ORIGINAL ARTICLE

Development, validation of liquid chromatography-tandem mass spectrometry method for simultaneous determination of rosuvastatin and metformin in human plasma and its application to a pharmacokinetic study

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ABSTRACT

A new, simple and accurate liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for simultaneous determination of rosuvastatin (ROS) and metformin (MET) in human plasma was developed. The assay procedure involved simple protein precipitation with acetonitrile. Following precipitation, fraction of supernatant was decanted and evaporated under gentle stream of nitrogen at 40°C. The residue was reconstituted in mobile phase and injected. The chromatographic separation was achieved with Thermo Hypurity C18 column (50 mm × 4.6 mm, 5 μ) using a mobile phase composition containing 0.1% v/v formic acid in water and acetonitrile (30:70, v/v) at a flow rate of 0.4 mL/min. The total run time was 3.5 min. The method showed good linearity in the range 0.5–200 ng/mL for ROS and 2–2000 ng/mL for MET with correlation coefficient (r) >0.9994 for both the analytes. The intra and inter-day precision values for ROS and MET met the acceptance criteria as per regulatory guidelines. The battery of stability studies viz., bench-top, freeze-thaw and long term stability were performed. The developed method was applied to a pharmacokinetic study.

Key words: Liquid chromatography-tandem mass spectrometry, metformin, method validation, pharmacokinetics, rosuvastatin

INTRODUCTION

Diabetes mellitus is a complex long-term metabolic disorder, which induces malfunctioning of cholesterol biosynthesis. As per the current American Diabetes Association guidelines, all the adults with diabetes should be managed to achieve a low density lipoprotein (LDL) cholesterol less than 100 mg/dl employing statins as first-line therapy.[1] Among the existing statins (viz., atorvastatin, simvastatin and pravastatin), rosuvastatin (ROS) [Figure 1] was found to be more effective in reducing LDL cholesterol in hypercholesterolemia patients.[2-4] In general, adults diagnosed with diabetes have high or borderline total cholesterol, hence treatment regimen should include lipid lowering drugs e.g. statins to maintain the cholesterol level and to reduce the risk of heart attack.

Majority of therapies for diabetes includes combination medication with metformin (MET) [Figure 1]. A recent patent application "Pharmaceutical composition comprising MET and ROS" claiming the advantage of reducing side effects caused by statins, enhances safety and patients convenience and compliance with its one-per-day dosage.[5] To study the pharmacokinetics of the new combined formulation, a sensitive and specific method that allows simultaneous determination of ROS and MET in human plasma is needed.

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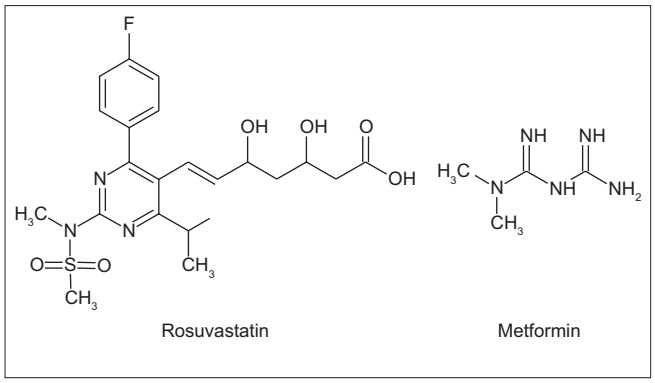

Figure 1: Chemical structures of rosuvastatin and metformin

Literature survey reveals, several liquid chromatography -tandem mass spectrometry (LC-MS/MS) methods have been reported for the determination of ROS[6-12] and MET[13-22] individually or with some other drugs in biological matrices. No methods were traced for simultaneous determination of ROS and MET in biological matrices by LC-MS/MS. In this work we proposed a method for simultaneous determination of ROS and MET in human plasma by LC-MS/MS.

