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RESEARCH ARTICLE

Simultaneous Quantitation and Monitoring of Gemifloxacin and Rosuvastatin by Liquid Chromatography with UV-detection

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Abstract

Rapid, sensitive and economical liquid chromatographic methods have been developed for simultaneous quantitation of gemifloxacin and rosuvastatin in Active Pharmaceutical Ingredients (API), pharmaceutical formulations and in human serum. At first, method was developed with chromatographic system consisting methanol and water in the ratio of 90:10 as mobile phase (pH adjusted 3 with o-phosphoric acid) passing through stationary phase of prepacked Purospher Star ® C18 (5 µm, 25 × 0.46 cm) column at 1.0 mLmin^{-1}. Drug analytes eluted at 1.9 and 3.042 minutes simultaneously at isosbestic point of 263 nm. Linear calibration curves were obtained for 0.125-7.5 µgmL^{-1} gemifloxacin and 0.25-15 µgmL^{-1} rosuvastatin with $r^2 > 0.998$. Inter and intra day precision studies were within 2%. Accuracy of method from recovery studies were within 97.62-102.44%.

The method was further optimized and validated for the same chromatographic conditions at individual λ_{max} of each drug at 346 nm for 0-2.8 min for gemifloxacin and 243 nm for 2.9-3.6 min for rosuvastatin by programming the detector to match maximum absorbance of individual chromophores. The method followed linearity over a range of 0.03125-7.5 µgmL^{-1} gemifloxacin and 0.0625-15 µgmL^{-1} rosuvastatin for which the LOQ values shifted to more sensitive level of 05 from 16 ngmL^{-1} rosuvastatin and 06 from 09 ngmL^{-1} gemifloxacin as compared to prior method. LOD values also shifted from 5 to 2 ngmL^{-1} rosuvastatin and 3 to 2 ngmL^{-1} gemifloxacin. Precision was less than 2% RSD and accuracy of method was 99.21-103.21 % for both drugs. Robustness and ruggedness parameters were also carried out for validation of methods. Both methods were applied on tablet formulations and human serum samples and followed ICH guidelines.

The methods are applicable to pharmacokinetic and pharmacodynamic drug-drug interactions studies, can be utilized in clinical, forensic and drug research centres. Use of conventional UV detectors for sensitive determination of compounds will help in valuable research in developing countries.

Introduction

Gemifloxacin (figure 1) is the antibiotic used for the treatment of acute bacterial exacerbation of chronic bronchitis and mild-to-moderate pneumonia [1]. It is active against staphylococcus pneumoniae, haemophilus influenzae, haemophilus parainfluenzae, moraxella catarrhalis, chlamydia pneumoniae and klebsiella pneumonia bacteria [2]. Patients of hypercholesterolemia may have added risk of cardiovascular toxicity due to long term

medication of chronic bronchitis. Statins play a vital and efficient role in controlling the toxicity simultaneously produced during quinolone treatment [3]. Rosuvastatin is considered super-statin for cholesterol reduction for proper heart function. This combination of drugs needs to be evaluated simultaneously.

Spectrophotometry using ion-pair complexation technique [4] and spectroflourometry [5] have been utilized for determination of gemifloxacin in human plasma. HPLC technique has also been utilized for its determination alone [6] and in combination with other drug classes [7-9] in past years. Our research group has long been involved in this research area and has developed methods for simultaneous quantitation of gemifloxacin on HPLC with H_2-receptors [10], diuretics [11] and NSAIDs [12]. Rosuvastatin has also been determined with variety of drug classes [13], ACE inhibitors [14], NSAIDs [15], NIDDMs [16] ciprofloxacin [17]. The combination of rosuvastatin and gemifloxacin of treatment still has not been considered in the literature.

The present work is based on the development of method for simultaneous determination of rosuvastatin and gemifloxacin by reverse phase chromatography. Two approaches have been practiced and validated on raw bulk drugs and further applied on formulations, and human serum. One of the two methods developed is approached conventionally that has later been sensitized by programming the detector response to wavelength with respect to time so that maximum absorbance of individual analyte's chromophore is achieved.

