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STABILITY INDICATING ASSAY METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF RACECADOTRIL AND OFLOXACIN IN TABLET DOSAGE FORM BY REVERSE PHASE-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A simple, accurate, precise and specific spectrophotometric method has been developed for simultaneous determination of racecadotril and ofloxacin in its combined tablet dosage form by using methanol as a solvent. The method involves absorbance correction based on measurement of absorbance at two wavelengths at 231 nm and 323.40 nm. Method follows Beer's linearity in the range of 10-24 µg/ml for racecadotril and 20-48 µg/ml ofloxacin both. The mean % recoveries were found to be in the range of 99.22 – 100.66 % and 99.62 100.19 % for racecadotril and ofloxacin respectively. LOD and LOQ were found to be 0.1922µg/ml and 0.5826 µg/ml for racecadotril and 0.1063 µg/ml and 0.3223 µg/ml for ofloxacin respectively. Assay results of market formulation were found to be 99.24 % and 99.25 % for racecadotril and ofloxacin respectively. The proposed method has been validated as per ICH guidelines and successfully applied to the estimation of racecadotril and ofloxacin in their combined Tablet dosage form.

Keywords: Racecadotril, Ofloxacin, Method Validation, Development and Estimation.

INTRODUCTION

Pharmaceutical examination is a part of practical chemistry that includes a progression of process for ID, assurance, measurement and cleansing of a substance, detachment of the segments of an answer or blend, or assurance of structure of concoction mixes [1]. The substance might be a solitary compound or a blend of mixes and it might be in any of the dose shape. The substance utilized as pharmaceuticals are creatures, plants, micro-organisms, minerals and different manufactured items [2]. Analytical chemistry is a branch of chemistry that deal with the identification of compounds and mixtures (qualitative analysis) or the determination of the scope of the constituent (quantitative analysis) [3]. Racecadotril is a novel antidiarrhoeal agent indicated in the treatment of acute diarrhoea. Ofloxacin acts on DNA gyrase and toposiomerase IV, enzymes which, like human topoisomerase, prevents the excessive supercoiling of DNA during replication or transcription. By inhibiting their function, the drug thereby inhibits normal cell division [4].

Materials and Methods

Materials

Pure drugs samples of ofloxacin and racecadotril were obtained as a gift samples from MSN Laboratories, Hyderabad, T.S, India. HPLC grades of Methanol and acetonitrile procured from Merk. All the chemicals used in the study are analytical grade.

Methods

Selection of solvent

Based on the solubility profile of the selected drugs, the solvents are to be selected. The solvents used for performing the tests are water, methanol, ethanol, chloroform, acetonitrile, 1N NaOH, and 1N HCl. These results are even helpful in the selection of mobile phase [5].

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Preparation of solutions

Preparation of mixed standard stock solutions (200µg/mL&100µg/mL)

Accurately Weighed and transferred 5mg of ofloxacin and 2.5 mg of racecadrotril working Standards into a 25ml clean dry volumetric flask, add 3/4th volume of diluent, sonicated for 5 minutes and make up to the final volume with diluents [6].

Preparation of standard working solutions (20µg/mL&10µg/mL) (100% solution)

1ml from the above two stock solutions was taken into a 10ml volumetric flask and made up to 10ml.

Selection of detection wavelength

The sensitivity of method that uses UV-VIS detector depends upon the proper selection of wavelength. An ideal wavelength is that gives maximum absorbance and good response for both the drugs to be selected [7].

Identification of the peaks

Standard solution (100 µg/ml) of ofloxacin was injected into HPLC at ambient temperature using a mixture of methanol and water in the ratio of 55:45v/v/ as mobile phase and discovery C8 (4.6 x 250mm, 5µm) column as the stationary phase at a detection wavelength of 295nm and 1ml/min flow rate. The chromatogram was recorded. At the same chromatographic conditions, 100 µg/ml of racecadotril was also injected and the chromatogram was recorded [8].