MATERIALS AND METHODS

Standards and reagents

Rosuvastatin, MET hydrochloride, ROS -d6 and MET-d6 reference standards were purchased from Clearsynth Labs Pvt. Ltd., Mumbai, India. Formic acid was purchased from Merck. HPLC grade acetonitrile and methanol were obtained from J.T Baker, USA. Milli-Q water was collected from the Milli-Q system. The control human plasma with K_2 EDTA anticoagulant was procured from Navjeevan Blood Bank, Hyderabad, India.

Instrumentation

A Shimadzu (LC-20AD) HPLC system equipped with degasser, binary pump along with auto-sampler was used to inject samples. The chromatographic separations were performed on a Thermo Hypurity C18 column (50 mm × 4.6 mm, 5 µ) using isocratic mobile phase, a mixture of 0.1% v/v formic acid in water and acetonitrile (30:70, v/v) delivered at a flow rate of 0.4 mL/min.

Quantitation was achieved by MS/MS detection in positive ion mode for both the analytes and internal standards, using an AB Sciex API 4000 mass spectrometer equipped with ESI source. The source temperature and ion spray voltage were set at 400°C and 5000 volts, respectively. The other source dependent parameters viz., nebulizer gas (GS1), drying gas (GS2) and curtain gas, were set at 35, 45 and 20 psi respectively. The compound dependent parameters viz., declustering potential (DP), entrance potential (EP), collision energy (CE) and collision exit potential (CXP) for ROS, ROS-d_6 were 55, 10, 40, 11 V and for MET, MET-d_6, DP, EP, CE and CXP were 50, 10, 25, 9 V respectively. Detection of ions was performed in multiple reaction monitoring (MRM) mode, the mass transitions of m/z 482.1 → 258.1 for ROS, 130.0 → 60.0 for MET, 488.2 → 258.2 for ROS-d6 (IS) and 136.2 → 60.1 for MET-d6 (IS) were used.

Preparation of standard stock solution, calibration and quality control samples

The primary standard stock solutions (1.0 mg/ml) of ROS and MET were prepared in HPLC grade methanol. These stock solutions were successively diluted with 50% methanol in water to prepare combined working solutions of ROS and MET. The appropriate working solution was spiked in to pooled plasma (5% v/v) to give final concentrations of ROS/MET for calibration standards (0.5/2, 1/4, 5/20, 25/100, 50/500, 100/1000, 150/1500 and 200/2000 ng/mL). Quality control samples with following concentrations were prepared at four different levels: 0.5/2, 1.5/6, 75/750 and 170/1700 ng/mL. The individual internal standard stock solutions (0.5 mg/mL) were prepared in methanol and combined (ROS-D6/MET-D6) spiking solution of concentration 300/1000 ng/mL was prepared in 50% methanol in water. All the solutions were stored at 4°C. The calibration and QC samples were stored at −70°C.

Sample processing

To an aliquot of 200 µL human plasma sample in a 2 mL micro-centrifuge tube, 50 µL of diluent containing 300/1000 ng/mL of internal standard (ROS-d_6/MET-d_6) was added and vortex mixed for 30 s. To this sample mixture 750 µL of acetonitrile was added, vortex mixed for 5 min and centrifuged at 14,000 rpm for 5 min. From the supernatant, an aliquot of 0.7 mL of was transferred into a fresh tube and evaporated at 40°C. Samples were reconstituted with 0.2 mL of mobile phase and vortex mixed. Transfer the samples into autosampler vials for injection.

Method validation

The method was validated according to guidance for industry, bioanalytical method validation, USFDA.[23]

Selectivity

The selectivity was evaluated by analyzing six different lots of human plasma to investigate the interference from endogenous plasma components. The acceptance criteria was, at least four out of six lots should have percentage interference <5 of the LLOQ level response in the same matrix.

Calibration curve

The calibration curves were constructed by plotting the peak area ratios of analyte-IS against the nominal concentration of calibration standards in human plasma. The results were fitted to linear regression analysis using $1/x^2$ (x = concentration) as weighing factor. The calibration

curve should have correlation coefficient (r) ≥0.99. The acceptance criteria for each back-calculated standard concentration were ± 15% deviation from the nominal value except at LLOQ, which was set at ± 20%.

