

Development of a Validated RP-HPLC Method for Simultaneous Estimation of Metformin Hydrochloride and Rosuvastatin Calcium in Bulk and In-House Formulation

Murthy TGK and Geethanjali J*

Bapatla College of Pharmacy, Bapatla, Andhra Pradesh, India

Abstract

A simple, precise, rapid, selective, and economic reversed phase high-performance liquid chromatography (RP-HPLC) method has been established for simultaneous analysis of Metformin hydrochloride and Rosuvastatin in bulk powder and In-House Formulation on a Phenomenex C18 (250×4.6 mm i.d) chromatographic column equilibrated with mobile phase containing Acetonitrile/0.02 M Sodium dihydrogen o-phosphate. Experimental conditions such as pH of mobile phase, ratio of organic phase, flow rate, wavelength, etc. were critically studied and the optimum conditions were selected. Efficient chromatographic separation was achieved with mobile phase containing combination of Phosphate buffer pH 2.8 and Acetonitrile in ratio of 65:35(v/v) adjusted to pH 3.8 at flow rate of 1.0 ml/min and eluents were monitored at 252 nm. 20 µl of sample was injected into chromatographic system and the total run time was 10 min. The retention time for Metformin and Rosuvastatin were 2.147 min and 3.80 min respectively. The method was linear in the range of 5µg mL⁻¹ to 30µg mL⁻¹ and 0.4 µg mL⁻¹ to 2.4µg mL⁻¹ for Metformin and Rosuvastatin respectively. The proposed method was successfully applied to the analysis of Metformin and Rosuvastatin in bulk and in-house formulation without interference from other additives. The developed method was validated according to ICH guidelines. Linearity, regression value, recovery and % RSD of intra and interday precision values were found within the limits and the method was found to be satisfactory. This validated HPLC procedure is economic, sensitive, user-friendly and less time consuming than other chromatographic procedures.

Keywords: RP-HPLC; Rosuvastatin; Metformin

Introduction

Diabetes mellitus, a metabolic disorder characterized by increased blood sugar levels also causes malfunctioning of major organs of the body and even enhances cholesterol biosynthesis and causes dyslipidemia. Hyperglycemia, increased levels of reactive oxygen species, production of advanced glycation end products and glycation of lipoproteins, and lipid abnormalities, such as increases in the levels of VLDL and their remnants, lead to diabetes induced dyslipidaemia. Diabetes is often associated with accelerated atherosclerosis and subsequent cardiovascular disease, people with diabetes are at two to fourfold greater risk of developing cardiovascular disease than people without diabetes, and the majority of type 2 diabetic patients die of atherosclerotic disease [1]. For many patients with diabetes induced dyslipidaemia, monotherapy with an oral antidiabetic agent is not sufficient to reach target glycemic goals and lipid levels therefore, a combination of 2 drugs i.e., an oral antidiabetic drug and an anti hyperlipidemic drug preferably a statin may be necessary to achieve adequate control. In such cases combination of metformin (MET) and Rosuvastatin (ROS) is used that lowers the blood glucose and lipid levels [2]. Metformin is an oral anti-diabetic drug in the biguanide class. It is the first-line drug of choice for the treatment of type-2

diabetes, particularly in overweight and obese people [3]. Rosuvastatin is a selective, competitive inhibitor of HMG-CoA reductase, the rate limiting enzyme that converts 3-hydroxy-3-methylglutaryl-coenzyme A to mevalonate, a precursor of sterols, including cholesterol. Hence, the combination of Rosuvastatin and Metformin complement each other and provides reduction in plasma cholesterol along with glycemic control thereby providing a comprehensive control of diabetes and associated dyslipidemia (Figures 1 and 2).