Development and optimization of HPLC method

The method development was focused on the selection of a suitable HPLC column, optimization of the composition of the mobile phase, investigating the impact of flow rates, wavelength and fine tuning the conditions of the final elution profile. Based on the nature of the sample, the reverse phase HPLC was selected for the initial separation because of its simplicity and suitability. From the knowledge of properties of the selected drugs, the following initial conditions were set and then optimized by making deliberate changes in the parameters like flow rate, injection volume, working concentration and even the mobile phase composition. The trials were run for the mixed working standard at 100 µg/ml [9].

System suitability

The chromatographic conditions were set as per the optimized method and mobile phase was allowed to equilibrate with stationary phase and was indicated by the steady base line. Six replicate injections of a single working mixed standard of 50µg/ml solution were injected and the chromatograms were recorded [10].

Method validation

The developed analytical method was validated by using following parameters specificity, linearity, precision, accuracy, robustness, and limit of detection, limit of quantitation, system suitability and solution suitability.

Linearity

The working mixed standard solution of concentrations 25, 50, 75, 100, 125 and 150 µg/ml were prepared by appropriate dilutions of the mixed standard stock solution with the mobile phase. The solutions thus prepared injected at the optimized chromatographic conditions and the chromatograms were recorded. The linearity of the drugs was accomplished from the regression equation [11].

Accuracy

To evaluate the accuracy of the proposed method, recovery studies were carried out by standard addition method, where a pre analyzed test sample solution was spiked with mixed standard solution at three levels of 50%, 100%, 150% of the ofloxacin and racecadotril. At each level recovery studies were carried out in triplicate and expressed as percent recoveries [12].

Precision

The precision of analytical method is a method of the random error and is defined as the agreement between replicate measurements of the sample. It is expressed as the percentage coefficient of variation (%CV) or relative standard deviation (RSD) of the replicate measurements. The solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits [13].

Robustness

Robustness of the method was demonstrated by making deliberate changes in the optimized conditions of the developed method. From the mixed standard stock solution, a six let of standard solutions were prepared by appropriate dilutions and were treated in the similar manner as that of working standards. Six replicate injections were given and the effects of variations were observed in the respectively recorded chromatograms and the %RSD of the peak areas were calculated for both the drugs at each of the following conditions [14].

A. Effect of variation in flow rate

A study was conducted to determine the effect of variation in flow rate. Standard solution of 30µg/ml concentration was prepared and injected into HPLC system by keeping flow rates 0.8ml/min, 1.2ml/min, and 1.4ml/min. The effect of variation of flow rate was evaluated.

B. Effect of variation in mobile phase composition

A study was conducted to determine the effect of variation in Mobile phase ratio by changing the ratio of organic phase i.e., Acetonitrile: Buffer by ±2ml standard solution of 65µg/ml concentration was prepared and injected into HPLC system and the chromatograms were recorded. %RSD of the peak areas and retention times were calculated for both the drugs.

C. Effect of variation of detection wavelength

Standard solution of 60µg/ml concentration was prepared and injected to HPLC system by keeping the wavelength at 293nm and 298nm. The effect of variation of detection wavelength was evaluated [15 & 16].

Forced degradation studies

A. Acid degradation studies

To 1 ml of stock solution ofloxacin and racecadrotril 1 ml of 2N HCl was added and refluxed for 30mins at 60^0c.The resultant solution was diluted to obtain (20ppm &10 ppm) solution and 10µl solutions were injected into the system and the chromatograms were recorded to assess the stability of sample.