Precision and accuracy

The intra-day precision and accuracy was measured by analyzing six replicates at four different levels. The inter-day precision and accuracy was determined by analyzing six replicate samples at four different levels in three different runs. The criteria for acceptability of the data included accuracy within ± 15% deviation from the nominal values and a precision of within ± 15% relative standard deviation (RSD), except for LLQC, where it should not exceed 20%.

Matrix effect

The effect of matrix components over ionization of analytes and IS was determined by comparing the responses of fortified postextracted samples with responses of neat samples at equivalent concentrations. Matrix effect was determined at low and high QC levels with six different blank matrix lots.

Recovery

The recovery was determined by comparing the responses of the analytes and internal standards extracted from six replicate samples at three levels to neat samples (nonextracted) at equivalent concentrations. Recoveries of ROS and MET were determined at 1.5/6, 75/750 and 170/1700 ng/mL. The acceptance criteria for % coefficient of variation (CV) of recovery across concentrations should not exceed 20%.

Stability experiments

The stability of ROS and MET in the biological matrix during storage at ambient temperature (25 ± 3°C) on bench top was determined at low and high concentrations in six replicates. The stability of ROS and MET in human plasma following repeated freeze/thaw cycles was assessed using QC samples, which have undergone three freeze/thaw cycles. Long term stability of analytes in biological matrix was assessed by analyzing QC samples stored at −70°C. Samples were considered stable if assay values were within the acceptable limits of accuracy (i.e. 85–115% from fresh samples) and precision (i.e. ±15% RSD).

RESULTS

Method development

A series of experiments with different LC columns, mobile phase compositions and flow rates were checked to obtain optimal sensitivity, analytical speed and peak shape for both ROS and MET. The resolution of peaks was achieved with 0.1% v/v formic acid in water and acetonitrile (30:70, v/v) at a flow rate of 0.4 mL/min on Thermo Hypurity C18 column (50 mm × 4.6 mm, 5 µ) and was found to be suitable for the quantification of electro-spray response for ROS and MET.

In order to optimize electrospray ionization (ESI) conditions for ROS and MET, quadrupole full scans were carried out in positive ion detection mode. During a direct infusion experiment, the mass spectra for ROS, ROS-d6 and MET, MET-d6 revealed peaks at m/z 482.1, 488.2 and 130.0, 136.2 respectively, as protonated molecular ions, $[M + H]^+$. Following detailed optimization of mass spectrometry conditions, the MRM transitions m/z 482.1 → 258.1, 488.2 → 258.2 were used for quantification of ROS, ROS-d6 and m/z 130.0 → 60.0, 136.2 → 60.1 were used for quantification of MET, MET-d6, respectively.

Method validation

Selectivity

A typical chromatogram for the control human plasma (free of analyte and IS) and human plasma spiked with ROS and MET at LLOQ are shown in Figures 2 and 3, respectively. No interfering peaks from endogenous compounds are observed at the retention times of analytes and IS. The retention times of ROS and ROS-d6 (IS) was 2.0 min, MET and MET-d6 (IS) was 1.4 min. The total chromatographic run time was 3.5 min.

Calibration curve

The plasma calibration curve was constructed using eight calibration standards of 0.5–200 ng/mL for ROS and 2–2000 ng/mL for MET. The results were fitted to $y = mx + c$ using $1/x^2$ weighting factor. The average correlation coefficient ($n = 3$) was found to be ≥ 0.999. The percent accuracy observed for the mean of back-calculated concentration for three calibration curves was within 96.30–104.33 and 98.78–102.30 for ROS and MET respectively. The precision values (%CV) was ranged from 1.10 to 3.04 and 0.35 to 3.56 for ROS and MET respectively.

Precision and accuracy

Accuracy and precision data for intra- and inter-day plasma samples for ROS and MET are presented in Table 1. The assay values on both the occasions (intra- and inter-day) were found to be within the acceptable limits.