The aim of methods is generation of clinical data at modest cost than separate assays where two co-administered drugs of different classes can be determined on a single chromatographic system with great specificity, sensitivity, less solvent and time utilized. Either method developed can be applied to detect samples of low concentrations with conventional UV- detectors. They follow ICH (International Conference on the Harmonization of technical requirements for the registration of pharmaceuticals for human use) guidelines [18]. Methods are applicable for drug-drug interactions, pharmacokinetic and research studies.

Experimental

2.1. Materials and reagents

Gemifloxacin and rosuvastatin reference standards were gifted by Genix Pharma Pvt. Ltd and Pharm Evo Pvt. Ltd. Karachi, Pakistan respectively. Their pharmaceutical formulations were obtained from local pharmacy of same manufacturers; Grat® 320 mg and X-plended® 5 mg tablets. *O*-phosphoric acid, methanol, and acetonitrile (ACN) of HPLC grade were purchased from Merck, Darmstadt, Germany. Double distilled de-ionized water was used throughout the experiments and was freshly prepared daily.

2.2. Apparatus

Shimadzu-10A and 20A facilitated with LC-20-AT HPLC pump, a SPD-20A Shimadzu UV visible detector, CBM-102 communication Bus Module Shimadzu aided with Shimadzu Class-GC 10 software (version 5.03) in both the systems were employed for the analyses. Separation of components was carried out using Purospher STAR® C_{18} (5 µm, 250 x 0.46 cm) of (Merck Millipore); and Discovery® C_{18} (5 µm, 250 x 0.46 cm) of (Sigma-Aldrich, St Louis, MO). Shimadzu UV-Spectrophotometer UV-1800 was used to determine spectral behavior of drugs prior to analysis. Moreover STEDEC CSW-300 deionizer (Stedec (Pvt) Ltd., Karachi, Pakistan) and Elma Ultrasonic LC 30 H sonicator (Elmer, NY) were used for deionizing water and degassing mobile phase respectively for sample preparation and analysis.

2.3. Preparation of standard solutions

100 µgmL^{-1} of standards stock solutions of API of gemifloxacin and rosuvastatin were prepared in diluents of mobile phase selected. These stock solutions were stored at 4^{o}C and used throughout for sample preparation of working standards accordingly. Calibration curves were drawn for a range of working solutions prepared from 0.125-7.5 µgmL^{-1} for gemifloxacin and 0.25-15 µgmL^{-1} for rosuvastatin.

2.4. Chromatographic conditions

With the aim to attain suitable compositions, pH, flow rates, shortest retention times and best resolution among the peaks of the analytes the optimum conditions were achieved for simultaneous analysis of drugs through HPLC. Isocratic mode on Shimadzu-20A HPLC system was used with reverse phase analysis using Purospher STAR® C_{18} (5 µm, 250 x 0.46 cm) column as stationary phase. Mobile phase for this system was 90:10 v/v methanol and water, pH adjusted to 3.0 eluted at a flow rate of 1.0 mL min^{-1} used after filtration through membrane filter of 0.45 micron

porosity; and degassing. 20 µL samples were injected for single run through rheodyne sample loop. Method was first developed and validated at 263 nm, the isosbestic point of drugs then at their individual λ_{max} i.e, 346 and 243 nm (detected from UV scan) at 200-400 nm (figure 2) by programming UV-detector for 0–2.8 min, and 2.9–3.6 minutes respectively at a flow rate of 1 $mLmin^{-1}$. Retention times followed by drugs were 2.0 and 3.2 minutes respectively.

2.5. Preparation of solutions of gemifloxacin and rosuvastatin in pharmaceutical formulations

Contents of ten tablets of Grat® 320 mg and X-plended® 5 mg were finely ground separately. An accurately weighed powdered sample of each drug was transferred to a 100 mL volumetric flask to make 100 μgmL^{-1}. The volume was adjusted with diluents and the resultant solution was sonicated for 5 min. Working solutions of 0.125-7.5 μgmL^{-1} for gemifloxacin and 0.25-15 μgmL^{-1} for rosuvastatin were prepared from this stock solution. A portion of the solution was then filtered through a 0.45 µm millipore filter paper. Aliquot (20 µL) of each solution was injected into the column.