B. Alkali degradation studies

To 1 ml of stock solution ofloxacin and racecadrotril 1 ml of 2 N sodium hydroxide was added and refluxed for 30mins at 60^0c. The resultant solution was diluted to obtain (20ppm & 10ppm) solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

C. Dry heat degradation studies

The standard drug solution was placed in ovenat105^0c for 6h to study dry heat degradation. For HPLC study, the resultant solution was diluted to (20ppm & 10ppm) solution and 10µl were injected into the system and the chromatograms were recorded to assess the stability of the sample**.**

D. Photo stability studies

The photochemical stability of the drug was also studied by exposing the (200ppm & 100ppm) solution to UV light by keeping the beaker in UV chamber for 7days or 200 Watt hours/m^2 in photo stability chamber. For HPLC study, the resultant solution was diluted to obtain (20ppm & 10ppm) solutions and 10µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

E. Neutral degradation studies

Stress testing under neutral conditions was studied by refluxing the drug in water for 6hrs at a temperature of 60ºc. For HPLC study, the resultant solution was diluted to (20ppm & 10ppm) solution and 10µl were injected in to the system and the chromatograms were recorded to assess the stability of the sample [17, 18 & 19]**.**

Estimation of stability of drug solutions

Stability was estimated for standard (ofloxacin 150 µg/ml & racecadotril 300µg/ml) solution. The standard solution was injected after preparation and the peak areas were recorded. After 24 hours, the solution was prepared in the similar way and was injected thrice (in order to minimize errors) along with the solution of the initial day and the peak areas were recorded. The same procedure was followed at an interval of 24 hours until there was a significant change (due to degradation) in the peak area values [20 & 21].

Results and Discussion

Selection of solvent

Based upon the solubility results, methanol was selected as the solvent to be used (which is economic and easily available when compared to acetonitirile). The results are shown in Table 1.

Selection of detection wavelength

The standard solutions of ofloxacin and racecadotril (100 µg/ml) were scanned in the UV range (200-400nm) and the spectrums obtained were overlain. From the overlain spectrum 295nm was selected as the detection wavelength for the present study. The results are shown in Figure 1.

Development and optimization of HPLC method

The method was developed and optimized chromatographic conditions are mobile phase 55% 0.01N Kh2po4 (4.8): 45% acetonitrile, flow rate was maintained at 1ml/min, column specifications are discovery C8 (4.6 x 250mm, 5µm), detector wave length was found to be 295nm, the sample injection volume is 10µL, run time of samples was 6 min. The resultant peaks showed good resolution, tailing factor, theoretical plate count and resolution. These ofloxacin and racecadrotril were eluted at 2.767min and 3.795 min respectively with good resolution. Plate count and tailing factor was very satisfactory, so this method was optimized and to be validated. The optimized chromatogram was shown in Figure 2.

System suitability

System suitability parameters of ofloxacin and racecadotril of all replicate injections were recorded and were tabulated. By performing system suitability test, it ensures that both methodology and instrumentation were performing within expectations. The results are shown in Table 2.

Linearity

Linearity established over the concentration range 25-125µg/ml for both the drugs and correlation was found to be 0.999 for ofloxacin and racecadotril. The results are shown in Table 3 and Figure 3 & 4.

Accuracy

The mean percentage recoveries of ofloxacin found to be within the limit i.e.98-55% and racecadotril found to be 98.85% hence the method was considered to be accurate. The results are shown in Table 4 & 5.

Precision

The %RSDs were found to be within in the acceptance limits (%RSD < 2%) at all the levels of precision i.e., system precision and method precisions. Hence the proposed method was said to be precise. The results are represented in Table 6 & 7 and Figure 5& 6.

Robustness

Even though deliberate changes were made in flow rate, wavelength, and the organic phase, there were no significant changes observed in the chromatograms and the %RSD for the peak areas (%RSD <2%) and system suitability parameters of both the drugs were found to be within the acceptance limits. Hence the proposed method was considered to be robust. The results are summarized in Table 8, 9 & 10.

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

The LOD values were found to be for ofloxacin and racecadotril were found to be 3.3 × 471/15183.67=0.10µg/ml and 3.3 × =391.3/18829=0.06µg/ml. The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined. The results of limit of quantitation (LOQ) of ofloxacin and racecadoril were found to be 10×l8806/1456 = 0.31µg/ml and 10 × 391.3/18829= 1.21µg/ml.

Estimation of stability of drug solutions

The drug solutions were found to be stable for the day (24hrs) from the time of preparation. On the day, significant degradation (nearly 2%) of the drugs was observed. The results of the stability data was summarized in Table 11.