Matrix effect

The matrix effect was determined at two levels (low and high QC) with six different blank matrix lots. No significant ion suppression or enhancement of the analyte or IS signal due to endogenous components was observed at the two tested concentrations. The variability expressed as %CV was between 4.5% and 7.4% for ROS and 6.6 and 7.9% for MET at low and high QC levels investigated.

Recovery

Recovery was found to be 95.02 ± 4.01%, 93.66 ± 3.26% and 94.81 ± 4.65% at LQC, MQC and HQC respectively for ROS. For MET at LQC, MQC and HQC was found to be

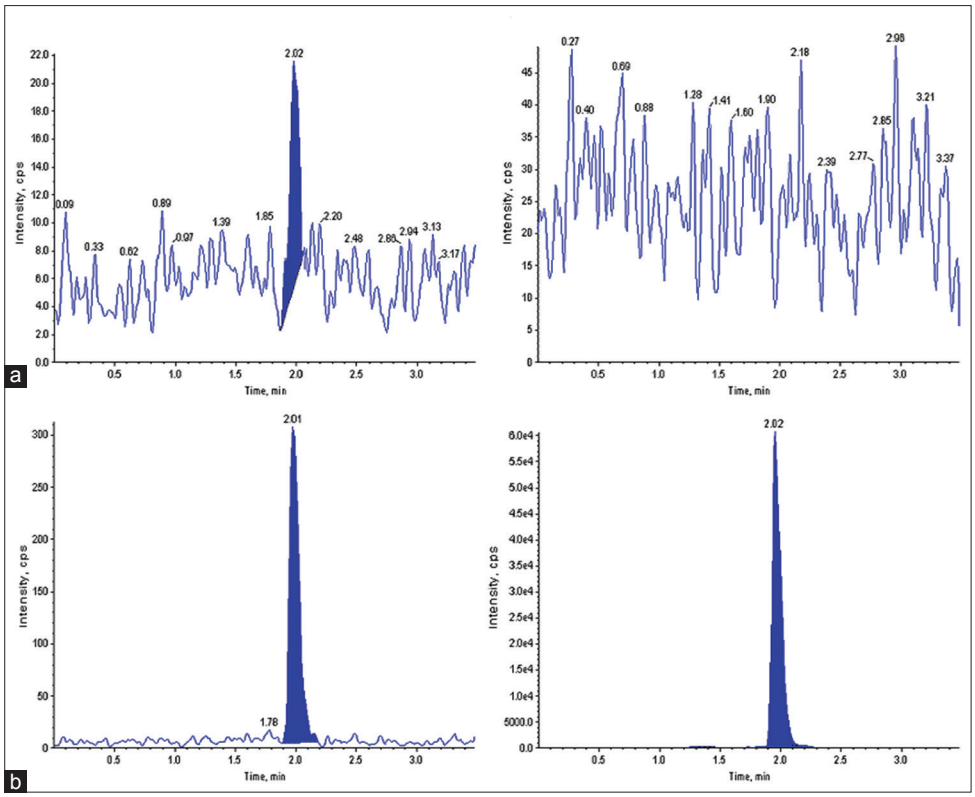

Figure 2: Typical multiple reaction monitoring chromatograms of ROS (left panel) and IS (right panel) in (a) blank human plasma, (b) blank plasma spiked with ROS at LLOQ level