2.6. Preparation of solutions of gemifloxacin and rosuvastatin in human serum

Blood sample (3 mL) of a healthy volunteer (aged 24 years; not involved in any medication, smoking, and strenuous activity) was collected in an evacuated glass tube through an indwelling cannula placed on forearm vein at Fatmid foundation, Pakistan. Blood was centrifuged at 10,000 rpm for 10 min to separate out serum from plasma. 9 mL ACN was added to 1 mL plasma and centrifuged at 10,000 rpm for 10 min to deproteinate it [19]. The supernatant serum thus obtained from this plasma was filtered and used for analysis and stored at -20^{o}C. Working solutions of 0.125-7.5 μgmL^{-1} for gemifloxacin and 0.25-15 μgmL^{-1} for rosuvastatin were prepared by spiking stock solutions into the serum maintaining the ratio 1:1 (drug stock diluted by diluent:serum v/v). Triplicate injections were made for each working solution for the analysis in serum.

Results and discussion

3.1. Method Development and Optimization

Optimized chromatographic detection of gemifloxacin and rosuvastatin was achieved at isosbestic point and individual λ_{max} of drugs, by varying rest of chromatographic parameters such as mobile phase composition, pH and flow rate. The ratios of 50:50, 60:40, 70:30, 75:25, 80:20, 85:15, and 90:10 v/v for methanol: water composition were tested. Peak broadening was experienced with increase of water content and retention times. 90:10 v/v for methanol: water was considered the ratio of choice for its sharper peaks and least retention times. Isosbestic point was determined for maximum absorbance of analytes at the same wavelength. Results were compared with programmed detection of analytes at their maximum wavelengths (figure 2).

1 $mLmin^{-1}$ flow rate was so adjusted that above this flow rate detection of analytes was so rapid for analysis and below this flow rate they took longer retention times. The variation of pH of mobile phase with *o*-phosphoric acid from 2.5 to 4.0 showed that sharper and well resolved peaks are produced at pH 3.0.

Further validation of analytical procedures was carried out according to the ICH guidelines [18] and United States Pharmacopeia (USP) [20].

3.2. Method Validation

The standard ICH guidelines recommend the methods of validation by suitability test, specificity, detection and quantitation limits, linearity, accuracy, precision, ruggedness and robustness of the method.

Specificity

For specificity studies, the chromatograms of blank serum, excipients and filler of tablet, spiked serum sample and raw drug were analyzed (figure 3). 10% solutions of commonly present excipients were prepared in methanol and analyzed. Excipients included lactose monohydrate, magnesium stearate, sucrose, talc and starch. The chromatograms so obtained for each category show that the chromatographic system is specific for the drug analytes and does not show any hindering peak.

System suitability

The system suitability studies were carried out by equilibrating the HPLC system with initial mobile phase composition and analyzing the detector response. The evaluation was carried out by calculating the parameters including capacity factors (k'), theoretical plates (N), tailing factor (T), resolution (Rs), and separation factor (α).

Results are tabulated in table 1. System suitability studies show the compatibility of method with the column packing.

Linearity

Regression statistics was linear over the concentration range of 0.125-7.5 μg mL^{-1} for gemifloxacin and 0.25-15 μg mL^{-1} for rosuvastatin at isosbestic point for raw drug and spiked samples in human serum.

Results were compared with those at programmed analysis where concentration range of analytes were more 0.03125 – 7.5 for gemifloxacin and0.0625 – 15 μg mL^{-1} for rosuvastatin. Linearity was very well achieved in both cases however lower limit of concentration could be measured through the programmed analysis. This condition was true for spiked samples in human serum (table 2). Calibration curves were constructed for analytes in mobile phase and spiked serum samples by plotting concentration vs. area and regression characteristics were calculated.