Table 1: Solubility study data

Drug	Solvent				
	H_2O	MeOH	CH_3CN	1N NaOH	HCl
Olfloxacin	-	+	+	-	-
Racecadotril	-	+	+	-	-

{Whereas (+) indicates solubility (-) indicates insolubility}

Table 2: System suitability data of ofloxacin and racecadotril

S.No	Ofloxacin				Racecadotril			
	RT	Peak area	Theoritical plate	Tailing factor	RT	Peak area	Theoretical plate	Tailing Factor
1	2.742	314957	6771	1.19	3.790	184856	12317	1.01
2	2.752	314404	6345	1.11	3.791	183514	11891	1.01
3	2.753	312910	6539	1.13	3.796	184540	12142	1.01
4	2.754	314621	6791	1.14	3.796	180522	12237	1.00
5	2.755	311855	6816	1.18	3.796	182554	12455	1.01
6	2.756	311728	6708	1.12	3.804	183440	12302	1.00
Mean		**313413**				**183238**		
Std.D	-	**1438.4**	-	-	-	**1566.8**	-	-
%RSD	-	**0.5**	-	-	-	**0.9**	-	-

Table 3: Linearity data of ofloxacin and racecadotril

S.No	Ofloxacin		Racecadotril	
	Concentration (µg/ml)	Peak area	Concentration (µg/ml)	Peak area
1	25	80270	25	50541
2	50	148513	50	98479
3	70	228358	70	139111
4	75	312733	75	188109
5	100	378803	100	235931

6	125	454663	125	285337
Statistical analysis	**Slope : 15181** **y-intercept : 1340** **correlation coefficient(R^2) : 0.999**		**Slope : 18806** **y-intercept : 1456** **correlation coefficient(R^2) : 0.999**	

Table 4: Accuracy study data of olfloxacin

Specification level (%)	Area	Amount added (ppm)	Amount found (ppm)	% Recovery	Mean recovery
50%	456594 455491 456298	10	9.99	99.92%	**98.55%**
100%	603372 606808 605530	20	19.65	98.28%	
150%	753433 754018 753864	30	29.57	98.57%	

Table 5: Accuracy study data of racecadotril

Specification level (%)	Area	Amount added (ppm)	Amount found (ppm)	% Recovery	Mean recovery
50%	282312 283075 282675	5	4.96	99.28%	**98.85%**
100%	375583 376085 377121	10	9.95	99.59%	
150%	469343 467253 469748	15	14.87	99.19%	

Table 6: System precision data of ofloxacin and racecadotril

S.No	Ofloxacin	Racecadotril
	Peak area	**Peak area**
1	314957	184856
2	314404	183514
3	312910	184540
4	314621	180522
5	311855	182554
6	311728	183440
Mean **SD** **%RSD**	**313413** **1438.4** **0.5**	**183238** **1566.8** **0.9**

Table 7: Method precision data of ofloxacin and racecadotril

S.No	Ofloxacin		Racecadotril	
	Peak area	**% Assay**	**Peak area**	**% Assay**
1	312665	99.66	182435	99.46
2	313052	99.79	182841	99.68
3	312887	99.73	183320	99.94
4	312301	99.55	182546	99.52

5	312598	99.64	181920	99.18
6	310598	99.00	182892	99.71
Mean **SD** **%RSD**	**312350** **896.0** **0.3**	**99.56** **0.29** **0.29**	**182659** **476.1** **0.3**	**99.58** **0.2596** **0.3**

Table 8: Robustness study data (change in flow rate) ofloxacin and racecadotril

Flow rate	**Peak area**	
	Ofloxacin	**Racecadotril**
0.8ml/min	353570	210179
	358035	213408
	358124	211537
	357554	217069
	349298	213668
	358096	214676
Mean : **Std.dev :** **%RSD :**	**355779** **3633.2** **1.0**	**213423** **2408.4** **1.1**
Flow rate	**Peak area**	
1.2ml/min	368914	221482
	367690	224185
	371064	220012
	369881	222030
	364610	221451
	364166	224716
Mean : **Std.dev :** **%RSD :**	**367721** **2813.5** **0.8**	**222313** **1793.7** **0.8**