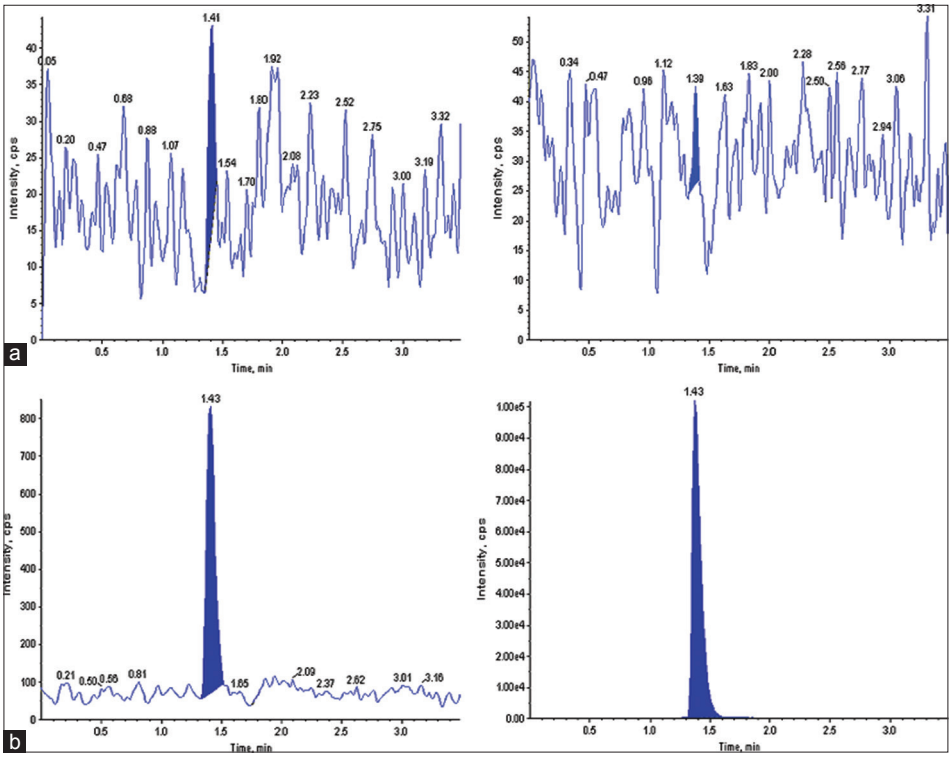

Figure 3: Typical multiple reaction monitoring chromatograms of MET (left panel) and IS (right panel) in (a) blank human plasma, (b) blank plasma spiked with MET at LLOQ level

74.39 ± 2.78%, 76.18 ± 1.75% and 76.78 ± 2.44% respectively. The RSDs for all recoveries were less than 4.9% throughout the concentration ranges for both the analytes.

Stability experiments

The calculated concentrations for both the analytes at LQC and HQC samples was not deviated by ± 15% of the nominal concentrations in a battery of stability tests viz., bench-top (22 h), repeated three freeze/thaw cycles, processed sample (28 h) and long-term storage at − 70°C for at least for 26 days [Tables 2 and 3].

Pharmacokinetic study

The method was applied to the analysis of plasma samples obtained from pharmacokinetic study in rats. ROS and MET were co-administered by oral gavage at a dose of 25 mg/kg each. The detailed pharmacokinetic parameters (C_{max}, T_{max}, area under the curve $[AUC]_{0-t}$ and $AUC_{0-\infty}$) of ROS and MET are presented in Table 4. The pharmacokinetic profile of ROS and MET are presented in Figure 4.

DISCUSSION

To the best of our knowledge, we have developed for the first time the LC-MS/MS method for simultaneous determination of ROS and MET in biological matrix, using economic protein precipitation. Although both the analytes have distant physico-chemical properties, method development was judiciously carried out to fit the analysis of both the analytes in single assay method.

Table 1: Summary of precision and accuracy results of ROS and MET

Analyte	Nominal concentration (ng/mL)	Intra-day (*n*=6)			Inter-day (*n*=18)		
		Calculated concentration (mean±SD, ng/mL)	Precision (% CV)	Accuracy (%)	Calculated concentration (mean±SD, ng/mL)	Precision (% CV)	Accuracy (%)
ROS	0.5	0.492±0.015	3.02	98.39	0.497±0.016	3.22	99.40
	1.5	1.504±0.035	2.31	100.27	1.498±0.052	3.47	99.87
	75	76.839±1.363	1.77	102.45	76.546±1.678	2.19	102.06
	175	171.600±2.84	1.66	98.06	172.560±3.034	1.76	98.61
MET	2	2.007±0.059	2.93	100.33	2.042±0.079	3.87	102.1
	6	5.997±0.152	2.53	99.95	6.002±0.231	3.85	100.03
	750	763.005±16.046	2.13	101.73	759.495±12.235	1.61	101.27
	1750	1722.953±8.791	0.51	98.45	1729.56±7.834	0.45	98.83