Accuracy

The measure of correctness of results near the standard value is its accuracy. It was tested on pharmaceutical formulations of both analytes in the same system of mobile phase and spiked serum sample at above mentioned concentrations. Recovery of drug analytes was calculated by the following formula:

% Recovery = (Peak response of sample at specific concentration / peak response of reference standard at specific concentration) × 100

Three concentrations of 80, 100 and 120 % tablet formulations in mobile phase were prepared. Accuracy was found for both criteria of methods i.e., isosbestic point and programmed analysis at individual λ_{max} (table 3).

Precision

Six calibration standards ranging from 0.125-7.5 μg mL^{-1} for gemifloxacin and 0.25-15 μg mL^{-1} for rosuvastatin at isosbestic point; in bulk drug and pharmaceutical formulation and three serum samples were analyzed in five replicates on three separate days. The inter-day and intra-day precision of the method was assessed at the same concentrations.

$$\% \text{ RSD } (= SD / M \times 100)$$

where SD and M are the standard deviation and mean of the peak area at specific concentratio where SD and M are the standard deviation and mean of the peak area at specific concentration (table 4).

Detection and quantitation limits

LOD and LOQ are the values of lowest concentration that the developed method can detect and quantitate on the system. In other words these terms explain the sensitivity of method for the whole system. Formulae used were:

$$LOD = 3.3 \times SD/\alpha \quad \text{and} \quad LOQ = 10 \times SD/\alpha$$

where SD and α are the standard deviation and slope of the calibration curve respectively (table 2).

Robustness and ruggedness

Another means of testing the reproducibility of method are robustness and ruggedness. These are the test to validate the method on rough and tough use. For this purpose applicability of method were achieved by introducing minor changes in chromatographic parameters intentionally, like mobile phase composition was changed to ±2 mL, pH up to ±2 and flow rate was varied to 0.1 mL min^{-1} and their effect was observed on analytical results. This test is called robustness (table 5). % RSD values show no significant difference in the results. This shows method is reproducible in case when parameters are lit bit disturbed.

The whole procedure when followed on another system and column of similar configuration on different place with analytes of different supplier, the method was subjected to ruggedness (table 5). Shimadzu LC 10 AT HPLC and column of Discovery® C_{18} (5 μm, 250 x 0.46 cm) were utilized for this purpose. The instrument was placed in Research Institute of Pharmaceutical Sciences, University of Karachi, Karachi. Relative standard deviation is less than 2% that shows reproducibility of method.

Determination of rosuvastatin and gemifloxacin in pharmaceutical formulations

Accuracy studies were carried out on tablets of each formulation. Known amounts of each analyte were added to corresponding tablet formulations. Three levels of concentration (80%, 100% and 120%) were studied table 3.

Precision studies were also carried out on tablet formulations (table 4). %RSD values for inter-day assay were less than 2%. It showed the method is applicable on tablet formulations also.

Determination of ROS and NSAIDs in human serum

Calibration curves were prepared by analyzing the compounds after spiking them in human serum. 0.125-7.5 μg mL^{-1} for gemifloxacin and 0.25-15 μg mL^{-1} for rosuvastatin were added in the serum and assayed in triplicates at eight different concentrations. Calibration curves were linear over the spiked range with good correlation coefficient. The table 2 shows the values of r^2 were less than 0.999. The sensitivity of method was also considerable that LOQ values for rosuvastatin and gemifloxacin were 23 and 15 $ngmL^{-1}$ and LOD for the same were 8 and 5 $ngmL^{-1}$. Comparison in behavior of chromatograms can be visualized in figure 3.

The accuracy and inter-day precision studies were also performed in drug-free serum samples spiked with concerned drugs afterwards. The same concentration range was selected for precision studies as was considered for calibration curves. Analysis was carried out for three days. % RSD values are less than 2%. Values are shown in comparison with values for bulk drugs in table 3. Spiked serum samples with 120, 100 and 80 % concentrations of drug compounds were analysed for accuracy and recovery studies of drugs from serum. 96.96-99.85% recovery of both compounds show that the method is accurate for the compounds in serum samples also.

