Original Article

DEVELOPMENT AND VALIDATION OF CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS ESTIMATION OF ROSUVASTATIN AND FENOFIBRIC ACID IN HUMAN PLASMA USING RP-HPLC/UV: OPTIMIZATION OF VARIOUS EXPERIMENTAL PARAMETERS

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ABSTRACT

Objective: A novel, accurate, precise and rapid RP-HPLC method has been developed for the simultaneous estimation of rosuvastatin and fenofibric acid in human plasma using atorvastatin as internal standard.

Methods: The chromatographic separation was achieved on Hibar C18 stationary phase (150mm X 4.6mm i.d; 5μ) with a mobile phase consisting of 0.025mM ammonium acetate pH 4.0 adjusted with glacial acetic acid and acetonitrile in gradient elution mode at a flow rate of 1.2ml/min. The UV detection was carried out at 250nm based on isobestic point of both the drugs. The retention time of rosuvastatin and fenofibric acid were found to be 4.6min and 9.7min respectively.

Results: The developed chromatographic method was validated as per ICH guidelines for linearity, sensitivity, accuracy, precision and recovery. The calibration curves were found to be linear over concentration ranges of 2.0-24ng/ml for rosuvastation and 0.2 – 20μg/ml for fenofibric acid. The limit of detection (LOD) and limit of quantification (LOQ) for rosuvastatin were found to be 1.46ng/ml and 4.44ng/ml respectively while for fenofibric acid were 2.5ng/ml and 7.6ng/ml, respectively.

Conclusion: The proposed method can be successfully applied for the simultaneous analysis of rosuvastatin and fenofibric acid in human plasma for the pharmacokinetic study, bioequivalence, and bioavailability.

Keywords: Fenofibric acid, Human plasma, Rosuvastatin, RP-HPLC, Validation.

INTRODUCTION

Rosuvastatin (Figure.1a), chemically (3*R*,5*S*,6*E*) -7- [4- (4-fluorophenyl) -2- (*N*-methyl methanesulfonamido) -6- (propan-2-yl) pyrimidin-5-yl]-3,5-dihydroxy hept-6-enoic acid calcium salt belong to the class of statins and fenofibric acid (Figure.1b), belong to the fibrate class, which are used as antilipemic agents employed in the combination therapy to reduce hypercholesterolemia in patients with cardiovascular disease and to prevent the risk of developing atherosclerosis. Rosuvastatin acts by inhibiting the hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase that catalyzes the conversion of HMG-CoA to mevalonic acid which is the rate-limiting step in biosynthesis of cholesterol[1-3] on the other hand fenofibric acid is an active of fenofibrate a fibric acid derivative lowers the lipid levels by activating peroxisome proliferator-activated receptor alpha (PPARα) which in turn activates lipoprotein lipase and reduces apoprotein CIII, thus increasing the lipolysis and elimination of triglycerides from plasma. It is official in USP [4] and BPmonographs [5].
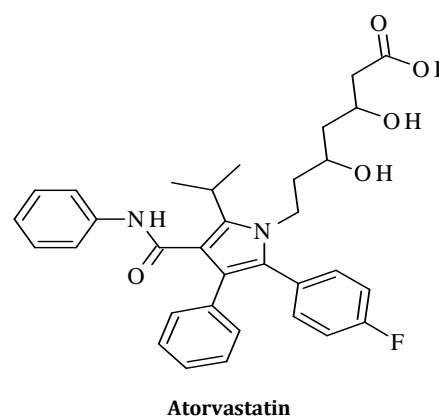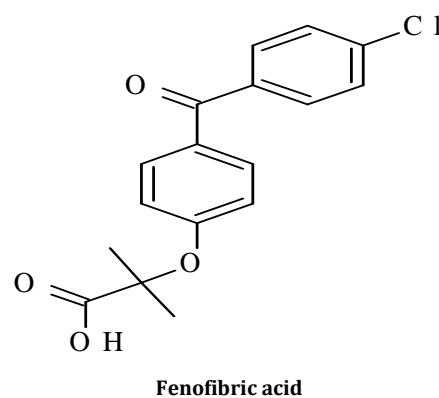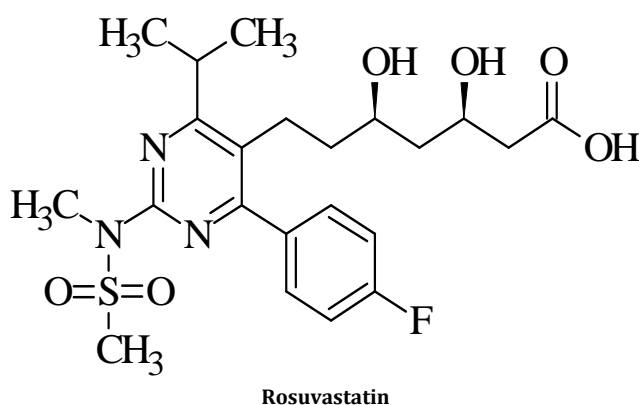