The literature survey reveals several analytical methods for quantitative estimation of Metformin alone in body fluids and in pharmaceutical formulations. Those methods include spectrophotometry, electrochemical methods, HPLC, liquid chromatography-electrospray ionization tandem mass spectrometry and electrophoresis [3-6]. Rosuvastatin was estimated in body fluids and in pharmaceutical formulations by spectrophotometry, HPLC methods and resonance light scattering technique [7-9]. Formulations containing combination of Metformin and statin derivative such as Atorvastatin are available in market. But the combinations of Metformin and Rosuvastatin are not yet available though this combination can be used to provide comprehensive control of Diabetes and associated

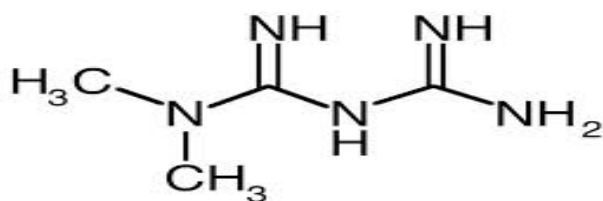


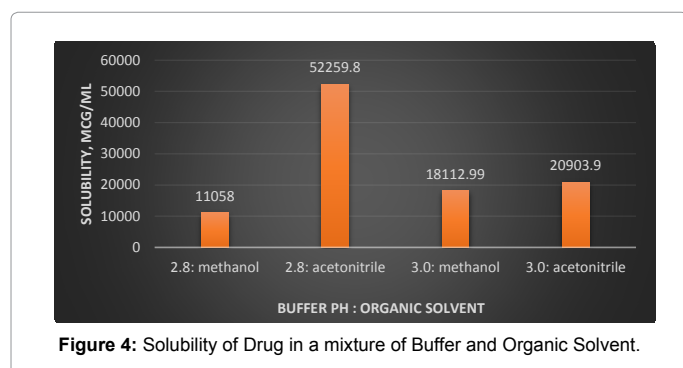
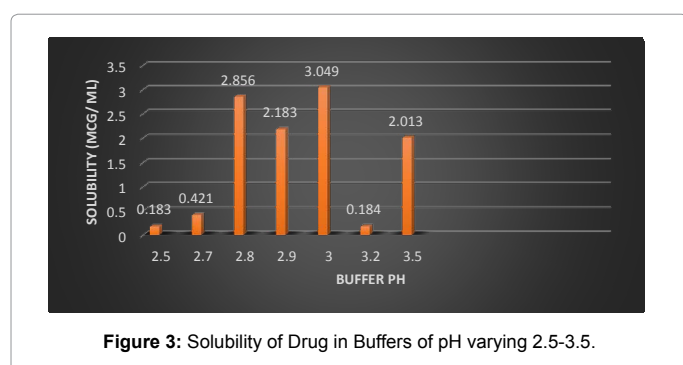
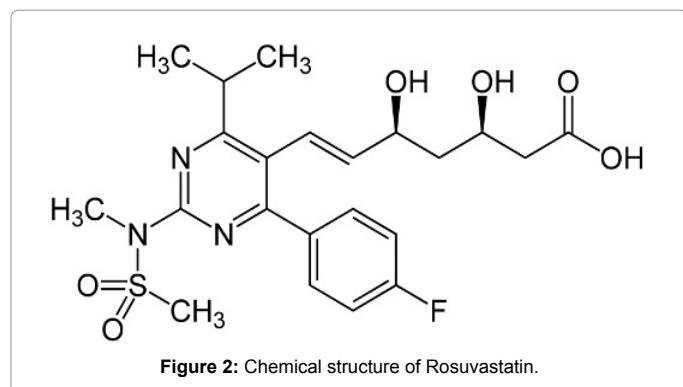
Figure 1: Chemical structure of Metformin.

*Corresponding author: Geethanjali J, Bapatla College of Pharmacy, Pharmaceutical Analysis and Quality Assurance, D.No: 6-10-48/1, Srungarapuram, Bapatla, Guntur district, Bapatla, Andhra Pradesh, 522101 India, Tel: +919494946016/+919440836754; E-mail: geethanjaliyakki@gmail.com

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dyslipidemia. Up to our knowledge, no method was described for the simultaneous estimation of these drugs in bulk or laboratory prepared mixtures. Therefore an attempt was made to develop a simple, accurate isocratic HPLC method for simultaneous determination of Metformin and Rosuvastatin in bulk and In-House formulation.

