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DEVELOPMENT AND VALIDATION OF STABILITY-INDICATING RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF ROSUVASTATIN AND GLIBENCLAMIDE

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ABSTRACT

A convenient and rapid reverse phase-HPLC (RP-HPLC) method has been developed and validated for simultaneous estimation of rosuvastatin (RSV) and glibenclamide (GBC) according to international conference on harmonization (ICH) guideline. The separation of these two drugs was attained on C_{18} (ZORBAX Eclipse Plus 4.6 mm×150 mm, 5µm) with isocratic mobile phase containing methanol: acetonitrile: 0.02 M phosphate buffer pH 3.5 (60:20:20 v/v/v) and flow rate 1.0 ml/min with diode array detector at 237 nm. The rosuvastatin and glibenclamide were determined simultaneously with good linearity in a range of 5-22 µg/ml with R^2 value 0.999 and 0.998 respectively. Stability-indicating stress conditions (temperature, acid and hydrogen peroxide) were applied to this method as per ICH guidelines. This method was applied to pharmaceutical formulation (99.29±1.03% for RSV and 99.30±0.13% for GBC) and was also subjected to analysis of rabbit plasma-spiked with rosuvastatin and glibenclamide (recovery 96.97±0.69% for RSV and 97.68±0.05% for GBC). So the proposed RP-HPLC method is effective and accurate for estimation of rosuvastatin and glibenclamide in combined dosage-form and in biological sample.

KEYWORDS: RP-HPLC, rosuvastatin, glibenclamide, stability-indicating, rabbit plasma.

INTRODUCTION

Combination of drugs is widely used now a day. Hence it is essential to develop simple, precise and accurate method for simultaneous determination of drugs in combine dosage from. Reversed-phase high performance liquid chromatography (RP-HPLC) is a well-known technique for the qualitative and quantitative analysis of drugs. It is very useful for simultaneous determination of two or more drugs in pharmaceutical dosage forms. This technique is extensively used in different analytical lab for higher sensitivity and selectivity than other methods such as titration or UV spectrophotometric method. This paper describes a simple reverse phase high performance liquid chromatographic method (RP-HPLC) method for estimation of rosuvastatin (RSV) and glibenclamide (GBC) in tablet dosage form and in rabbit plasma.

Rosuvastatin (RSV) is a member of statin group, selective inhibitor of HMG-CoA reductase [Fig. 1]. It has been used for treatment of lipid abnormalities and cardiovascular disease. In the liver, rosuvastatin increases the number of hepatic LDL receptor on the cell surface and enhances LDL uptake and catabolism. It also inhibits the hepatic synthesis of VLDL, thereby reducing the total number of VLDL particles. [1-2] Literature survey showed that UV spectrophotometric methods have been developed using dye for determination of statins from chloroform extraction [3-4]. Moreover, some methods have been developed based on their absorption maxima with different wavelength. [5-8] High-performance liquid chromatography (HPLC) method has also been developed for identification of statins in pharmaceutical and biological fluid. [9-10] Few RP-HPLC methods for RSV determination have been developed in pharmaceutical dosage and human serum analysis. [11-13] HP-TLC method and chromatography stability indicating methods have also been developed for estimation of RSV or ant diabetic drug in pharmaceutical preparation or biological fluids. [14-18]