Fenofibric acid

Atorvastatin

Rosuvastatin

Fig. 1: Chemical structures of rosuvastatin, fenofibric acid and atorvastatin (I.S)

Till today many methods have been developed for the estimation of rosuvastatin and fenofibrate either alone or in combination with other drugs in different biological matrices. Liquid chromatography coupled with MS/MS methods reported for estimation of rosuvatstatin in different biological matrices [6-11]either alone or in combination with other drugs such as fenofibric acid [12]. High performance liquid chromatographic methods have also been reported for rosuvastatin in rat plasma [13], in pharmaceutical dosages forms [14], in human serum in combination with atorvastatin [15], timolo maleate, diclofenac sodium in pharmaceuticals and physiological fluids [16] and in human plasma along with the gemfibrozil[17]. HPLC-UV analytical methods have been published for the simultaneous estimation of five statins namely lovastatin, atorvastatin, pravastatin, simvastatin and rosuvastatin in dosage form[18] and in combination with ezetimibe in tablet dosage from [19].

Similarly, fenofibrate has been determined along with its metabolites in various matrices by LC-MS/MS [20], UP-LC/MSMS [21], UPLC stability indicating method for the simultaneous estimation of atorvastatin and fenofibrate and their degradation products in tablets[22], RP-HPLC method for fenofibrate in combination with atorvastatin, metformin in bulk and tablet dosage [23,24]was reported. Spectrophotometric method for the simultaneous estimation of fenofibrate and atorvastatin in bulk and dosage form [25] has also been reported.

As far as of our knowledge no HPLC-UV method has been reported for the simultaneous estimation of rosuvastatin and fenofibric acid in human plasma. So we aimed to develop rapid, precise, sensitive method and its validation according to the international guidelines.

MATERIALS AND METHODS

Materials

Rosuvastatin and fenofibric acid were kindly provided by Zim laboratories, Nagpur, India. Acetonitrile (HPLC grade) was procured from Merck limited (Mumbai India). Ammonium acetate (AR grade) was purchased from SD Fine chemicals (Mumbai, India). Water HPLC grade obtained from Milli-Q RO system was used in preparing of buffer and sample solutions.

Instrumentation

A double beam UV-Visible spectrophotometer (Shimadzu-1700, Japan) was used for Spectral studies. Low pressure gradient high performance liquid chromatographic system from Shimadzu (Kyoto, Japan) equipped with SPD M10 A UV detector, LC-10 AT-VP solvent delivery system, LC-2010 A HT auto sampler, Rheodyne 9725*i* with a loop volume of 100μl. All the data were processed by using Class VP data station. The chromatographic separation was achieved on Hibar C18 column (150mm X 4.6mm; 5μ).Perkin Elmer guard column cartridge RP18 (30mm X4.6mm, 10μm) was used for protection of all the columns.

Optimized chromatographic conditions

All the chromatographic determinations were carried out at ambient temperature. The gradient mode was used for the separation of compounds using 0.025mM ammonium acetate (pH 4.0 adjusted with glacial acetic acid) and acetonitrile as mobile phase. The organic phase was kept at 50% for the first 5 min, increased to 85% for next 5 min and then brought back to 50% in next 3 min. The mobile phase was filtered through 0.45μm nylon membrane filter and degassed using ultra-sonication before use. The injection volume of 20μl was used and the mobile phase flow rate was kept constant at 1.2ml/min. The detection was carried out at 250nm based on the isobestic point of rosuvastatin and fenofibric acid.