Materials and Methods

Instrumentation

HPLC system (Agilent technology, 1200 series) consisting of gradient pump, Auto sampler, column oven and photodiode array detector (PDA, Agilent technology) was employed for analysis. The analytical column employed was Phenomenex C₁₈ (250×4.6 mm, 5.0 μm). Data acquisition was performed on EZ Chrom Elite software. An ELICO UV – spectrophotometer SL 164 was used for measuring absorbance of samples. A Mettler Toledo MP 225 pH meter 3510 pH/mV was used for pH adjustment. A vacuum filtration unit was employed to filter the mobile phase through 0.45 μm filter. A degasser, Crest Ultrasonics was used. Systronics electronic balance was used to weigh the samples.

Reagents and chemicals

Metformin Hydrochloride and Rosuvastatin active pharmaceutical ingredient were supplied by Cadila Healthcare Limited (Ahmadabad, India). The HPLC grade Acetonitrile, Methanol and Ortho Phosphoric acid were purchased from Sigma Gmbh, Germany. Analytical reagent grade disodium hydrogen phosphate and triethylamine were used.

Chromatographic Conditions

Column: Phenomenex C₁₈ (250×4.6 mm, 5.0 μm)

Mobile phase: Acetonitrile and Phosphate buffer pH 2.8 in the ratio of 65:35 (total pH of mobile phase was adjusted to 3.8 using triethylamine)

Flow rate: 1.0 ml/min

Wavelength: 252 nm

Column temperature: ambient

Injection volume: 20 μl

Run time: 10 min

Preliminary Studies

Selection of mobile phase: A suitable mobile phase composition was selected based upon maximum solubility of drug in solvents employed viz., buffers and organic solvents like acetonitrile and methanol. Metformin is a hydrophilic drug which is freely soluble in most of organic solvents and buffers. Therefore, a suitable mobile phase was selected based on solubility of Rosuvastatin. Solubility of Rosuvastatin was determined by shake-flask method [10]. In this method, an excess amount of drug was added to the solvent medium so as to make a saturated solution in equilibrium with the solid phase. Then the two phases were separated by filtration and concentration of drug in saturated solution was determined by UV spectrophotometric method. Suitable solvents employed for determining solubility of Rosuvastatin were selected based upon pka of the drug (pka of Rosuvastatin: 4) [11]. Initially the solubility was tested in a series of buffers of pH ranging 2-5 which showed maximum solubility of the drug in buffers of pH 2 and 3. The solubility of drug was then determined in buffers ranging pH 2.5-3.5, which indicated maximum solubility of drug in buffers of pH 2.8 and 3. Solubility of drug was again determined in a mixture of buffer and organic solvent in a ratio of 1:1. The results of solubility studies were represented in the form of graphs (Figures 3 and 4) from the data obtained, it was concluded that Acetonitrile: 2.8 pH buffer was an appropriate mobile phase for simultaneous estimation of Metformin and Rosuvastatin.

Method development and optimization: Several trials were performed using Acetonitrile and 50 mm phosphoric acid (sodium) buffer solution (pH=2.8) in different proportions, under different pH conditions, by varying wavelength and flow rate conditions and a range was obtained for each factor and optimized chromatographic conditions were selected from fractional factorial design. The optimization parameters were listed in (Table 1).

S.NO	FACTOR	RANGE
1.	Mobile phase pH	3.8-3.9
2.	Organic phase proportion	60-65 %v/v
3.	Wavelength	251-253 nm
4.	Flow rate	1.0-1.2 ml/min

Table 1: Optimization parameters.

Formulation	Composition of tablet	Analyte	Label claim (mg)	% label claim estimated (*mean of 6 determinations)	Standard deviation
In-House Tablet formulation	Metformin Hydrochloride -79.6 % Rosuvastatin Calcium - 6.37 %	Metformin	500	98.5 %	0.950 %
	Poly Vinyl Pyrrolidone - 5 % Sodium Starch Glycolate - 5 % Magnesium Sterate - 2 % Talc - 2 %	Rosuvastatin	40	99.2 %	1.547 %

Table 2: Analysis of In-House tablet formulation.

