

Rosuvastatin calcium Quantification in Rat Serum with the aid of RP-HPLC: Method Development and Validation.

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Abstract: The objective of the present study is to develop simple, sensitive, accurate and reproducible RP-HPLC method for Rosuvastatin calcium (RST) estimation in rat serum. The developed method has been applied to determine RST concentrations in rat serum samples for a pharmacokinetic study. The procedure involves simple liquid-liquid extraction of RST and internal standard (IS, Atorvastatin) from rat serum directly into acetonitrile which is injected onto a Kromasil KR 100-5C₈ column (4.6 × 250mm, 5µm) at 30°C. Mobile phase consisting of orthophosphoric acid and acetonitrile (45:55, v/v) was used at a flow rate of 1.0mL/min for the separation of RST and IS. The detection of the analyte peak was achieved by monitoring the eluate using a PDA detector set at 241nm. The ratio of peak area of analyte to IS was used for quantitation of serum samples. Retention times of RST and IS were 4.704 and 9.344 min respectively. The standard curve for RST was linear ($r^2 > 0.99$) in the concentration range of 0.1 - 32µg/mL. Absolute recoveries of RST and IS were 90 - 110% and >95% respectively from rat serum. The lower limit of quantitation of RST was 0.03 µg/mL. The inter-day and intra-day precisions in the measurement of different concentrations of 0.5 - 4.0µg/mL were in the range 0.653 - 2.696 % relative standard deviation (RSD) and 0.125 - 1.010 % RSD, respectively. Accuracy in terms of % error in the measurement of different concentration samples was in the range 0.1 - 4.9 of the spiked nominal values. Stability studies results indicated that both analyte and IS were stable on bench top, autosampler and freeze-thaw cycles. Thus, developed method was simple, specific, accurate, precise and robust with good recovery.

Keywords: Method development, Pharmacokinetic study, Rosuvastatin Calcium, RP-HPLC, Validation.

I. Introduction

Atherosclerosis, coronary artery disease, and peripheral vascular disease are caused by hyperlipidemia. It is a disorder of lipid metabolism, also called hyperlipoproteinemia, which results in abnormally high levels of cholesterol, triglycerides, and lipoproteins in the blood circulation. Rosuvastatin [1] is a synthetic lipid lowering agent that blocks the production of cholesterol in the body, effective in lowering LDL cholesterol and triglycerides developed for the treatment of dyslipidemia [2]. It is a reversible competitive inhibitor of HMG-CoA reductase, which prevents or controls the conversion of 3-hydroxy-3-methylglutaryl coenzyme A to mevalonate, a precursor to cholesterol [3, 4]. Chemically rosuvastatin calcium is (3R, 5S, 6E)-7-[4-(4-fluorophenyl)-6-(1-methylethyl)-2-[methyl (methylsulphonylamino)]-5-pyrimidinyl]-3, 5-dihydroxy-6-heptenoic acid calcium [5] and structure elucidated in Fig. 1. Extensive literature survey reveals that few analytical methods were reported for estimation in pharmaceutical dosage forms by HP-TLC [6], UV spectroscopy [7-10], assay by RP-HPLC [11-15], capillary electrophoresis [16], Mass spectrometry [17-19], LC-MS/MS [20] and simultaneous determination with atorvastatin by mass spectrometry [21], a stability-indicating assay method for determination of ROSV in the presence of its degradation products using high performance liquid chromatography [22], stability-indicating RP-UPLC method for ROSV and its related impurities in pharmaceutical dosage form [23] and one method reported for estimation of rosuvastatin in plasma [24]. The limitation with the reported method is tedious sample preparation technique.

The main objective of the study is to establish a simple, sensitive, accurate, reproducible, improved and fast RP-HPLC method for determination of Rosuvastatin calcium in rat serum, which is most recently approved drug in the statin family for treating various dyslipidemic disorders. The method describes the application of the assay to determine the pharmacokinetic disposition after a single oral dose to rats.

II. Materials and Methods

Rosuvastatin calcium and Atorvastatin were kind gift from MSN Laboratories, Hyderabad, India.

2.1 Experimental Methods

The HPLC Waters system Model no. 2695 auto injector, with a PDA detector Model no. 2996 was used for analysis. The data were acquired and processed using EMPOWER 2.0 software. The analytical column was a Kromasil KR 100-5C₈ column 4.6 × 250mm, 5µm particle size. Isocratic mobile phase consisting of orthophosphoric acid and acetonitrile (45:55, v/v) was run at a flow rate of 1.0mL/min. The eluate was monitored by a PDA detector set at 241nm, at which the maximum absorption was observed both for Rosuvastatin and adequate for Atorvastatin (Internal standard).