Preparation of standard solutions

The standard stock solutions of rosuvastatin, fenofibric acid were prepared by dissolving appropriate amount in acetonitrile and the atorvastatin (I.S) in methanol to obtain a concentration of 1mg/ml. All the standard stock solutions were stored at 2-4°C. The standard stock solutions of rosuvastatin and fenofibric acid were further diluted to give a sequence of standard mixtures ranging

concentrations from 2.0-24ng/ml and 0.2-20μg/ml, respectively. A working standard solution of atorvastatin was also prepared having a fixed concentration 20μg/ml and added to all the standard solution mixtures and plasma samples.

Preparation of sample solutions

A simple protein precipitation method was carried out for the extraction of drugs from human plasma samples. The standard working solutions of rosuvastatin, Fenofibric acid and atorvastatin (0.1ml each) were transferred separately into clean dry centrifuge tubes. Human plasma (0.5ml) was added to each tube. These samples were vortexed for approximately 1min before adding 0.4ml of acetonitrile and centrifuged at 4500rpm for 10min. The supernatant obtained was separated and loaded into the vials before injecting into the HPLC system.

Method validation

The developed bioanalytical method was validated as per United States food and drug administration (USFDA) guidelines [26] with respect to various parameters like linearity, accuracy, precision, recovery, LLOD, LLOQ, specificity/selectivity and robustness.

Specificity/selectivity

Selectivity is the capability of an analytical method to discriminate and quantify the analyte in the presence of other endogenous matrices in the sample and it is investigated by confirming resolution and separation of all desired analytes peaks in the blank human plasma and mobile phase.

Linearity

Linearity of the method is the relationship between detector response and known concentrations of the analytes. The curves were plotted based on expected concentration in the plasma. In the present study calibration range for rosuvastatin and fenofibrate acid were 2.0-24ng/ml and 0.2-20μg/ml respectively was chosen and a working solution of internal standard 20μg/ml were spiked in to the human plasma in sextuplicate. The standard curves for spiked human plasma samples and standard drug solutions were acquired by plotting response factor against the concentrations. The least square regression analysis was applied to determine correlation coefficient (r), slope (m), intercept (c) and standard error (SE).

Precision

Precision of the developed method was determined in terms of injection repeatability and intermediate precision (intra-day and inter-day). The repeatability of the injection performed by spiking 12ng/ml of rosuvastatin and 20μg/ml of fenofibric acid in plasma were injected 10 times into the system and the results were expressed in terms of mean concentration and % rsd values. Similarly, for the intermediate precision (intra-day and inter-day), plasma samples spiked at three different concentrations were analyzed and three times on the same day over three consecutive days and expressed as mean concentration found and % rsd.

Accuracy

Accuracy of the method was determined based on the percent recovery. Blank plasma spiked with the three different concentration levels of analytes (3.6, 7.2, 12ng/ml of rosuvastatin and 4.0, 12, 20μg/ml of fenofibric acid) keeping the internal standard atorvastatin concentration constant (20μg/ml). Another set of standard drug mixtures were prepared at same concentration levels as that of spiked plasma samples in the mobile phase. The analytes were extracted using protein precipitation method and injected onto the system. Percentage recoveries of rosuvastatin and fenofibric acid were calculated using the % Analyte recovery= (X/Y) x 100 Where X is the response ratio of analyte with respect to the I.S in plasma sample, Y is the response ratio of I.S in standard drug mixture.

LLOD and LLOQ

Lower limit of detection and quantification were determined based on the standard deviation of the response and the slope. Detection limit and quantification limit were calculated by ((3.3x σ)/S and (10x σ)/S

respectively. Where 'σ' is the standard deviation of the response and 'S' is the slope of the calibration curve.

Stability

The stability of rosuvastatin and fenofibric acid spiked plasma were carried out over a period of 72hrs at 20°C (room temperature), 2-8°C (refrigeration temperature) and -70°C (deep freezer) and standard drug solutions for a month at 2-8°C.

Robustness

Robustness of the developed bioanalytical method was investigated by studying the influence of minor deliberate changes in procedure variables such as flow rate (±0.1ml/min) and pH of the buffer (±0.2 units).