Procedure for analysis of tablets: The tablet containing 500 mg of Metformin and 40 mg of Rosuvastatin were prepared by wet granulation technique. The composition of tablet was given in (Table 2). Not fewer than 10 tablets were weighed and grounded to fine powder. Accurately weighed portion of this powder equivalent to 25mg of Metformin and 2mg of Rosuvastatin and transferred to a 100 mL volumetric flask containing 50 mL of mobile phase. The contents of the flask were allowed to stand for 10 minutes with intermittent sonication to ensure complete solubility of the drugs and made up to volume with mobile phase. The solution was then filtered through a 0.45 µm membrane filter. 1.0 mL of the filtrate solution was transferred into a 10 mL volumetric flask, and volume was made up to the mark with mobile phase. From this solution appropriate dilutions were made with mobile phase to obtain concentration in calibration range for both the drugs and this solution was used for estimation.

With the optimized chromatographic conditions, a steady baseline was recorded, the mixed working standard solution was injected and the chromatogram was recorded. The retention times of Metformin and Rosuvastatin were found to be 2.147 and 3.800 min respectively. The results of analysis shows that the amount of drug was in good agreement with the label claim of formulation (Table 2).

Validation of the Proposed Method

The proposed method was validated according to the International Conference on Harmonization (ICH) guidelines [10-15].

Specificity

The specificity of the method was evaluated by assessing whether excipients present in the formulations interfered with the analysis. A placebo for each tablet was prepared by mixing the respective excipients, and solutions of required concentrations were prepared.

Linearity

It is the ability of the method to obtain test results which are directly proportional to the concentration of analyte in the sample within a given range. Different levels of standard solutions were prepared and injected into the HPLC and the chromatograms were recorded. The peak area corresponding to different concentrations of selected drugs was observed and the data was analyzed for linearity by fitting the data in regression equation, $y = mx + b$.

Accuracy

The accuracy of the method was determined by recovery experiments. Known concentration of working standard was added to the fixed concentration of the pre-analyzed tablet solution. Percent recovery was calculated by comparing the area before and after the addition of working standard. For both the drugs, recovery was performed in the same way. The recovery studies were performed in triplicate. This standard addition method was performed at 80%, 100%, 120% level and the percentage recovery was calculated.

Precision

The precision of the method was demonstrated by inter day and intraday variation studies. In the intraday studies, solutions of standard and sample were repeated thrice in a day and percent relative standard deviation (%RSD) for response factor was calculated. In the interday variation studies, injections of standard and sample solutions were made on three consecutive days and % RSD was calculated.

Limit of detection and limit of quantification

The Limit of detection and quantification were calculated using standard deviation of the response (σ) and slope (S) of calibration curve.

$$\text{Limit of Detection, LOD} = 3.3 \sigma / S$$

$$\text{Limit of quantification, LOQ} = 10 \sigma / S$$

Robustness

Robustness of the method was checked by making slight deliberate changes in chromatographic conditions like mobile phase pH, organic phase ratio, and flow rate and detection wavelength.

Results and Discussion

A simple isocratic high-performance liquid chromatographic method was developed for the determination of Metformin and Rosuvastatin in pure form and in In-House tablet formulations using Phenomenex C column. The mobile phase consisted of acetonitrile and buffer at pH⁸ 3.8 (65: 35 %v/v). The mobile phase was chosen after several trials to reach the optimum stationary/mobile-phase matching. The optimized chromatographic conditions were selected based on results obtained from fractional factorial design.

Fractional factorial design

The efficiency of chromatographic method may be influenced by pH of mobile phase, organic phase composition of mobile phase, flow rate and detection wavelength. So to study the effect of these parameters at 2 different levels on the efficiency of method, fractional factorial design with 3 replications was employed. The performance parameters such as asymmetry, efficiency and resolution of Metformin and Rosuvastatin peaks were observed for each experiment and the results were analyzed with MINITAB 16 version software. The effect of each factor was analyzed by observing the coefficients of polynomial equation and presented in the (Tables 3 and 4).

The basic Polynomial equation is given below:

$$Y = B_0 + B_1 X_1 + B_2 X_2 + B_3 X_3 + B_4 X_4 + B_{12} X_1 X_2 + B_{13} X_1 X_3 + B_{14} X_1 X_4$$

Where,

$$X_1 = \text{pH of mobile phase } B_0 = \text{Constant } B_4 = \text{Coefficient of } X_4$$

$$X_2 = \text{organic phase ratio } B_1 = \text{Coefficient of } X_1, B_{12} = \text{Coefficient of } X_1 \text{ and } X_2$$