ROS: Rosuvastatin, MET: Metformin, SD: Standard deviation, CV: Coefficient of variation

Table 2: Stability data of ROS

Stability experiment	Nominal concentration (ng/mL)	Calculated concentration (*n*=6, mean±SD, ng/mL)	Precision (*n*=6, % CV)	Accuracy (%)
Bench top stability (for 22 h)	1.5	1.487±0.014	0.94	99.13
	175	171.729±3.425	1.99	98.13
Freeze-thaw stability (3 cycles)	1.5	1.493±0.022	1.47	99.53
	175	170.393±4.574	2.68	97.37
Long term stability (at −70°C for 26 days)	1.5	1.488±0.021	1.41	99.2
	175	172.741±4.138	2.4	98.71
Processed sample stability (for 28 h)	1.5	1.496±0.027	1.8	99.73
	175	174.873 ± 3.143	1.8	99.93

SD: Standard deviation, CV: Coefficient of variation, ROS: Rosuvastatin

Table 3: Stability data of MET

Stability experiment	Nominal concentration (ng/mL)	Calculated concentration (*n*=6, mean±SD, ng/mL)	Precision (*n*=6, % CV)	Accuracy (%)
Bench top stability (for 22 h)	6	6.012±0.204	3.39	100.2
	1750	1741.841±5.64	0.9	99.53
Freeze-thaw stability (3 cycles)	6	5.947±0.148	2.49	99.12
	1750	1739.424±15.73	0.9	99.4
Long term stability (at−70°C for 26 days)	6	5.982±0.122	2.04	99.7
	1750	1748.124±10.43	0.6	99.89
Processed sample stability (for 28 h)	6	6.025±0.106	1.76	100.42
	1750	1755.339±14.408	0.82	100.31

SD: Standard deviation, CV: Coefficient of variation, MET: Metformin

Table 4: Mean pharmacokinetic parameters of ROS and MET

Pharmacokinetic parameter	Mean±SD (*n*=3)	
	ROS	MET
C_{max} (µg/mL)	0.5±0.1	2.1±0.3
T_{max} (h)	0.8±0.2	1.4±0.4
AUC_{0-t} (µg h/mL)	5.9±1.8	9.7±3.5
$AUC_{0-\infty}$ (µg h/mL)	6.1±2.2	9.8±3.7

SD: Standard deviation, ROS: Rosuvastatin, MET: Metformin, AUC: Area under the curve

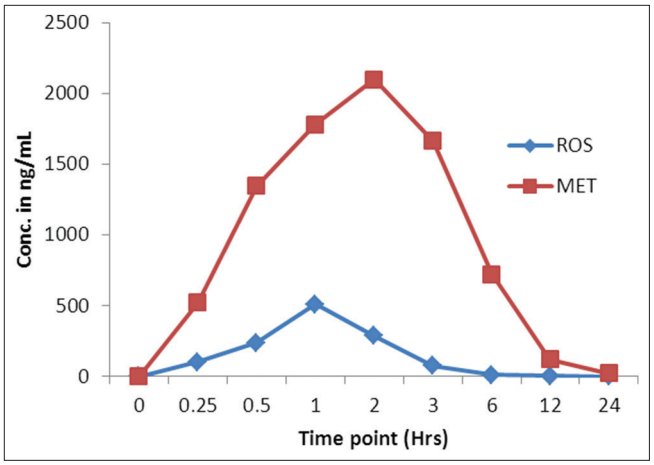

Figure 4: Mean pharmacokinetic profile of rosuvastatin and metformin

The method was validated according to guidelines from regulatory agencies, method showed good reproducibility during the entire process. The method was successfully applied to pharmacokinetic study in rats. From the results of nonclinical pharmacokinetic study, it is evident that, this method can be used for clinical pharmacokinetic study.

CONCLUSION

In summary, the validated LC-MS/MS described herein for the simultaneous determination of ROS and MET in human plasma is specific, accurate, precise, and reproducible. The simultaneous estimation has helped in rapid turnaround time.

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