RESULTS AND DISCUSSIONS

Method optimization

Various solvent systems such as ACN-Water; Methanol-Water; Buffer-Methanol; Buffer-ACN mixtures in different ratios, having variable pH range (3-6) and flow rates (0.8-1.5ml/min)were evaluated. While optimizing the method various pH were tried and the effect of pH on the retention factor was studied (Figure. 2). Best separation and resolution was obtained using ammonium acetate buffer pH 4.0 and acetonitrile in gradient elution mode with a flow rate of 1.2ml/min and the reason for selection of gradient mode is that the fenofibric acid require minimum 80% of organic phase for elution but while assessing in isocratic mode, rosuvastatinpeak was interfering with the atorvastatin and plasma interferences. So we have decided to go for gradient chromatographic separation mode, where we have achieved well separated peaks with good resolution within 10min. The detection wavelength 250nm was chosen based on the isobestic point of both the drugs. Besides atorvastatin various internal standards were tried including metformin, simvastatin and gimlipride. Poor peak shape and poor resolution between the drugs were observed. Therefore, atorvastatin was selected as internal standard on the basis as it belongs to class of statins, which has structural similarity with that of rosuvastatin and also it showed good resolution and symmetrical peak shape. Three different types of HPLC columns were tested including both C18 and C8 having lengths 150mm and 250mm, respectively from various manufacturers viz. Phenomenex C18 (250mm X 4.6mm, 5μ), Hibar C8 (150mm X 4.6mm, 5μ), Jones Chromatography C18(150mm X 4.6mm, 3μ), Hibar C18 (150mm X 4.6mm, 5μ). Hibar C18 column was selected as best on the basis of excellent peak parameters such as (Separation, asymmetry, tailing, retention and resolution). In case of other columns Phenomenex C18 column (250mm) also showed better efficiency in separation and resolution due to its greater

length retention time of fenofibric acid was observed at 15.0min which results in larger run time analysis. Retention factors (k') were plotted against various analytical HPLC columns. (Figure. 3)
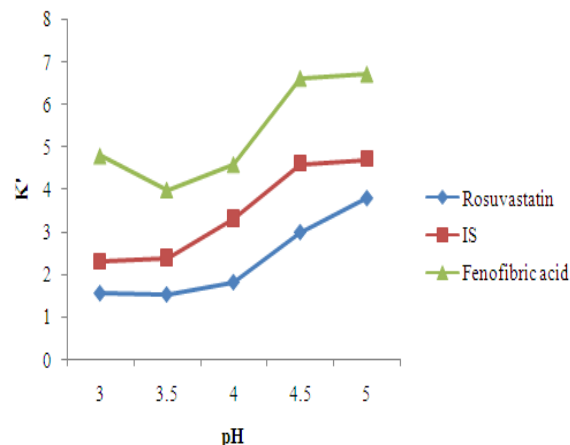

Fig. 2: Effect of variation in pH of mobile phase on the capacity factor (*k'*) of the analytes
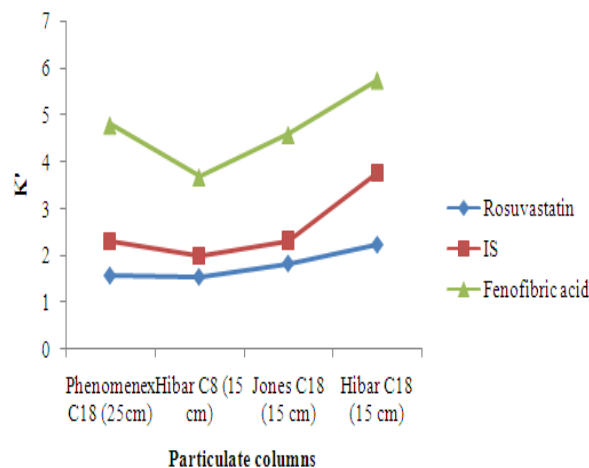