PARAMETER	EQUATION
Metformin peak asymmetry	$y=23.9-11.87X_1+3.61X_2+17.65X_3-1.04X_4-0.93X_1X_2-4.78X_1X_3+0.29X_1X_4$
Rosuvastatin peak asymmetry	$y=-45.69+14.28X_1+3.8X_2+38.43X_3-0.93X_4-0.99X_1X_2-10.22X_1X_3+0.23X_1X_4$
Metformin peak efficiency	$y=1474114-376578X_1+5651X_2-13967X_3-7164X_4-1456X_1X_2+3250X_1X_3+1837X_1X_4$
Rosuvastatin peak efficiency	$y=2223749-560236X_1+5697X_2-26259X_3-10115X_4-1484X_1X_2+5991X_1X_3+2565X_1X_4$
Resolution of Metformin & Rosuvastatin peaks	$y=23802-6136X_1-27X_2-272X_3-56.6X_4-7.12X_1X_2+67.32X_1X_3+22.36X_1X_4$

Table 3: Polynomial equations obtained from factorial design.

ANALYTICAL PARAMETER	COEFFICIENTS							
	B ₀	B ₁	B ₂	B ₃	B ₄	B ₁₂	B ₁₃	B ₁₄
Metformin Peak Asymmetry	23.9	-11.9	3.6	17.7	-1.04	-0.9	-4.8	0.92
Rosuvastatin Peak Asymmetry	-45.6	14.2	3.8	38.4	-0.92	-0.98	-10.2	0.23
Metformin Peak Efficiency	1474114	-376578	5651.2	-13967	-7164	-1456	3250	1536
Rosuvastatin Peak Efficiency	2223743	-560238	5697.7	-26259	-10115	-1484	5991	2565
Resolution	23802.7	-6136.8	-27.5	-272	-86.6	4.12	67	22.3

Table 4: Effect of various parameters on different analytical parameters.

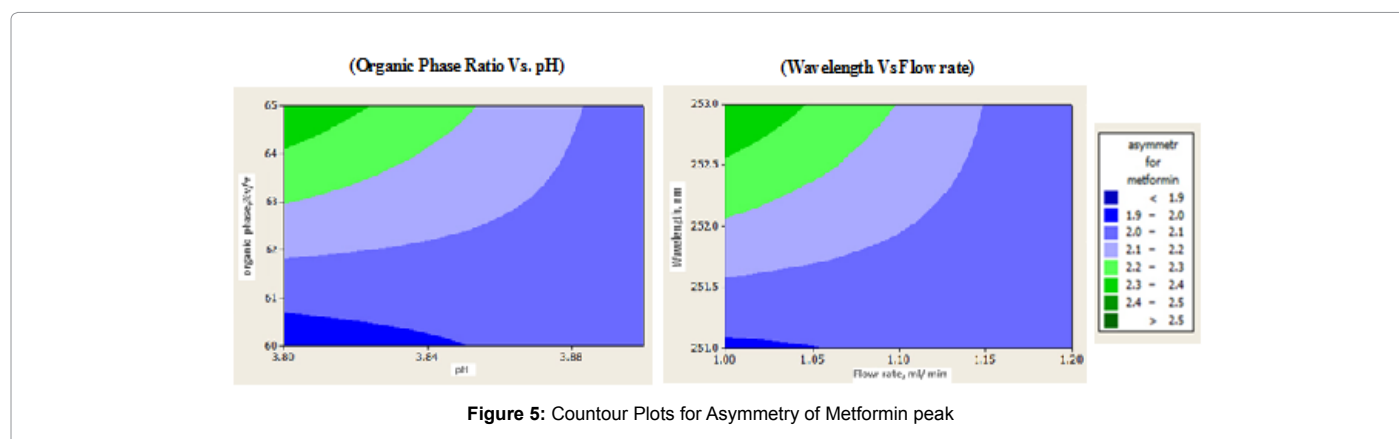


Figure 5: Countour Plots for Asymmetry of Metformin peak

X_3 = flow rate B_2 = Coefficient of X_2 B_{13} = Coefficient of X_1 and X_3

X_4 = detection wavelength B_3 = Coefficient of X_3 B_{14} = Coefficient of X_1 and X_4

From the (Tables 3 and 4), the following conclusions were drawn:

The main factors, pH of the mobile phase and wavelength are having negative effect and organic phase ratio and flow rate are having positive effect on asymmetry of Metformin peak. The interaction effect existing among the factors pH of mobile phase-organic phase ratio, pH of mobile phase- flow rate are having negative effect and the interaction effect existing among the factors pH of mobile phase-detection wavelength was found to have positive effect.