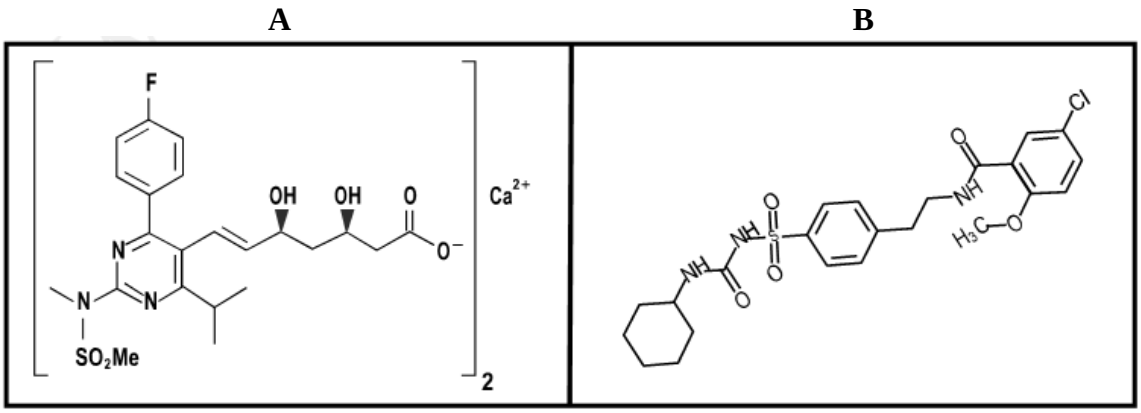

Fig. 1: Chemical structure of (A) rosuvastatin calcium (B) glibenclamide.

Glibenclamide (GBC), known as glyburide, a second generation sulfonyl urea, is used for the treatment of type 2 diabetes mellitus as well as in gestational diabetes.[19] Different HPLC methods had been described for determination of GBC in plasma. In this method the analytes were either injected directly onto the column after suitable extraction from human plasma-spiked with anti-diabetic drugs or internal standard.[20-21] Furthermore, HPLC analytical methods for GBC have been reported using different column and flow rate.[22-25] Moreover, several LC-MS/MS methods have been reported for analysis of sulfonyl ureas using an ion-trap detector in human plasma[26-27] and also for bioequivalence of GBC.[29] Liquid chromatography/tandem mass spectrometry (LC-MS/MS) method has been developed and validated for the simultaneous quantitation of antidiabetic drugs in human plasma.[30-32] However, few RP-HPLC methods were reported for analysis of GBC or combination with other anti-diabetic drug.[33-34] Capillary zone electrophoretic (CZE) methods were also used for quantitative analysis of GBC.[35] But the mentioned methods are little bit expensive and need special instrumentation.

To our knowledge, simple stability-indicating reverse phase isocratic HPLC (RP-HPLC) method for simultaneous estimation of RSV and GBC has not been reported in literature although these two drugs are using simultaneously in different disease conditions. Therefore, the aim of the present study was to develop and validate RP-HPLC method for the analysis of RSV and GBC as per international conference on harmonization (ICH) recommended guidelines.

EXPERIMENTAL

Standards and reagents

Rosuvastatin (potency 99.3%) was obtained from Melody Healthcare Ltd., Mumbai, India. Glibenclamide (potency 99.3%) was gifted from Incepta pharmaceutical, Bangladesh. HPLC grade acetonitrile, methanol, hydrochloric acid, sodium hydroxides were purchased from scharlau, Spain. Potassium dihydrogen phosphate, di potassium hydrogen phosphate and ortho-phosphoric acid were purchased from Merck, Germany. All other chemicals and reagents were used of analytical reagent (AR) grade.

HPLC chromatographic condition

RP-HPLC system was used for this study. The Agilent 1260RP-HPLC system was equipped with a 1260 Bin pump VL, 1260 HiP ALS auto sampler, 1290 thermostat column oven and aprogrammable 1260 DAD VL detector (Agilent technologies, USA). The software for data

analysis was LAB CDS chem Station. The identification of rosuvastatin and glibenclamide were achieved on Agilent C_{18} (ZORBAX Eclipse Plus 4.6 mm×150 mm, 5µm) column as stationary phase. The flow rate and detector were set at 1.0 ml/min and 237 nm respectively. The column temperature was maintained at 30^0 C and injection volume was 20 µL. The total run time was 15 min.

Solution preparation for method development

2.218 g potassium di-hydrogen phosphate (KH_2PO_4) and 3.484 g di-potassium hydrogen phosphate (K_2HPO_4) were accurately weighed dissolved in water to prepare 0.02 M phosphate buffer. Ortho-Phosphoric acid was added to adjust pH 3.5 and final volume was 1000 ml. To prepare isocratic mobile phase methanol, acetonitrile and phosphate buffer were mixed with the ratio of 60:20:20.Then the mobile phase was filtered through 0.45 µm filter with the help of vacuum pump. Acetonitrile and mobile phase were used as diluent to dissolve different samples.