Fig. 3: Effect of different columns on the capacity factor (*k'*) of the analytes
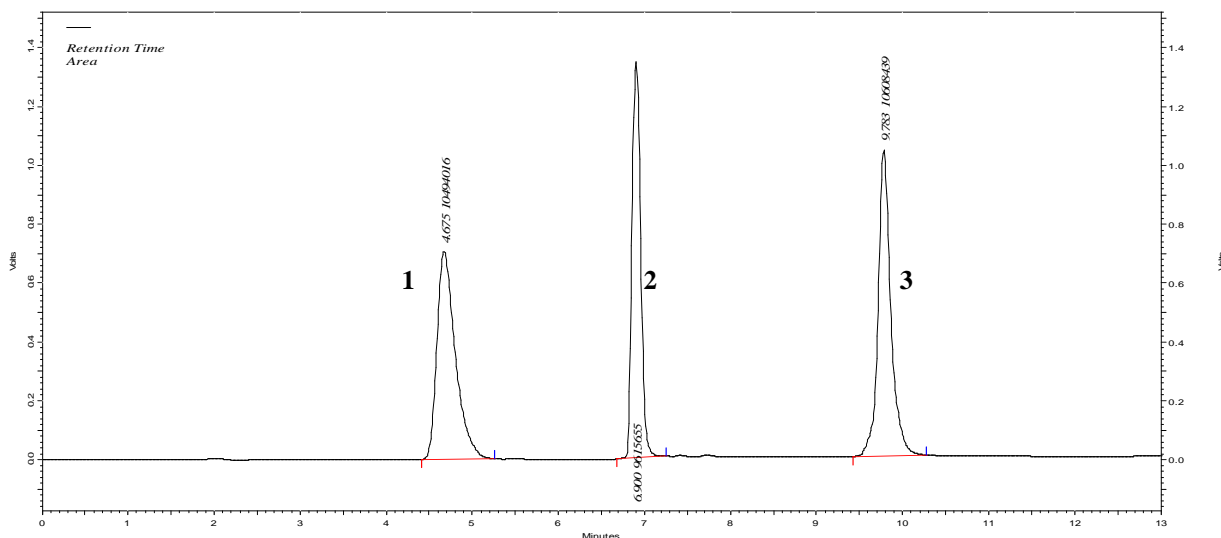

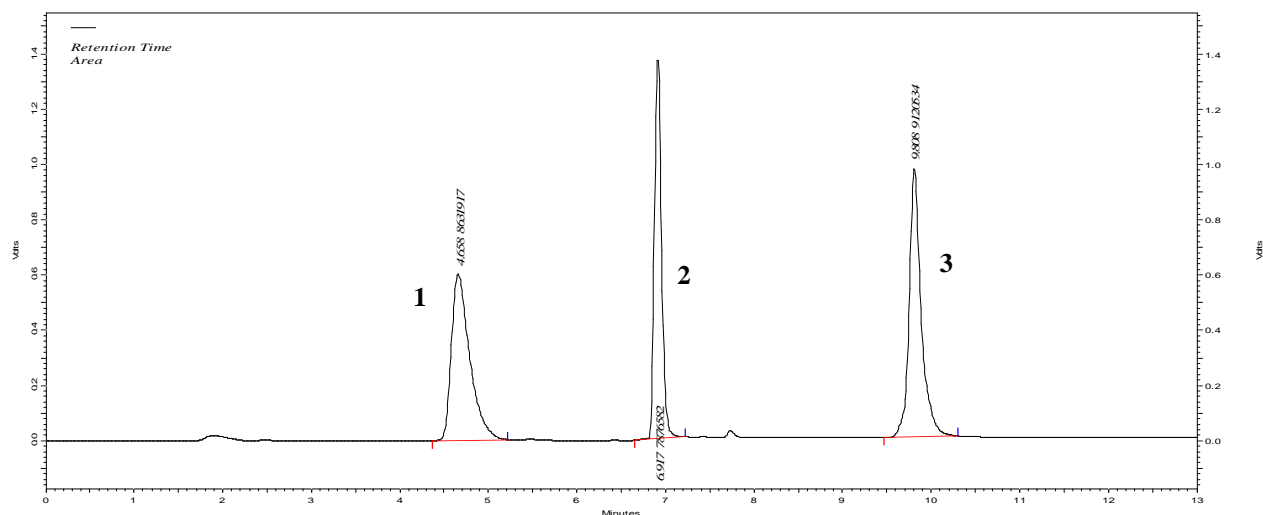**Fig. 4 (a): Standard chromatograms of rosuvastatin (1), atorvastatin (2) and fenofibric acid (3)**