The main factor detection wavelength is having negative effect and the factors, pH of the mobile phase, organic phase ratio and flow rate are having positive effect on asymmetry of Rosuvastatin peak. The interaction effect existing among the factors pH of mobile phase-organic phase ratio, pH of mobile phase- flow rate are having negative effect and the interaction effect existing among the factors pH of mobile phase-detection wavelength was found to have positive effect.

The main factors viz., pH of the mobile phase, flow rate and detection wavelength are having negative effect and organic phase ratio is having positive effect on efficiency of Metformin peak. The interaction effect existing among the factors pH of mobile phase-organic phase ratio is having negative effect and the interaction effect existing among the factors, pH of mobile phase- flow rate, pH of mobile phase-detection wavelength was found to have positive effect.

The main factors viz., pH of mobile phase, flow rate, detection wavelength are having negative effect and factor, organic phase ratio is having positive effect on efficiency of Rosuvastatin peak. The interaction effect existing among the factors pH of mobile phase-organic phase ratio is having negative effect and the interaction effect existing among the factors pH of mobile phase- flow rate, pH of mobile phase-detection wavelength was found to have positive effect.

The main factors viz., pH of mobile phase, organic phase ratio, flow rate, detection wavelength is having negative effect on resolution of Metformin and Rosuvastatin peaks. The interaction effect existing among the factors viz., pH of mobile phase-organic phase ratio, pH of mobile phase- flow rate, pH of mobile phase-detection wavelength was found to have positive effect.

Optimizing of parameters based on countour plots

To optimize the selected factors, the observed response with respect to the selected factors was represented as countour plots (Figures 5-9).

Interpretation obtained from countour plots

The required asymmetry for the Metformin chromatogram peak (<2) is possible by maintaining the pH between 3.8-3.85, organic phase ratio between 60-60.5 %, flow rate at 1.0 ml/ min and wavelength at 251 nm.

The required asymmetry for the Rosuvastatin chromatogram peak (<2) is possible by maintaining the pH between 3.8-3.9, organic phase ratio between 60-64.5 %, flow rate between 1.0-1.2 ml/ min and wavelength between 251-253 nm.

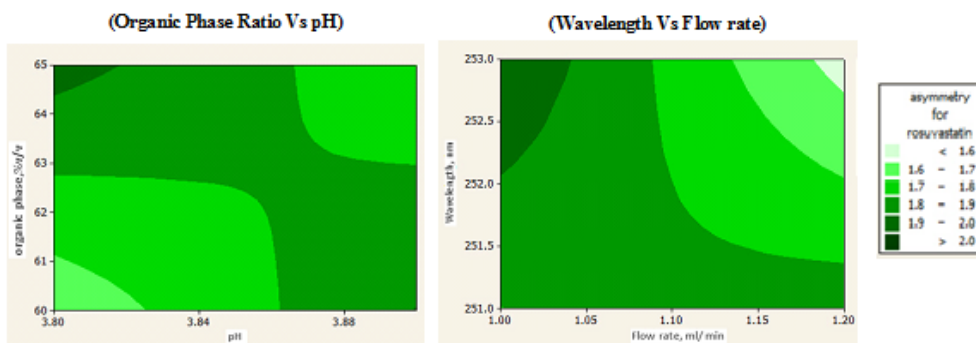


Figure 6: Countour Plots for Asymmetry of Rosuvastatin peak.