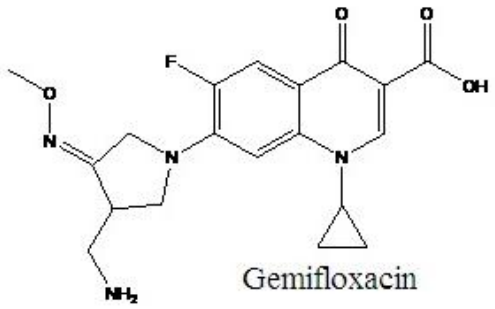
Analysis under program detector

Approach of simultaneous determination of drug analytes by detection at their own λ_{max} rather than isosbestic point was also useful and informative for routine analysis. However results with isosbestic point are in nanogram measurements, this approach has produced results with finer results in nanogram measurements.

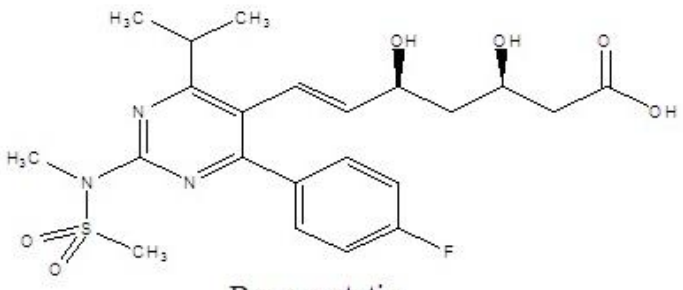
The developed method was processed at detector response of λ_{max} of each analyte and validated. Calibration curves obtained for linearity of same optimized concentration range showed that there were big gaps among the peak areas of each analyte in chromatograms. Comparison in peak areas of drugs for two methods is shown in figure 4. r^2 with values 0.998 also showed linear response of the drugs. Concentration range increased towards lower limit. Optimized range of concentrations were 0.0625-15 μgmL^{-1} rosuvastatin and 0.03125-7.5 μgmL^{-1} gemifloxacin.

LOD values were within smaller quantifiable limits i.e., 2 ng mL^{-1} rosuvastatin and gemifloxacin. These values were far smaller than those detected at isosbestic point. Likewise LOQ values also reached to lower values from 16 to 5 $ngmL^{-1}$ rosuvastatin and from 9 to 6 $ngmL^{-1}$ gemifloxacin respectively (table 2).

Accuracy (99-101%) and inter-day precision (%RSD <2) studies were carried out as previously done and the results met the ICH guidelines. Robustness and ruggedness studies were also performed and they met system suitability parameters. Specificity and system suitability parameters are shown in table 1 and figure 2.