Preparation of rosuvastatin (RSV) and glibenclamide (GBC) stock solution

To prepare rosuvastatin and glibenclamide stock solution 100 mg equivalent of RSV and GBC were weighted and taken into two separate 100 ml clean and dried volumetric flask. 50 ml acetonitrile was added and then mixed by sonication process for 15 minutes and diluted up to 100 ml with acetonitrile (concentration 1mg/mL). Subsequently different concentration of RSV and GBC were prepared by diluting with mobile phase as per requirement in the study.

Method development

For method development, several variable parameters have been studied by changing different types of organic solvent, buffer of different pH, HPLC column, temperature, flow rate and wave length. Mobile phase has been optimized by applying methanol, acetonitrile and 0.02 M phosphate buffer at different ratio to find out the optimum resolution of rosuvastatin and glibenclamide peak at different wavelength, flow rate and pH. The satisfactory resolution and retention time was found when the ratio of methanol: acetonitrile: phosphate buffer was 60:20:20 (v/v/v).

Method validation

RP- HPLC analytical method was validated in compliance to ICH guideline for system suitability, selectivity, accuracy, linearity, limit of detection (LOD) and limit of quantitation (LOQ), precision (repeatability and Intermediate precision) and robustness.

System suitability

System suitability study was conducted by the assay of samples containing RSV and GBC. The samples were prepared by diluting stock solution and injected six times. Average value, SD and % RSD for different parameters (retention time, peak area, theoretical plate, tailing factor and capacity factor) were calculated and compared.

Selectivity

The selectivity of HPLC method was determined by repeated injections of three different concentrations of RSV and GBC (8, 10, 12 µg/mL) with and without placebo. The variations of peak areas were determined by calculating % of RSD of peak areas for selectivity study.

Accuracy

Accuracy of the proposed method was evaluated at three levels of RSV and GBC (80%, 100% and 120% of the working level concentration) with and without placebo, each level were injected in triplicates. Percent recovery (RC) for each concentration was calculated for the determination of accuracy.

Linearity

To establish the linearity of rosuvastatin and glibenclamide standard solutions of RSV and GBC were prepared (5.0-22.0 µg/ml) and injected to get peak area for each solution. A graph was plotted by placing concentration on X-axis versus peak area on Y-axis. The correlation coefficient and Y-intercept for RSV and GBC were then calculated.

LOD and LOQ

Limit of detection (LOD) and limit of quantification (LOQ) of the developed RP-HPLC method were determined as per published method and ICH guideline. [36] To determine LOD and LOQ, blank samples (samples without rosuvastatin and glibenclamide) were injected in triplicate and the peak area of these blank samples was recorded. LOD and LOQ were determined from the slope (S) of the calibration curve and the standard deviation (SD) of the response by using the formulae: LOD = 3.3 × SD/S and LOQ = 10 × SD/S.

Precision

Precision of the proposed RP-HPLC method was determined as intraday and interday precision. Repeatability (intraday) studies were performed by analysis of three different concentrations of RSV and GBC (80%, 100% and 120%) in triplicate on the same day.

Intermediate precision (inter day) of the method was checked by repeating the studies on three different days at same concentration level. Solution of rosuvastatin and glibenclamide was prepared and injected for peak area. Then average area and % RSD were calculated.

Robustness

The robustness of the method was determined to evaluate the effect of deliberate variation of chromatographic conditions on determination of rosuvastatin and glibenclamide. The target concentration RSV and GBC (10 µg/mL) was selected for these studies. Robustness of rosuvastatin and glibenclamide were determined by changing the mobile phase ratio from (60:20:20) to (58:20:22), flow rate from 0.9 to 1.10 ml/min and temperature from 28 to 32^0 C.

Solution stability and forced degradation

Solution stability-indicating method (SSIM) is defined as accurately and precisely measures active ingredients (drug substance or drug product) free from potential interferences like degradation products, process impurities, excipients, or other potential impurities. Stability testing of drug substance accomplished to confirm that their quality does not differ with time under the effect of a diversity of environmental issues for instance temperature, humidity, and light. Furthermore, stability studies were characteristically done using RP-HPLC methods, to conclude the re-test period of a drug substance along with its suggested storage condition according to ICH guideline. To ensure the reliability of the results in relation to handling and storage condition, solution stability studies were performed at target concentration (10 µg/mL) by repeated analysis of the samples over a period of 72 h at ambient temperature (25 ± 1 °C) with relative humidity (60± 5%) and at the refrigerated temperature (5 ± 3) °C.