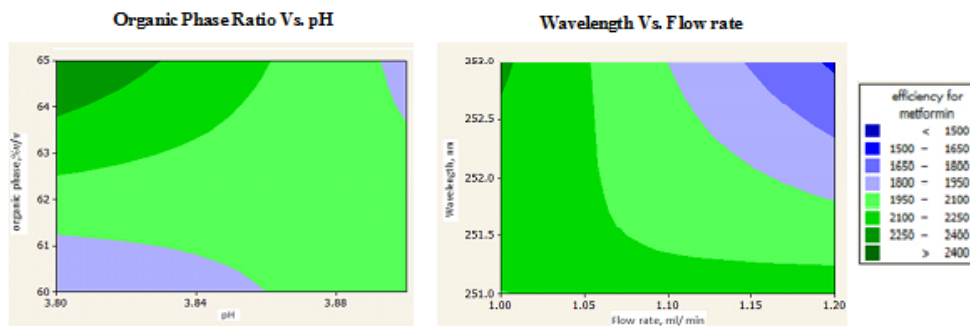


Figure 7: Countour Plots for Efficiency of Metformin peak .

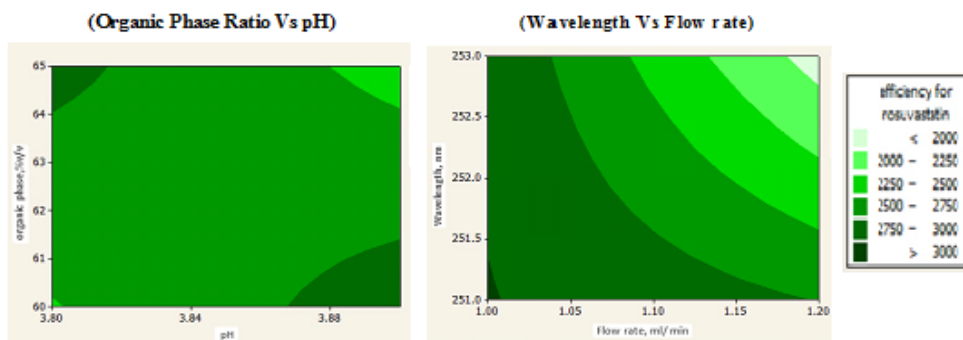


Figure 8: Countour Plots for Efficiency of Rosuvastatin peak.

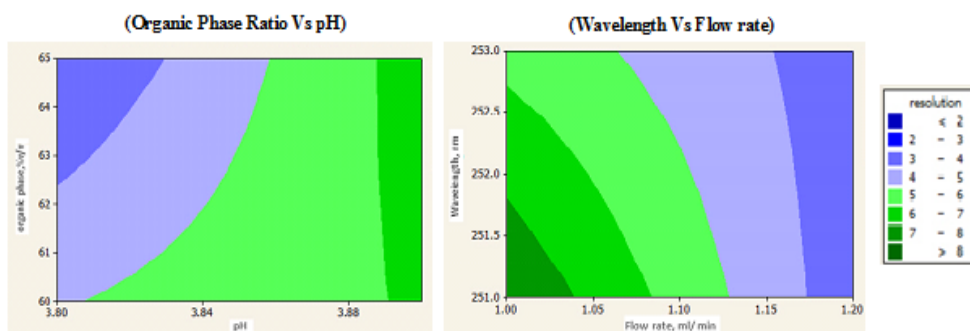


Figure 9: Countour Plots for Resolution of Metformin and Rosuvastatin peaks.

The required efficiency for the Metformin chromatogram peak (>2000) is possible by maintaining the pH between 3.85-3.9, organic phase ratio between 61-65 % v/v, flow rate at 1.0 – 1.1 ml/ min and wavelength between 251-253 nm.

The required efficiency for the Rosuvastatin chromatogram peak (>2000) is possible by maintaining the pH between 3.8-3.9, organic phase ratio between 60-65 % v/v, flow rate at 1.0 – 1.18 ml/ min and wavelength between 251-253 nm.

The required resolution for the Metformin and Rosuvastatin chromatogram peaks (>2) is possible by maintaining the pH between 3.8-3.9, organic phase ratio between 60-65 % v/v, flow rate at 1.0 – 1.2 ml/ min and wavelength between 251-253 nm.

Finally, based on the results obtained from fractional factorial design, Acetonitrile: phosphate buffer (65:35) pH adjusted to 3.8 with triethylamine at a flow rate of 1.0 ml/min was selected as an appropriate mobile phase and eluents were monitored at 252nm which gave good resolution, and acceptable system suitability parameters. The retention times of Metformin and Rosuvastatin were found to be 2.147 min and 3.800 min respectively.