Gemifloxacin



Rosuvastatin

Figure 1: Chemical structures of gemifloxacin and rosuvastatin

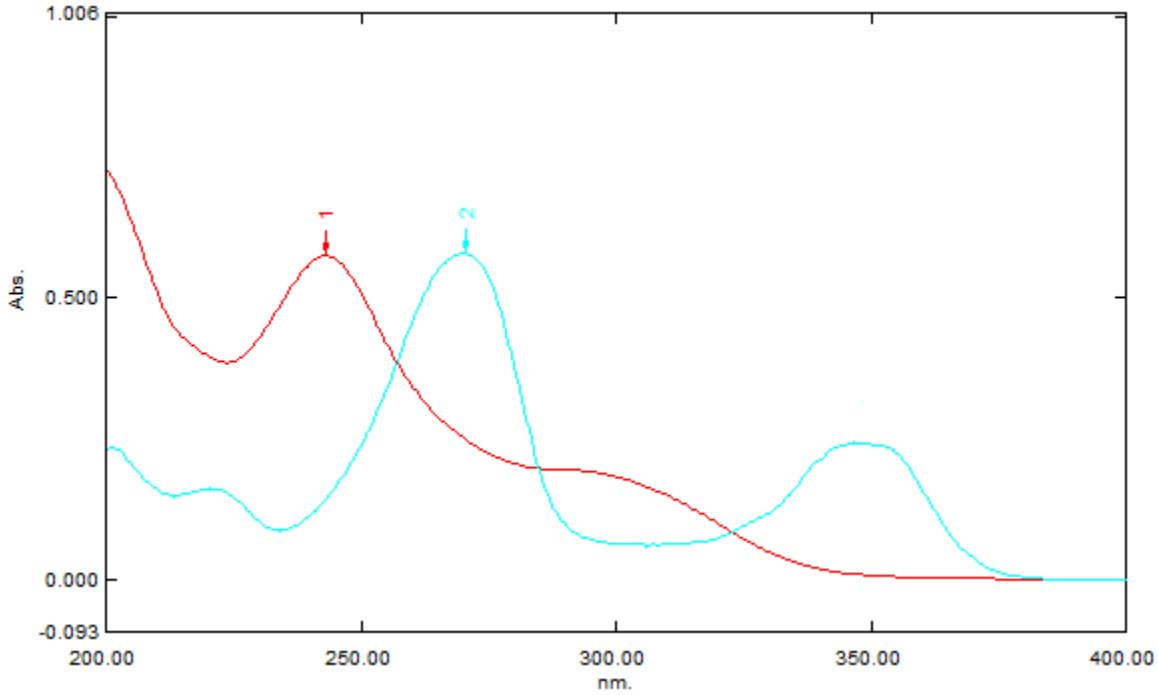


Figure 2: Representative UV spectra of rosuvastatin (1) and gemifloxacin (2)

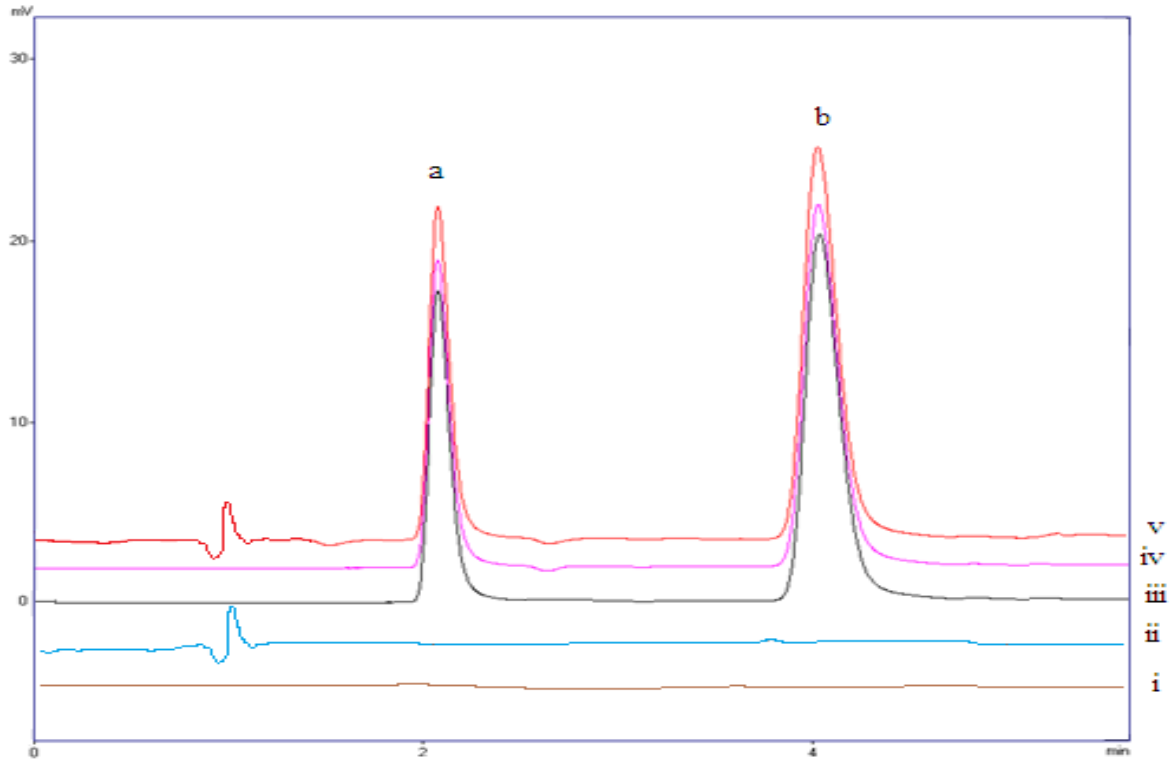


Figure 3: Representative chromatograms of placebo (i), blank human serum (ii), gemifloxacin (a) and rosuvastatin (b) in bulk (iii), pharmaceutical formulation (iv) and human serum sample spiked with standards (v).