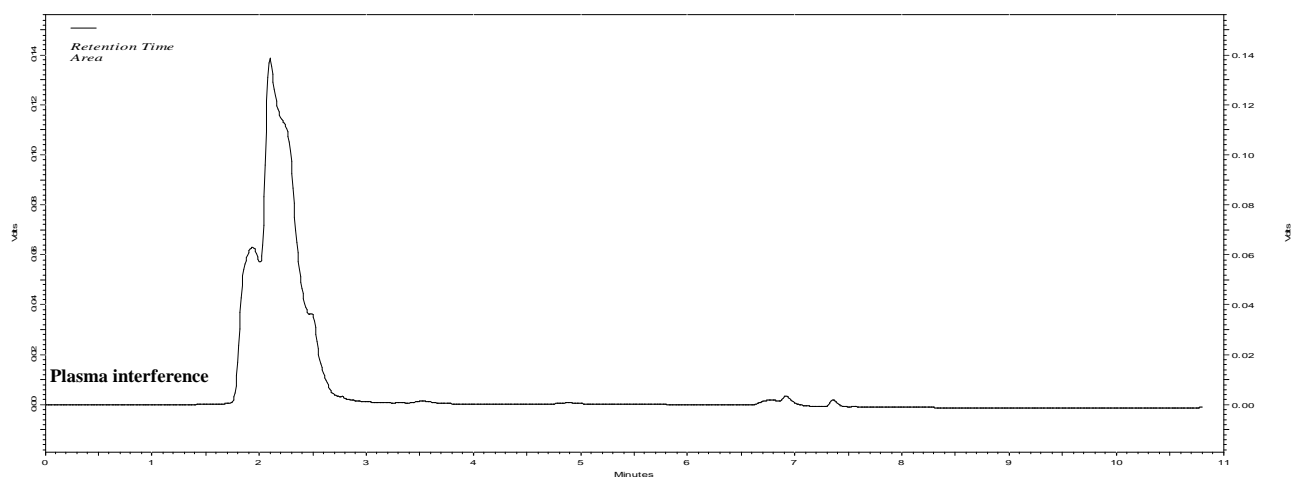**Fig. 4(b): Plasma spiked chromatograms of rosuvastatin (1), atorvastatin (2) and fenofibric acid (3)**

Fig. 4(c): Blank plasma chromatogram

Method validation

Accuracy and recovery

Mean percent recovery for rosuvastatin and fenofibric acid were found to be greater than 97.0 and 96.0% respectively, while the % relative standard deviation (rsd) values for rosuvastatin and fenofibric acid were less than 1.1% indicating accuracy of the developed method.

Linearity

The detector response for rosuvastatin and fenofibric acid was found to be linear over a concentration range of 2.0-24ng/ml and 0.2-20μg/ml respectively.

The regression analysis showed a correlation coefficient of 0.999 for both the drugs. The standard errors and linearity equations for the standard curves of standard drug mixtures and spiked human plasma of rosuvastatin and fenofibric acid acid are presented in Table 1.

Precision studies

Precision results showing both repeatability and intermediate precision are summarized in the Table.1 and 2, respectively. The % rsd values for rosuvastatin and fenofibric acid were less than 1.8% which indicates that the proposed bioanalytical method is precise.

Specificity/selectivity

Representative chromatograms of blank plasma, plasma spiked with IS, rosuvastatin and fenofibric acid are shown in the Figure. 4(a), 4(b), 4(c)confirms the absence of interference of endogenous components of plasma. Rosuvastatin, atorvastatin (IS) and fenofibric acid were well resolved at retention times of 4.6, 6.9, 9.7min, respectively.

LLOD and LLOQ

The lower limit of detection for rosuvastatin and fenofibric acid was found to be 1.46ng/ml and 2.5ng/ml, respectively, while lower limit of quantification were found to be 4.44ng/ml and 7.6ng/ml, respectively, as shown in the Table. 1.