2.2 Preparation of standard solution

Standard solutions of Rosuvastatin (RST) and Atorvastatin (IS) are made in methanol. Appropriate dilutions of RST were made in methanol to produce working stock solution of 32,16,8,4,2,1,0.5,0.25,0.1µg/ml. The prepared solutions were stored at approximately 5°C. Working stock was used to prepare serum calibration standards. A working IS solution (1µg/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 100µL of control rat serum. Prior to spiking, methanol which is used to prepare standard solutions has to be evaporated by using vacuum oven followed by addition of 100µL of control serum to respective tubes. Samples for the determination of recovery, precision and accuracy were prepared by spiking control rat serum in appropriate concentrations into different tubes, and depending on the nature of the experiments were stored until further analysis.

2.3 Preparation of sample solution

To 100µL of serum sample, 100µL methanolic solution of (IS) was added and mixed on a cyclomixer (Remi instruments, Mumbai, India) for 1min followed by addition of 0.2mL acetonitrile, the mixer was vortex for 10mins followed by centrifugation (Remi instruments, Mumbai, India) for 10mins at 4000rpm. The supernatant was separated and 20µL is injected onto HPLC column.

2.4 Calibration curves

Calibration curves were acquired by plotting the peak area ratio of RST: IS against the nominal concentration of calibration standards.

2.5 Precision and accuracy

The intra-assay precision and accuracy were estimated by analyzing four replicates containing RST at four different levels, i.e. 4,2,1,0.5µg/mL. The inter –assay precision were determined by analyzing the four levels repeatedly for 4 times. The criteria for acceptability of the data included accuracy within ±15% deviation (DEV) from the nominal value and precision within 15% relative standard deviation (RSD) [25, 26].

2.6 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 24hr (in the autosampler at 5°C) after the initial injection. Stability of RST in the biomatrix during 6hr (bench-top) was determined at ambient temperature (25±3°C) at four different concentrations in quadruplicates. The stability of RST in rat serum following repeated freeze-thaw cycles (up to three) was assessed. The criteria for acceptability of the data included accuracy within ±15% deviation (DEV) from the nominal value and precision within 15% relative standard deviation (RSD) [24, 25].

2.7 Extraction recovery

Two sets of standard containing the analyte and IS at three different concentration were prepared, One in rat serum and the other set in methanol. The recovery was determined by comparing peak areas of both.

2.8 Animal study

The study was conducted at Albino research center, with following Registration No. 1722/RO/Ere/S/13/CPCSEA. Male albino wistar rats (180–200 g) used in the study had free access to food and water. Six animals were kept for overnight fasting prior to dosing and were administered with oral suspension of RST in sodium carboxymethyl cellulose (0.25%). At predetermined time intervals, blood samples (250 µL) were collected from retro orbital plexus into microcentrifuge tubes. The blood was allowed to clot, and the serum was separated by centrifugation at 10,000 rpm for 10 min in a microcentrifuge tube and used for further analysis.

2.9 Sample analysis

The serum samples were processed by protein precipitation method. Briefly, 100 μ L of serum sample was treated with 100 μ L of internal standard (1 μ g/mL of atorvastatin in methanol) and vortexed for 1 min. The drug was extracted with 0.2mL of acetonitrile, vortexed for 10mins followed by centrifugation at 4000rpm for 15mins and the separated organic layer was injected onto the HPLC (20 μ L). Rosuvastatin calcium content was quantified using the standard plot.

2.10 Pharmacokinetic parameters

The peak concentration (C_{max}) and its time (T_{max}) were obtained directly from the serum concentration vs. time profile. The area under the curve AUC_{0-t} was calculated by using trapezoidal rule method. The $AUC_{t-\infty}$ was determined by dividing the serum concentration at last time point with elimination rate constant (K_{el}).

III. Results

3.1 Specificity and chromatography

Specificity is defined as the absence of any endogenous interference at retention times of peaks as evaluated by chromatograms of blank rat serum and serum spiked with RST and IS and shown in Fig. 2.

3.2 Calibration curve

Peak area ratios of RST to the IS were measured and calibration graph of peak area ratio (RST to IS) vs. RST concentration was plotted and shown in Fig. 3.

3.3 Accuracy and precision

The data of which is given in the TABLE 1.

3.4 Stability

The data of which is given in the TABLE 2.

3.5 Extraction recovery

The percentage recovery indicates that the extraction method is effective and can be extended for studying drug release profile or in pharmacokinetic study using pharmaceutical formulations.