Acid induced degradation of standard stock solution was taken and transferred into a 50 ml falcon tube for maintaining different condition. Then 5 ml of 0.01 N HCl were added into volumetric flask. Before inject on HPLC, the solution was neutralized. Hydrogen peroxide-induced degradation of standard stock solution was taken and transferred into a 50 ml falcon tube. Then 5 ml of 4% H_2O_2 were added into volumetric flask. Thermal conditions by dry heat and moist heat induced degradation of rosuvastatin and glibenclamide with and without placebo were subjected to the conditions indicated. Stressed condition was maintained at 40 °C and 60 °C for 24h both for rosuvastatin and glibenclamide.

Analysis of RSV and GBC in tablet dosage form

The proposed method was used to determine RSV and GBC in tablets containing 5 mg of rosuvastatin and 5 mg of glibenclamide. Three replicate determinations were carried out for RSV and GBC.

Analysis of RSV and GBC in rabbit plasma

Male white rabbit (body weight 2.5 kg) were used in this experiment. They were kept under the ambient temperature and controlled humidity 12/12 h light-dark cycle. Animals were kept overnight fasting condition before the experiment. The animal experiment plan was followed according to animal care unit University of Asia pacific, Bangladesh. The animal was supposed to place in a clean place for blood collection. BD Syringe was applied to collect blood from the marginal ear vein of rabbit and plasma was collected. The extraction procedure was followed according to published method with slight modification. [37-39] Working solutions (100 µl) of rosuvastatin and glibenclamide were transferred to clean dry centrifugation tubes. Rabbit plasma (500 µl) was added to the tube. Then samples were mixed for 2 min before adding 400 µl of methanol. The supernatant was separated by centrifugation at 5000 rpm for 5 min and loaded into RP-HPLC vials before injecting 20 µl onto the HPLC system. The percent of recovery was calculated.

RESULTS AND DISCUSSION

Method development

Different solvent systems at different ratio were used to develop RP-HPLC method for simultaneous analysis of RSV and GBC with good resolution. Different ratio of phosphate buffer, acetonitrile and methanol were used as the mobile phase. To resolve the problem of poor asymmetric with a greater tailing factor, study was performed to get sharp peak with acceptable asymmetry and good sensitivity by applying the ratio of acetonitrile: methanol: phosphate buffer (60:20:20). Isocratic combination of this ratio was found to get sharp peak, suitable retention time and good asymmetry factor. The wavelength for detection of rosuvastatin and glibenclamide was set at 237 nm considering published reference wavelength of rosuvastatin and glibenclamide.[40-42] Therefore, the final mobile phase ratio of methanol: acetonitrile: phosphate buffer (60:20:20) was used to obtain a rapid and simple assay for rosuvastatin and glibenclamide with a reasonable run time 15 min [Fig. 2].

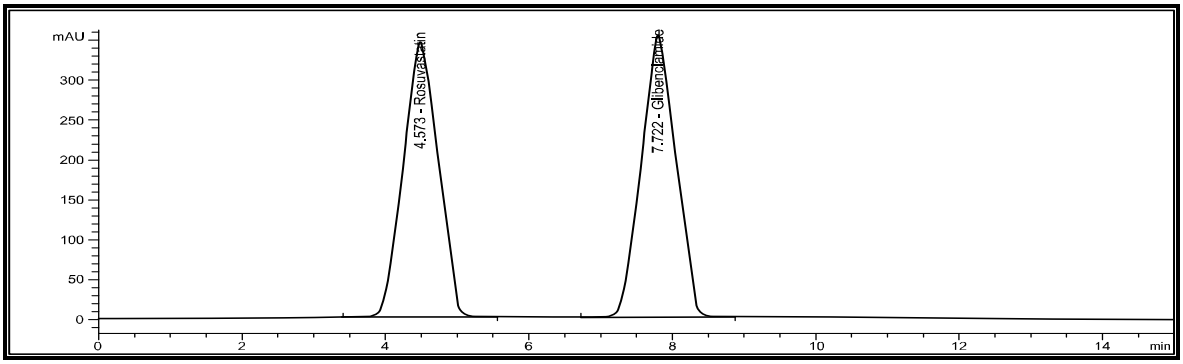

Fig. 2: RP-HPLC peak of rosuvastatin and glibenclamide.