S No	Area (mAU)	Area (mAU)	Other system suitability parameters		
			MET	ROS	
1	388135	80501			
2	390577	80580	USP Tailing	1.282	1.796
3	380323	79560			
4	380211	80508			
5	388563	78032	USP Plate count	6636	9051
6	398378	80520			
Avg	387698.7	79950.17			
StdDev	6837.32	1016.62	USP Resolution	-	3.833
% RSD	1.76	1.27			

S.No.	System Suitability Parameters	RESULTS	
		Metformin	Rosuvastatin
1	Tailing factor(T_r)	1.282	1.796
2	Resolution (Rs)	-	3.833
3	Retention time(Rt)	2.140	3.800
4	Theoretical plates(N)	6636.38	9051.47

Method Validation

The method was specific since excipients in the formulation did not interfere in the estimation of MET and ROS. The proposed method was found to be linear in the concentration range of 5 µg/ml to 30 µg/ml for Metformin and 0.4 µg/ml to 2.4 µg/ml for Rosuvastatin. Accuracy of the method was indicated by recovery values of 98.3 % for Metformin and 99.3 % for Rosuvastatin. Precision is reflected by %RSD values less than 2. The intraday %RSD of Metformin and Rosuvastatin were found to be 0.836 and 0.146 respectively. The interday% RSD for Metformin and Rosuvastatin were found to be 0.577 and 0.767 respectively. From the data obtained the developed RP-HPLC method was found to be precise. The LOD for Metformin and Rosuvastatin were found to be 0.32 µg/ml and 0.05 µg/ml respectively. The LOQ were 0.µg/ml and 0.16 µg/ml for Metformin and Rosuvastatin respectively. It was observed that there were no marked changes in chromatograms obtained by altering mobile phase composition, pH, flow rate and detection wavelength, which demonstrated that the developed RP-HPLC method was robust.

The results of analysis of In-House formulations were shown in (Table 2) and validation parameters were summarized in (Table 5). A typical chromatogram of Metformin and Rosuvastatin observed from formulation is shown in (Figure 10).

PARAMETER	METFORMIN	ROSUVASTATIN
Linearity (µg/ml)	5-30 µg/ml	0.4-2.4 µg/ml
Correlation Coefficient	0.986	0.993
Intra Day Precision (% RSD)	0.836	0.146
Inter Day Precision (% RSD)	0.597	0.767
Accuracy	80 % level	98.01 %
	100 % level	99.02 %
	120 % level	98.00%
Limit Of Detection (µg/ml)	0.32	0.05
Limit Of Quantitation (µg/ml)	0.99	0.16
Robustness	Robust	Robust

Table 5: Summary of Validation Parameters.

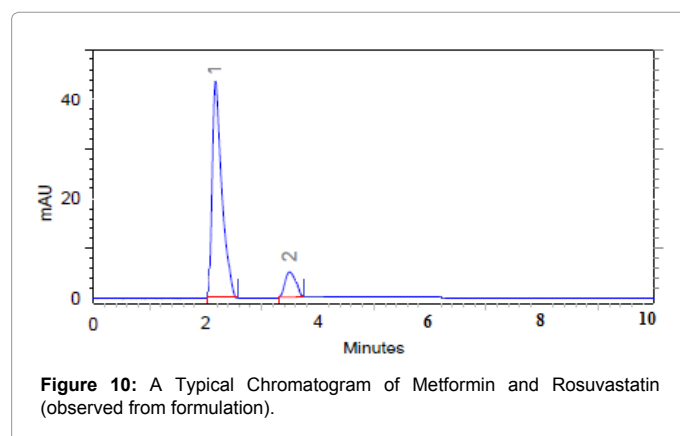


Figure 10: A Typical Chromatogram of Metformin and Rosuvastatin (observed from formulation).

Conclusion

This developed and validated method for simultaneous analysis of Metformin and Rosuvastatin is very rapid, accurate, and precise. The method was successfully applied for determination of MET and ROS in its In-House tablet formulations. The proposed method was simple and did not involve laborious time-consuming sample preparation. Moreover it has advantages of low costs of reagents used, short run time and the possibility of analysis of a large number of samples. Hence this method can be conveniently used for routine analysis and quality control of pharmaceutical preparations containing these drugs either as such or in combination.

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