3.6 Application of the method

After a single oral administration of 10mg/kg RST to rats, the concentrations were determined. The mean serum concentration vs. time profiles for RST is depicted in Fig. 4.

IV. Figures And Tables

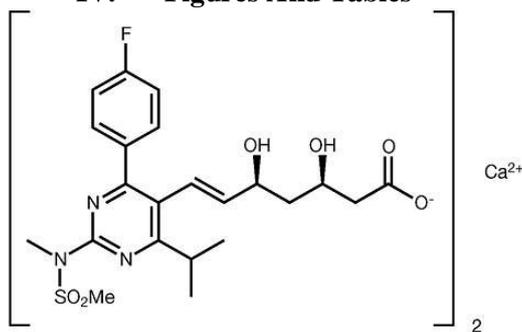


Fig. 1: Chemical Structure of Rosuvastatin calcium.

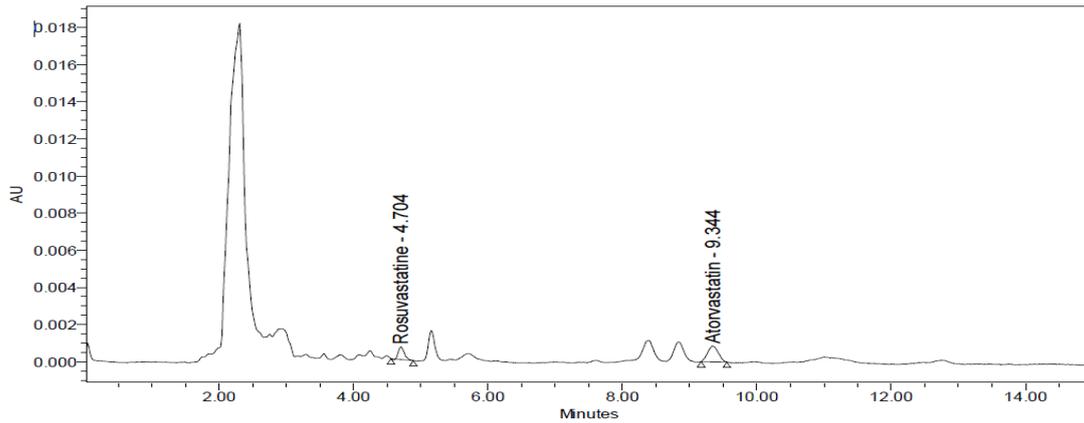


Fig. 2: Chromatograph with drug and internal standard eluted at their respective retention times.

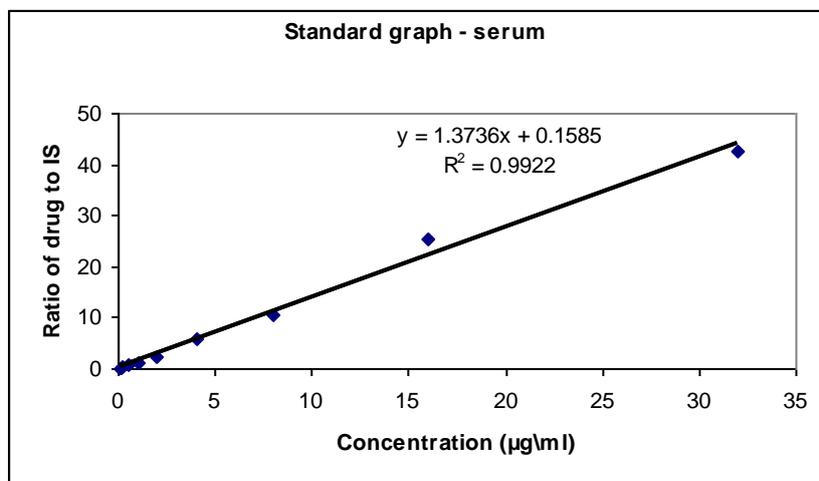


Fig. 3: Calibration curve was plotted against peak area ratio of RST & IS on Y-axis and Concentration on X-axis.

