VALIDATED RP-HPLC METHOD AND STABILITY STUDIES FOR SIMULTANEOUS ESTIMATION OF OFLOXACIN AND RACECADOTRIL IN BULK AND PHARMACEUTICAL FORMULATION

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ABSTRACT

A simple, accurate and precise stability indicating method for simultaneous estimation of ofloxacin and racecadotril in bulk and marketed formulation was developed and validated by RP-HPLC. The chromatographic separation of this method was carried out by Waters X Bridge Stainless steel C_{18} column (250 mm × 4.6 mm, 5 µm) packed with ODS using methanol: acetonitrile and water (40:40:20 V/V/V) as mobile phase. The pH of the mobile phase was 2.7, adjusted by orthophosphoric acid. The flow rate of the instrument was set at 0.8 mL min^{-1} and wavelength of UV visible detector was set at 210 nm at room temperature. The method showed good linearity and the correlation coefficients of ofloxacin and racecadotril were found to be 0.9966 and 0.9991, respectively. The mean recovery values were calculated to be 99.1 and 100.03, respectively. This method could be applied successfully for the estimation of both the drugs in bulk and pharmaceutical formulation. The stress studies for this method were performed to confirm that the specificity and stability was achieved.

Keywords: RP-HPLC, stability indicating, ofloxacin, racecadotril

INTRODUCTION

Drugs in combined form are nowadays mostly highly recommended as compared to drug in single dosage form, since they can target more than one disease in some cases. Tablet under the brand name Racigyl-O tab contains 200 mg of ofloxacin (OFL) and 100 mg of racecadotril (RAC) is used for acute symptomatic diarrhea treatment[1]. The aim of this method was to develop an accurate stability indicating analytical liquid chromatography approach, which may be served as assay method for OFL and RAC in their combined marketed formulation. A literature survey showed some available High Performance Liquid Chromatography (HPLC) analytical methods for the estimation of OFL and RAC individually or in combined form with other drugs as RP-HPLC is one of the most widely used and efficient analytical method for determining medicines for sensitivity and specificity. In addition to that, Food Drug Administration (FDA) and International Conference on Harmonization (ICH) guidelines recommend that the drug product shelf-life specification should also involve preservative content test, identification test, and limits for impurity substances. The majority of the currently available techniques rely on spectrophotometry[2-8]. To estimate the OFL and RAC simultaneously in a combination pharmaceutical dose form, however, there is no stability indicating method now available. In this work, an attempt was performed to establish an accurate, sample, and reliable method for simultaneous determination of these two drugs in their combined pharmaceutical dosage form. An isocratic reversed-phase HPLC technique for the simultaneous determination of OFL and RAC, as well as stability studies for the combined OFL and RAC was done in accordance with ICH guidelines; the method was validated.

Drug profile of ofloxacin

Ofloxacin is (±)-9-fluoro-3-methyl-10-(4-methylpiperazin-1-yl)-7- oxo-2,3-dihydro-7*H*-pyridol (1, 2, 3,-de)-1,4 benzoxazine-6- carboxylic acid[9] (Fig. 1), which belongs to the groun of synthetic broad-spectrum antibiotics. It kills bacteria by acting on topoisomerase IV and DNA gyrase which leads to prevent the excessive DNA from supercoiling during translation and transcription through inhibiting their function, it causes inhibition in

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the normal cell division[10]. It exists as yellow crystalline powder. OFL exhibits good solubility in glacial acetic acid, and has average solubility in water and methanol[11].