Stability

The data resulted from the stability of both standard drug solutions and spiked plasma samples indicated plasma spiked were stable for 48hrs when stored at 25°C (room temperature), 2-8°C (refrigeration temperature) and -70°C (deep freezer/frozen temperature), while the standard solutions of rosuvastatin and fenofibric acid were found to be stable for 1 month at refrigeration temperature.

Robustness

Small deliberate changes in the optimized chromatographic conditions such as flow rate (±0.1ml/min) and pH of the ammonium acetate buffer (±0.2 units) did not significantly affect the retention time, recoveries and peak area of both the drugs indicating the method developed is robust.

Table 1: Accuracy, calibration range, linearity, repeatability and sensitivity of the method

Parameter	Analytes	
	Rosuvastatin	Fenofibric acid
Accuracy (% mean recovery ± SD; % rsd)		
Concentration spiked -level 1[x]	97.8 ± 0.476; 0.486	96.83 ± 0.904; 0.93
Concentration spiked -level 2[x]	97.7 ± 0.483; 0.494	97.01 ± 1.024; 1.05
Concentration spiked -level 3[x]	98.0 ± 0.729; 0.744	97.35 ± 0.907; 0.931
Calibration range	2.0-24ng/ml	0.2-20µg/ml
Linearity		
Standard mixtures		
Slope (m)	0.162	0.101
Intercept (c)	0.003	-0.004
Correlation coefficient (r^2)	0.998	0.993
Standard Error (SE)	0.001	0.003
Spiked plasma samples		
Slope (m)	0.164	0.092
Intercept (c)	0.001	-0.001
Correlation coefficient (r^2)	0.999	0.999
Standard error (SE)	0.0004	0.0004
Repeatability		
Injection repeatability (% mean recovery ± SD; % rsd)		
Concentration spiked -level 3[y]	97.18 ±0.921; 0.948	97.83 ± 1.028; 1.051
Sensitivity		
LLOD (ng/ml)	1.46	2.5
LLOQ (ng/ml)	4.44	7.6

Concentration spiked –level 1= rosuvastatin: 3.6ng/ml and fenofibric acid: 4µg/ml, Concentration spiked –level 2= rosuvastatin: 7.2ng/ml and fenofibric acid: 12µg/ml, Concentration spiked –level 3= rosuvastatin: 12ng/ml and fenofibric acid: 20µg/ml,, [x]n=6, [y]n=10.

Table 2: Results of Intra-day and inter-day precision

Spiked Concentration level	Concentration found	
	Intra-day precision (mean ± SD; % rsd) (n=6)	Inter-day precision (mean ± SD; % rsd) (n = 6)
Rosuvastatin (ng/ml)		
3.6	3.518 ± 0.017; 0.74	3.506 ± 0.040; 1.74
7.2	7.095 ± 0.039; 0.55	7.081± 0.044; 0.63
12	11.76 ± 0.222; 1.90	11.65 ± 0.176; 1.511
Fenofibric acid (µg/ml)		
4	3.850 ± 0.083; 2.173	3.83 ± 0.081; 2.12
12	11.73 ± 0.136; 1.164	11.70 ± 0.109; 0.93
20	19.66 ± 0.186; 0.946	19.61 ± 0.231; 1.089

CONCLUSION

A novel, sensitive, rapid RP-HPLC method was developed for the simultaneous estimation of rosuvastatin and fenofibric acid in human plasma. The proposed method was validated as per international guidelines. The influence of various particulate columns was studied based on the retention factors of rosuvastatin, fenofibric acid and internal standard. In addition, the method employed a simple protein precipitation method for extraction of analytes from plasma. All the analytes were separated and well resolved within 10.0min. This method offers greater advantage for simultaneous estimation of rosuvastatin, fenofibric acid and atorvastatin in plasma in a single chromatographic run. There is no need of modifying the chromatographic conditions for the analysis of individual drug. The proposed method will be applied for assessing the drug-drug interactions and pharmacokinetic studies of individual drug with other prescribed drugs. This method could also successfully employ for the routine laboratory analysis with slight changes in extraction procedure. Over all, the developed method provides excellent accuracy, selectivity, sensitivity, precision and repeatability for the simultaneous estimation of rosuvastatin and fenofibric acid in human plasma.

ACKNOWLEDGEMENT

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