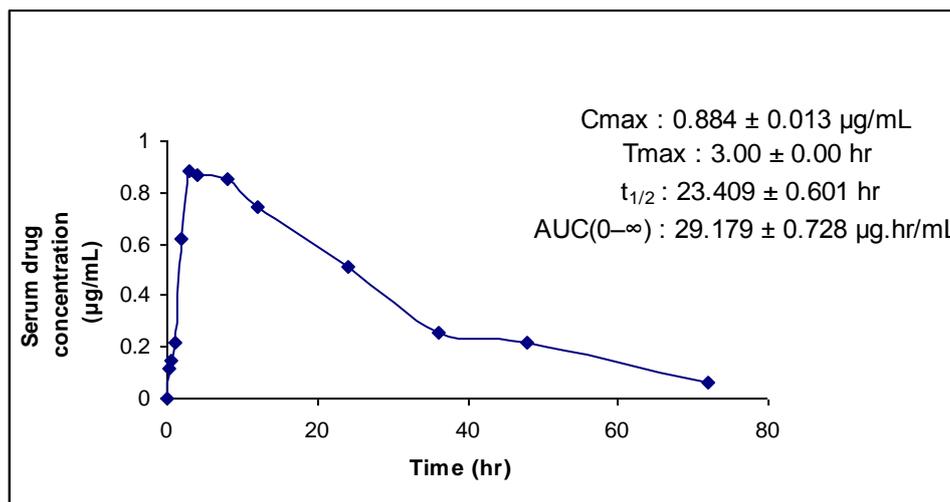


Fig. 4: Pharmacokinetic profiles of Rosuvastatin calcium in serum following oral administration (mean ± SD, n=6)

Table-1: Intra and Inter – day precision of determination of RST in rat serum

Theoretical concentration (µg/mL)	Measured Concentration (µg/mL)			
	Mean	SD	RSD	% error
Intra-day variation (four replicates at each concentration)				
0.5	0.495	0.005	1.010	1.0
1	0.997	0.005	0.501	0.3
2	1.977	0.009	0.455	1.2
4	3.997	0.005	0.125	0.1
Inter-day variation (four replicates at each concentration)				
0.5	0.487	0.005	1.026	2.6
1	0.987	0.009	0.970	1.3
2	1.975	0.0129	0.653	1.3
4	3.805	0.102	2.696	4.9

% Accuracy is expressed in terms of % Error

%Error= (Measured Value-Accepted value/Accepted Value) ×100

RSD= (SD/Mean) ×100

Table-2: Stability Data of RST in rat serum.

Spiked Concentration(µg/mL)	Stability	Mean*± SD (µg/mL) n=4	% Error	Precision (%CV)
0.5	0 h(for all)	0.495±0.005	1.0	1.01
	6h(BT)	0.492±0.0095	1.6	1.92
	6h(Injector)	0.492±0.0095	1.6	1.94
	3 F/T	0.487±0.0095	2.6	1.96
1	0 h(for all)	0.997±0.005	0.3	0.50
	6h(BT)	0.992±0.0095	0.8	0.96
	6h(Injector)	0.985±0.0129	1.5	1.31
	3 F/T	0.987±0.0095	1.3	0.96
2	0 h(for all)	1.977±0.009	1.15	0.45
	6h(BT)	1.98±0.015	1	0.75
	6h(Injector)	1.99±0.0081	0.5	0.41
	3 F/T	1.982±0.015	0.9	0.75
4	0 h(for all)	3.997±0.005	0.075	0.12
	6h(BT)	3.985±0.0173	0.375	0.434
	6h(Injector)	3.977±0.017	0.57	0.427
	3 F/T	3.957±0.017	1.075	0.431

* Mean of 4 replicates. % CV=Coefficient of Variance; F/T= Freeze-Thaw cycles; BT=Bench top

% Accuracy is expressed in terms of % Error.

%Error= (Measured Value-Accepted value/Accepted Value) × 100.

V. Conclusion

The developed method is a simple, sensitive, accurate and reproducible for the analysis of RST in rat serum. The method can be easily extended to quantitate RST in rat serum for routine monitoring of levels of RST. Both the analyte and IS were separated with good resolution and retention time of 4.704 and 9.344 min, respectively. The system suitability parameters were within the USP limits i.e., theoretical plate for RST is 11413 and for IS it is 16478 and USP tailing is 1.29 and 1.26 for RST and IS respectively. The concentration vs. peak area ratio plot was linear ($r^2 > 0.9922$) over the concentration range of 0.1 - 32µg/mL. The criteria for acceptability of the data included accuracy within ±15% deviation (DEV) from the nominal value and precision within 15% relative standard deviation (RSD). The limit of detection and quantification were 10 and 30ng/mL, respectively. The results for bench top, auto sampler and 3 freeze-thaw cycles were all within the nominal concentrations and are found to be within the specified variability of ±15%. The absolute recoveries ranged

from 85 to 110% across the concentration range of 4, 2, 1, 0.5µg/mL. The absolute recovery of internal standard at 1µg/mL was >100%.

Pharmacokinetic study reveals that the newly developed method has the required sensitivity to characterize the absorption, distribution and elimination phases of RST following oral dosing.

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