Method validation

Results of system suitability study are shown in Table 1. All the chromatograms showed uniform retention time for rosuvastatin (4.573 min with % RSD 0.029) and for glibenclamide (7.722 min with % RSD 0.055). The mean theoretical plate count, based on USP tangent calculations, was 3352 for rosuvastatin and 5136 for glibenclamide. % RSD is less than 2% for all the parameters.

Table 1: Summary of system suitability.

Parameters	RSV	GBC	Acceptance Criteria	Remarks
Retention Time (% RSD)	0.029	0.055	% RSD (n=6)< 2.00	Complies
Theoretical Plates (N)	3352	5136	Mean (n=6)>2000	Complies
Tailing Factor (TF)	1.33	1.03	Mean (n=6)<2.00	Complies
Capacity Factor (K')	2.12	2.78	K'>2	Complies
Peak Area (% RSD)	0.15	0.06	% RSD (n=6)< 2.00	Complies

RSV= Rosuvastatin; GBC= Glibenclamide

The calibration curve for pure RSV and GBC was found to be linear in the concentration range of 5.0-22.0 µg/ml. The correlation coefficient (R^2) for this calibration was found to be 0.999 and 0.998 respectively. The linear regression parameters for calibration curve of RSV and GBC are shown in table 2.

Table 2: Linear regression analysis for calibration curve of rosuvastatin and glibenclamide.

Parameter	Rosuvastatin	Glibenclamide
Concentration range (µg/mL)	5.0 -22.0	5.0-22.0
Slope	12977	15988
Intercept	21545	19833
Correlation coefficient (R^2)	0.999	0.998
LOD	0.733	0.674
LOQ	2.221	2.044

Limit of detection (LOD); Limit of quantification (LOQ)

LOD and LOQ for the proposed method were determined by the blank response method with the help of SD and slope. The values of LOD and LOQ were found to be 0.733 and 2.22 µg/mL for rosuvastatin and 0.674 and 2.04 µg/mL for glibenclamide respectively.

The selectivity of proposed RP-HPLC method was determined by repeated injections of RSV and GBC at the concentration range of 8-12 µg/mL. The mean peak area and % RSD of area were found satisfactory [Table 3 and 4]. So the method is found selective as placebo did not interfere the peak area and peak position.

Table 3: Selectivity study of rosuvastatin (RSV) and glibenclamide (GBC).

RSV without placebo			RSV with placebo	
Conc.	**peak area**		**Conc.**	**peak area**
8	127827±0.06		8	111649±1.45
10	151222±0.45		10	137692±0.92
12	180510±0.30		12	159182±0.25

Table 4: Selectivity study of glibenclamide (GBC).

GBC without placebo			GBC with placebo	
Conc.	**peak area**		**Conc.**	**peak area**
8	151843±0.32		8	122467±0.56
10	179459±0.34		10	151473±0.10
12	214492±0.85		12	174489±0.96

The accuracy of this method was determined for RSV and GBC by injecting samples with and without placebo at three different concentrations. The % recovery was calculated. Excellent recoveries range in pure form and with placebo was obtained at each concentration level prove that the method is accurate [Table 5].

Table 5: Accuracy for rosuvastatin (RSV) and glibenclamide (GBC).

Conc.	**% Recovery ±SD**			
	RSV without placebo	**RSV with placebo**	**GBC without placebo**	**GBC with placebo**
8	101±0.024	99.82±1.17	99.47±1.20	100.1±0.19
10	98.9 ±0.37	100.15±0.39	98.41 ±0.03	101.16±0.29
12	100 ±0.22	98.32±0.10	101.03±0.64	98.37 ±0.50

The RP-HPLC method was found to be precise for intraday and intermediate precision. The results of intraday and interday/intermediate precision were expressed in terms of area and % RSD at three different percentages 80%, 100% and 120% [Table 6].

Table 6: Precision for rosuvastatin (RSV) and glibenclamide (GBC).