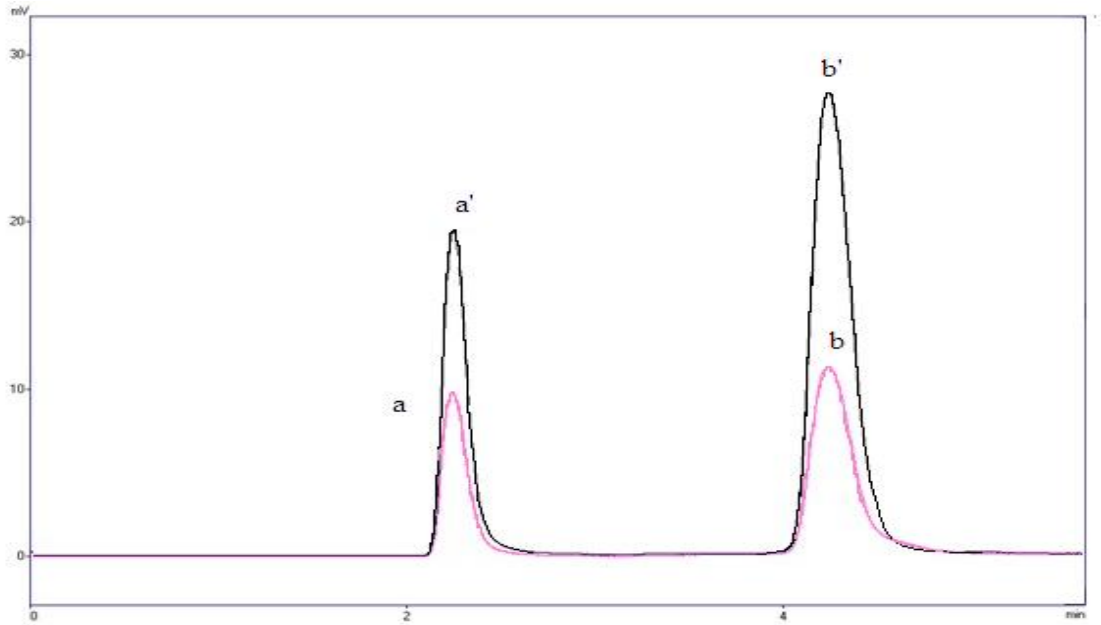


Figure 4: Representative chromatograms of gemifloxacin (a and a') and rosuvastatin (b and b') at isosbestic point and individual λ_{max} respectively.

Table 1: Comparison of system suitability parameters of two methods for gemifloxacin and rosuvastatin

At isosbestic point					
Analytes	t_R	K	N	T	Rs
GMI	1.815	1.12	1667	1.70	--
ROS	3.032	7.56	1985	1.72	5.41
At individual λ_{max}					
GMI	2.242	0.00	1453	1.53	1.01
ROS	4.241	0.89	1961	1.76	6.47
Retention time (t_R), Capacity factors (k'), Theoretical plates (N), Tailing factor (T), Resolution (Rs)					

Table 2: Comparison of regression statistics of two methods for gemifloxacin and rosuvastatin

Parameter	ROS	GMI	ROS	GMI
	At isosbestic point		At individual λ_{max}	
API				
Conc.(μgmL^{-1})	0.25 - 15	0.125 – 7.5	0.0625 - 15	0.03125 – 7.5
Slope	19139	38279	84723	17203
Intercept	5235	5235	17658	24226
LOD ($ngmL^{-1}$)	05	03	02	02
LOQ($ngmL^{-1}$)	16	09	05	06
r^2	0.999	0.999	0.999	0.999
Human serum				
Conc.(μgmL^{-1})	0.25 - 15	0.125 – 7.5	0.0625 - 15	0.03125 – 7.5
Slope	26529	38262	84720	17192

