ± 1.78		
	3 F/T	39.05 ± 0.78	90.1	1.99
	6 h (B.T.)	43.40 ± 3.02	100	6.96
	6 h (in-injector)	37.08 ± 2.57	85.6	6.94
	30 day at −80 °C	39.27 ± 1.72	90.6	4.38

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

3.5.3. Freezer stability

RST and FFA were 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%.

3.6. Plasma concentration–time profiles

The sensitivity and specificity of the assay were found to be sufficient for accurately characterizing the plasma pharmacokinetics of RST and FFA simultaneously in healthy

Table 7
Stability data of FFA quality controls in human plasma

QC (spiked) concentration (μg/ml)	Stability	Mean ± S.D.[a], $n = 4$ (μg/ml)	Accuracy (%)[b]	Precision (%CV)
0.50	0 h (for all)	0.57 ± 0.04		
	3 F/T	0.51 ± 0.09	89.5	16.9
	6 h (B.T.)	0.58 ± 0.01	102	1.39
	6 h (in-injector)	0.67 ± 0.07	118	11.0
	30 day at −80 °C	0.47 ± 0.02	82.5	3.37
1.50	0 h (for all)	1.42 ± 0.12		
	3 F/T	1.35 ± 0.11	95.1	8.40
	6 h (B.T.)	1.62 ± 0.05	114	3.22
	6 h (in-injector)	1.58 ± 0.07	111	4.23
	30 day at −80 °C	1.31 ± 0.01	92.3	1.15
8.00	0 h (for all)	8.39 ± 0.87		
	3 F/T	7.43 ± 0.41	88.6	5.52
	6 h (B.T.)	8.79 ± 0.29	105	3.29
	6 h (in-injector)	9.00 ± 0.64	107	7.17
	30 day at −80 °C	7.70 ± 0.05	91.8	0.55
15.0	0 h (for all)	16.30 ± 0.18		
	3 F/T	14.18 ± 1.48	90.1	7.90
	6 h (B.T.)	16.53 ± 0.25	101	1.52
	6 h (in-injector)	16.63 ± 1.36	102	8.19
	30 day at −80 °C	15.33 ± 1.42	94.0	9.60

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

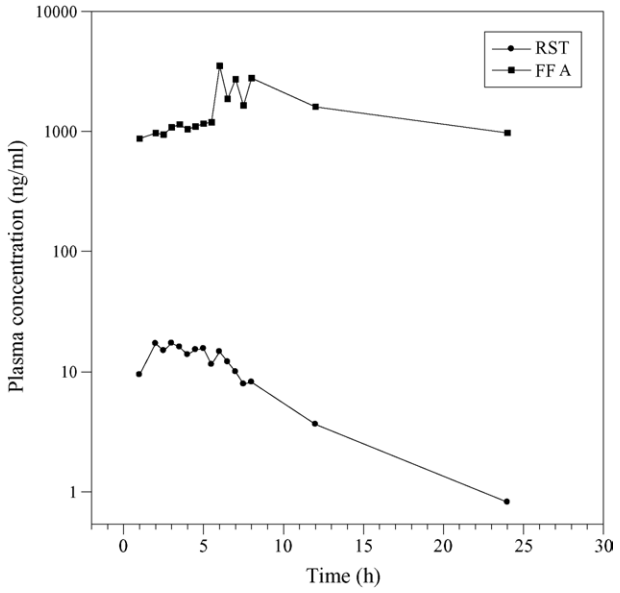

Fig. 3. Plasma concentration–time profiles of RST and FFA (released from FBT) in a volunteer following oral co-administration of RST and FBT tablets.

volunteers. The suitability of the developed method for clinical use was demonstrated by the simultaneous determination of RST and FFA in plasma samples from a volunteer following co-administration of RST (40 mg) and FFA (200 mg) tablets by oral route (Fig. 3). In this volunteer, the peak plasma concentration (C_{max}) of RST and FFA were attained at 3 and 6 h, respectively. The half-life for RST and FFA was found to be 5.0 and 11.5 h, respectively.

4. Conclusion

In summary, the validated LC–MS/MS described herein for the simultaneous determination of RST and FFA in human plasma is specific, accurate, precise and reproducible. In addition, the present method utilizes a single step liquid/liquid extraction method with a commercially available IS. The assay was successfully applied to determine the concentration–time profiles of RST and FFA simultaneously in a clinical pharmacokinetic study.

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