Conc.	Area mean ±% RSD			
	Intraday of RSV	Intraday of GBC	Inter day of RSV	Inter day of GBC
80%	128266±0.48	158319±0.7	126419±0.21	168665±0.64
100%	152339±0.52	176468±0.69	155209±0.33	174019±0.22
120%	181501±0.32	224467±0.88	182921±0.33	231566±0.028

Robustness of proposed RP-HPLC method was studied by changing mobile phase composition, wavelength and flow rate at a concentration level of 10 µg/ml of RSV and GBC. No significance changes in peak area were found. So the method is robust.

Stability-indicating forced degradation

Several degradation products have been anticipated to be formed during formal stability solution testing. Rosuvastatin and glibenclamide was found to be stable when stored for 72 h at ambient temperature (25 ± 1 °C) as well as under refrigeration (2-8 ± 0.5 °C) with mobile phase. More than 99% of rosuvastatin and glibenclamide was found to be unchanged, on the basis of comparison of peak areas with those obtained from a freshly prepared solution of rosuvastatin and glibenclamide. Furthermore, stability-indicating property and specificity of proposed RP-HPLC method was determined by exposing 10 µg/ml of RSV and GBC under various stress conditions. Results of forced degradation studies indicated that RSV and GBC was found to be degraded either slowly or moderately under all stress conditions in the presence of all degradation products. Under acid stress conditions (0.01 N HCl at 60^0 C), 85.74% of rosuvastatin and 83.33% of glibenclamide was found to be remained [Fig. 3]. The rosuvastatin and glibenclamide was also found to be degraded sufficiently under oxidative stress conditions (*i.e.* 4% H_2O_2 at 5^0 C) [Fig. 4]. Under oxidative stress, 90.09% of rosuvastatin and 94.24 % of glibenclamide was unchanged. However under thermal stress conditions 82.12 % for rosuvastatin (60^0 C) and 82.19% for glibenclamide (60^0 C) was unchanged [Fig. 5].

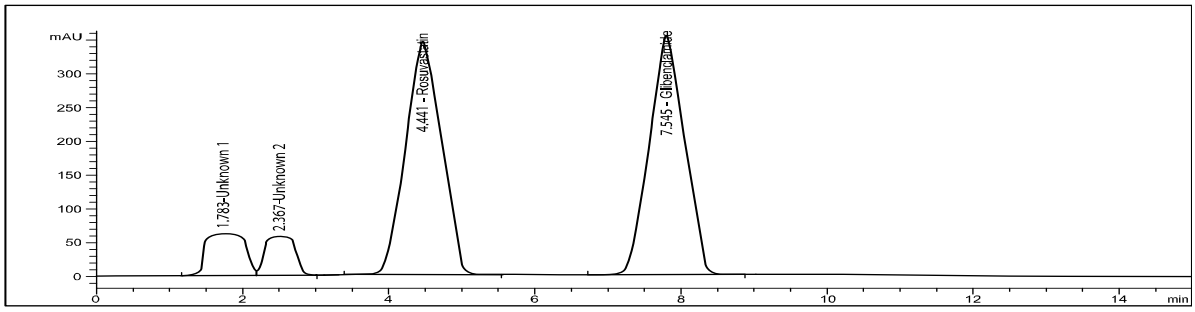

Fig. 3: Peak of rosuvastatin and glibenclamide after acid-induced stress condition (0.01 N HCl at 60 ^{0}C for24 h).

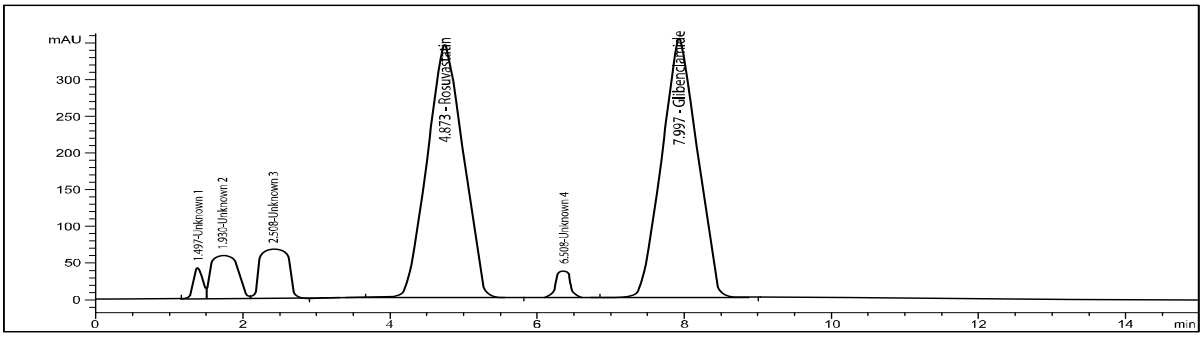

Fig. 4: Peak of rosuvastatin and glibenclamide after hydrogen peroxide-induced stress condition (4% H_2O_2 at 40^0 C for 24 h).