Intercept	4506	4850	16157	23226
LOD ($ngmL^{-1}$)	08	05	02	03
LOQ($ngmL^{-1}$)	23	15	06	08
r^2	0.999	0.999	0.999	0.999
Tablet formulations				
Conc.(μgmL^{-1})	0.25 - 15	0.125 – 7.5	0.0625 - 15	0.03125 – 7.5
Slope	26519	38238	84708	16827
Intercept	4766	5141	16926	16394
LOD ($ngmL^{-1}$)	06	04	02	03
LOQ($ngmL^{-1}$)	18	13	07	10
r^2	0.999	0.999	0.999	0.993

Table 3: Comparison of accuracy of two methods for gemifloxacin and rosuvastatin

	At isosbestic point			At individual λ_{max}		
conc.	% Recovery					
%	Bulk	Human serum	Tablet formulations	Bulk	Human serum	Tablet formulations
GMI						
80	99.56	97.62	99.11	103.01	100.48	100.55
100	102.44	98.71	96.88	100.04	101.68	100.36
120	100.09	96.96	99.38	99.38	102.90	100.28
ROS						
80	97.68	99.85	101.11	99.96	99.68	100.05
100	100.20	98.98	100.37	100.47	96.75	99.79
120	99.95	97.38	102.13	99.98	99.21	100.43

Table 4: Comparison of precision studies of two methods for gemifloxacin and rosuvastatin

Conc. e^{-3} $ngmL^{-1}$	%RSD											
	At isosbestic point						At individual λ_{max}					
	Bulk		Human serum		Tablet formulations		Bulk		Human serum		Tablet formulations	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
GMI												
0.25	0.31	0.40	0.25	0.36	0.31	0.23	0.05	0.31	0.05	0.25	0.06	0.17
07	0.20	0.18	0.04	0.17	0.13	0.09	0.04	0.40	0.12	0.17	0.07	0.01
15	0.06	0.07	0.02	0.03	0.02	0.02	0.01	0.25	0.01	0.40	0.01	0.17
ROS												
0.5	0.30	0.27	0.27	0.41	0.35	0.31	0.04	0.19	0.04	0.17	0.01	0.01
15	0.20	0.17	0.04	0.15	0.04	0.12	0.03	0.17	0.05	0.31	0.08	0.13
30	0.07	0.05	0.03	0.04	0.01	0.04	0.01	0.13	0.01	0.17	0.01	0.19

Table 5: Comparison of robustness and ruggedness of two methods for gemifloxacin and rosuvastatin

Drugs	T_R (min)	k’	N	T	Rs
pH ± 0.1					
GMI	1.815	1.12	1667	1.70	--

ROS	3.032	7.56	1985	1.72	5.41
Flow rate ± 0.2					
GMI	1.805	1.14	1665	1.68	0.01
ROS	3.035	1.10	1669	1.72	4.99
Mobile phase ratio ± 2					
GMI	1.815	1.12	1667	1.70	0.07
ROS	3.032	7.56	1985	1.72	5.41
Column					
GMI	1.815	1.12	1667	1.70	0.04
ROS	3.032	7.56	1985	1.72	5.41
System					
GMI	1.805	1.14	1665	1.68	0.01
ROS	3.032	7.56	1985	1.72	5.41
Retention time (t_R), Capacity factors (k'), Theoretical plates (N), Tailing factor (T), Resolution (Rs)					

Conclusion

The two developed methods are rapid, accurate and very sensitive for the estimation of rosuvastatin and gemifloxacin simultaneously. The methods are applicable in table formulations and human serum samples. Second method, the new approach of programming the detector for method development does not disturb the validity and stability of method parameters however sensitivity level has increased from the traditional approach. This approach also makes the method more economical with efficiency that conventional HPLC can be utilized for nano-level detection of drug analytes especially in third world countries. Both approaches are applicable in routine analysis, quality control, research and forensic studies.

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