Table 9: Robustness study data (change in wavelength) of ofloxacin and racecadotril

Wavelength	**Peak area**	
	Ofloxacin	**Racecadotril**
293nm	2955859	191687
	296494	190176
	294682	194167
	295485	191177
	296288	195464
	295016	190807
Mean : **Std.dev :** **%RSD :**	**295637** **711.1** **0.2**	**192246** **2090.8** **1.1**
Wavelength	**Peak area**	
298nm	297852	190632
	299333	194290
	298420	193754
	299718	192696
	291944	196797
	297686	191750
Mean : **Std.dev :** **%RSD :**	**297492** **2833.8** **1.0**	**193320** **2158.6** **1.1**

Table 10: Robustness study data (change in mobile phase) of ofloxacin and racecadotril

Mobile Phase	Peak area	
	Ofloxacin	**Racecadotril**
A:B=70:30	292315	181483
	294162	180089
	294841	180083
	292559	181999
	294400	182533
	295728	180218
Mean : **Std.dev :** **%RSD :**	**294001** **1326.3** **0.5**	**181068** **1080.4** **0.6**
Mobile phase	**Peak area**	
A:B=50:50	296757	194350
	294944	193509
	290335	197729
	298100	191958
	293689	196532
	293501	191403
Mean : **Std.dev :** **%RSD :**	**294554** **2729.0** **0.9**	**194247** **2499.1** **1.3**

Table 11: Stability study data of ofloxacin and racecadotril

S.NO	Ofloxacin			Racecadotril		
	R.Time	**Peak area**	**% Area**	**R.Time**	**Peak area**	**% Area**
1	2.676	300845	62.17	3.797	174335	37.83
2	2.682	300066	62.17	3.803	173839	37.83
3	2.683	302560	62.47	3.807	174065	37.53
4	2.683	303550	62.42	3.182	176028	37.58
5	2.685	302314	62.59	3.819	175514	37.41
6	2.689	303753	62.03	3.834	175264	13444
Mean **Std.Dev** **%RSD**		**302181** **1467.3** **0.5**		**Mean** **Std.Dev** **%RSD**	**175264** **1510.9** **0.9**	