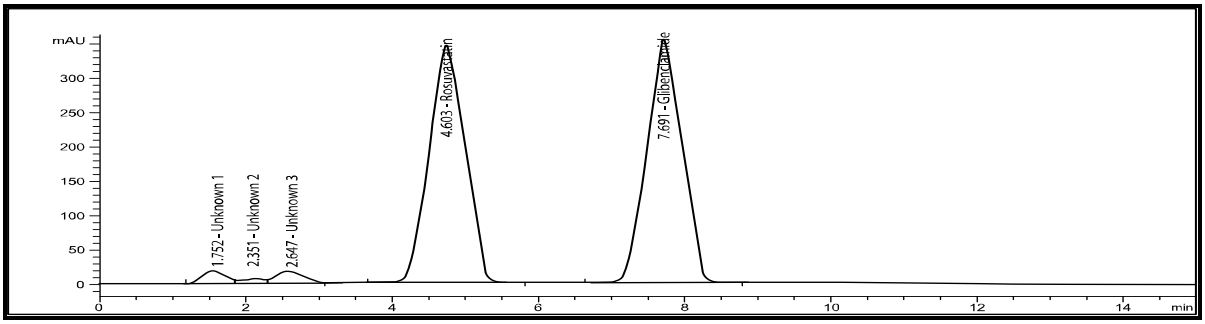

Fig. 5: Peak of rosuvastatin and glibenclamide after temperature and humidity induced stress condition (60 ^{0} C temperatures and 75 % humidity for 24 h).

In all stress condition, the degradation product does not interfere in this method for detection of rosuvastatin and glibenclamide. Overall these results indicated that proposed RP-HPLC method was specific and stability-indicating as it resolved the rosuvastatin and glibenclamide peak in the presence of all degradation products.

Application to commercial tablets and rabbit plasma

The proposed RP-HPLC method was found to be selective, precise, sensitive and stability-indicating for the quantification of rosuvastatin and glibenclamide in pure and dosage form. Furthermore, RP- HPLC method was applied for the quantitative analysis of rosuvastatin and glibenclamide in tablets and rabbit plasma. The amount of rosuvastatin and glibenclamide in tablets was found to be 99.29±1.03% and 99.30±0.13% respectively [Table 7].

Table 7: Determination of Rosuvastatin and Glibenclamide in tablet and rabbit plasma by HPLC.

Market product					Rabbit Plasma		
RSV		GBC			RSV		GBC
Strength (mg)	Potency (%)	Strength (mg)	Potency (%)		Spike Conc.	% Recovery	% Recovery
5	99.29±1.03	5	99.30±0.13		10 µg/ml	96.97±0.69	97.68±0.05

High recovery value of rosuvastatin and glibenclamide in tablets suggested that proposed method is suitable for routine analysis of rosuvastatin and glibenclamide in pharmaceutical dosage forms. There was no interaction between rosuvastatin and glibenclamide and various excipients present in tablets. Moreover, the extraction of rosuvastatin and glibenclamide from rabbit plasma showed suitable % of recovery [Table 7]. There was also no interaction between rosuvastatin and glibenclamide with other ingredients present in rabbit plasma.

CONCLUSION

The proposed stability indicating RP-HPLC method is simple, accurate and precise in analysis. The method is successfully applied for the assay of rosuvastatin and glibenclamide in pure, tablets and biological fluid. The proposed method is also suitable for routine analysis of rosuvastatin and glibenclamide. The proposed method can be applied for prediction of shelf life and half-life of rosuvastatin and glibenclamide in various formulations of commercial application because of its sustainable stability-indicating properties.

COMPETING INTERESTS

The authors declare no conflicts of interest.

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