Figure 1: Overlain UV spectra of ofloxacin and racecadotril

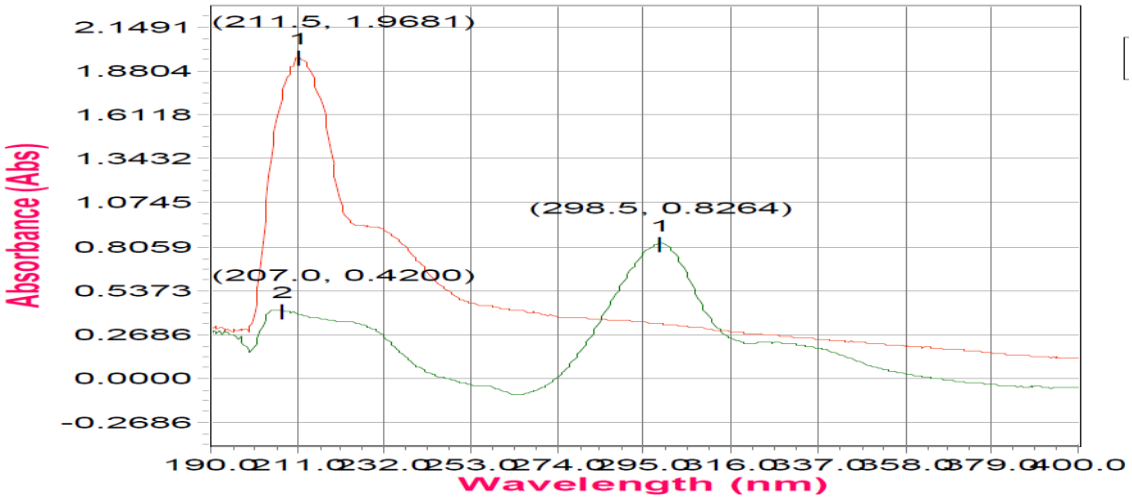


Figure 2: Optimized chromatogram of ofloxacin and racecadrotril

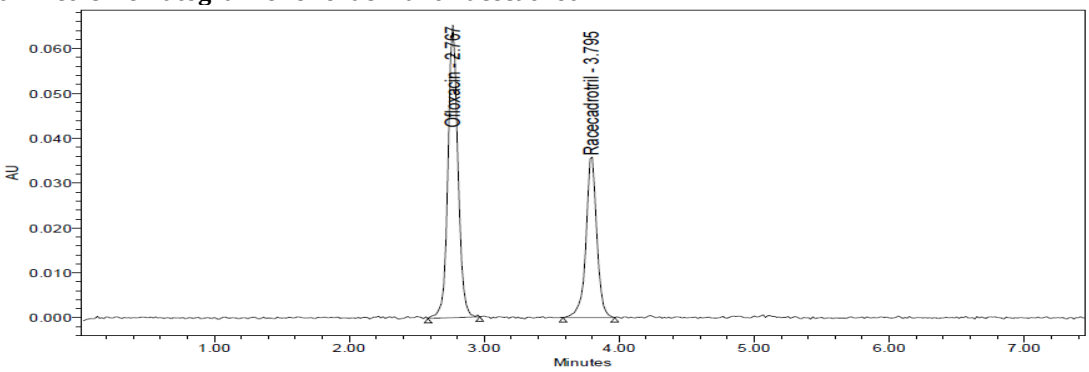


Figure 3: Linearity data curve for ofloxacin

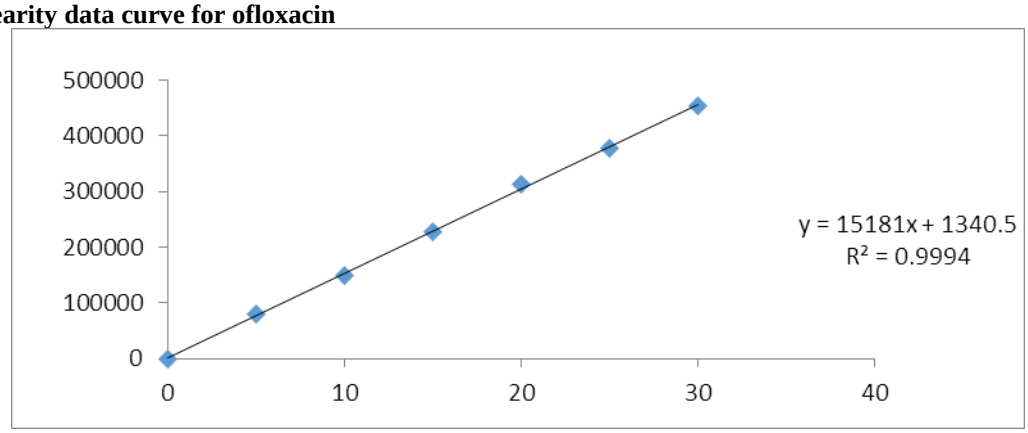


Figure 4: Linearity data curve for racecadotril

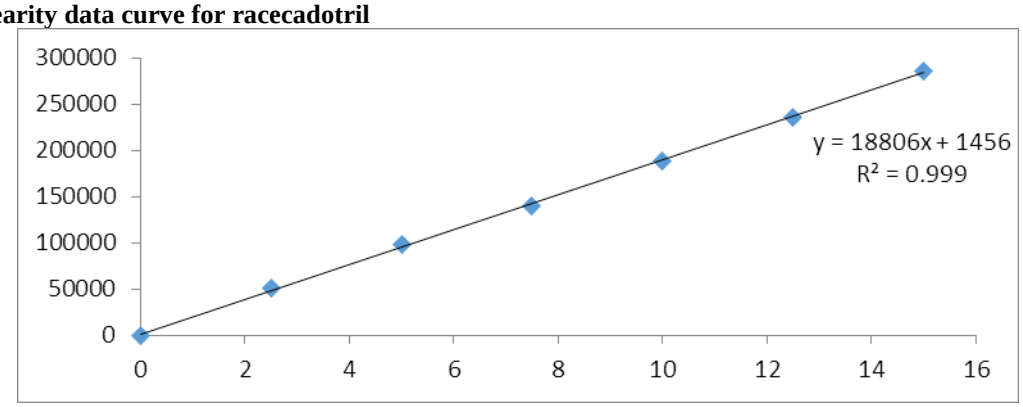


Figure 5: Overlain chromatogram for system precision

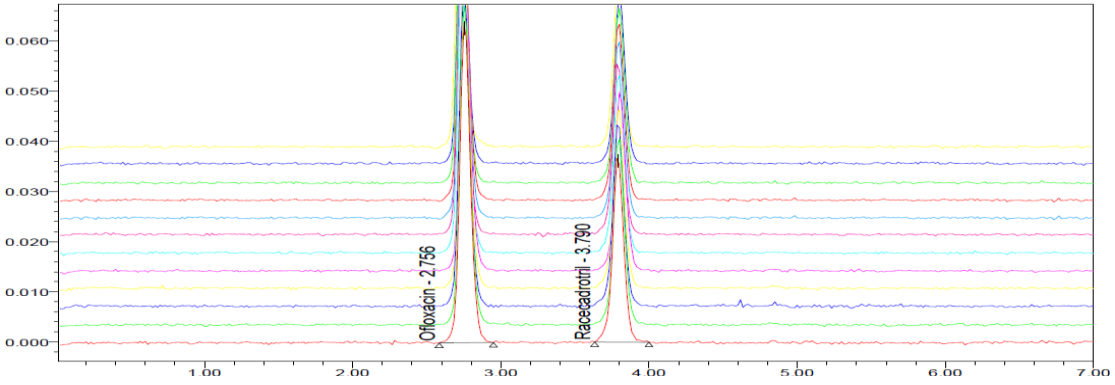
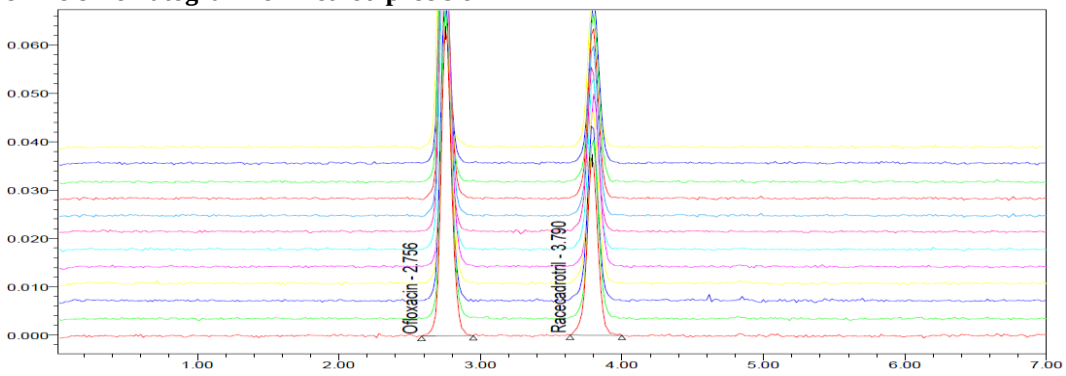


Figure 6: Overline chromatogram for method precision



CONCLUSION

A simple reversed phase HPLC method was developed and validated according to ICH guidelines for the Stability indicating RP-HPLC method for Simultaneous estimation of ofloxacin and racecadotril in tablet dosage forms. The developed RP-HPLC method was proved to be specific, accurate, robust, simple, rapid and reproducible. Because of its simplicity and short time consuming, the method can be used for routine analysis of these drugs in ear drops formulation in quality control laboratories.

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