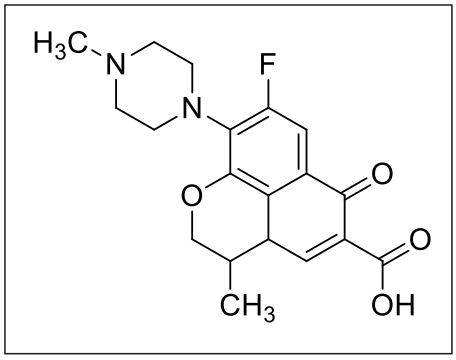

Fig. 1: Structure of ofloxacin

Drug profile of racecadotril

Racecadotril is *N*-[2-[(acetylthio) methyl]-1-oxo-3-phenyl-propanoyl)-glycine phenylmethyl ester[12]. RAC is a prodrug of the enkephalinase inhibitor type which has anti-secretory effects, causes reduction in the secretion of water and electrolytes from the intestine (Fig. 2). It also leads to reduce the amount of fluid lost from the body in the case of diarrhoea[12]. RAC has white color appearance and is freely soluble in methanol and dichloromethane[13].

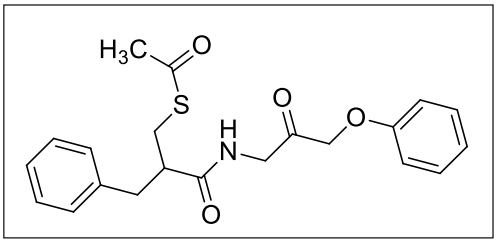

Fig. 2: Structure of racecadotril

EXPERIMENTAL

Chemicals and reagents

The standard drugs of OFL and RAC were procured from Estra Pharmaceuticals, Amritsar, Punjab and Leeford Healthcare Pvt. Ltd. Ludhiana. The RACIGYL-O tablets (Mankind), a pharmaceutical tablet dose of both drugs containing 100 mg of RAC and 200 mg of OFL, were purchased from the local market. Methanol (AR & HPLC), acetonitrile (HPLC Grade) and orthophosphoric acid (AR) were procured from SD Fine Chem. Ltd., all of the water used in this study was HPLC grade. The other chemicals were analytical- or HPLC-grade materials.

Instrumentation

The chromatographic separation was carried out by using WATERS, 2695 separation module HPLC instrument attached with Empower software and UV-VIS detector, version 2.0. The Waters X Bridge C_{18} column made of stainless steel (250 mm × 4.6 mm, 5 µm) packed with ODS chemically bounded porous silica particles were used as stationary phase for analysis. BL-220H analytical balance (Shimadzu Corporation, Japan), an ultrasonic cleaner (Frontline FS 4, Mumbai, India) and digital pH meter (LI 612 pH Analyzer, Elico Ltd., Ahmedabad), were utilized in this research.

Chromatographic conditions

The mobile phase consisted of methanol: acetonitrile and water (40:40:20 V/V/V). Adjustment of pH to 2.7 was there by the addition of few drops of orthophosphoric acid. The mobile phase was filtered through 0.22 µm membrane nylon filter and then sonicated in ultrasonic bath before its use. The injection volume was 20 µL and UV wavelength detection was set at 210 nm. All experiments were carried out at room temperature.

Standard and sample solutions preparation

Standard stock solution

The mobile phase and methanol were used as solvent system. Accurately weighed 200 mg of OFL and 100 RAC were transferred into a 100 mL volumetric flask individually and dissolved in the mobile phase to make standard stock solutions containing 2000 µg mL^{-1} and 1000 µg mL^{-1} of OFL and RAC, respectively.

Preparation of sample solutions

The individual equivalent weights of twenty tablets of OFL and RAC was evaluated and, based on average weight, required drug was transferred in volumetric flask to obtain 200 mg of OFL and 100 mg of RAC in single dilution i.e. which is based on the label claim of marketed formulation. Sonication and filtration by 0.22-micron filter paper were carried out.

Method development

The validation of optimized chromatographic conditions for this method was performed and the following parameters were evaluated, including: specificity, linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), robustness and system suitability parameters in accordance with the ICH guidelines Q2 (R1). To perform the range and linearity of this developed method, five different concentrated standard solutions

(160, 180, 200, 220, 240 µg mL^{-1}) and (80, 90, 100, 120, 140 µg mL^{-1}) of OFL and RAC, respectively, were diluted. The dilutions were prepared in triplicate. The values of peak areas of the previous dilutions were plotted against their respective concentrations. The evaluation of accuracy and precision was done by assay of spiked placebos at three concentration levels of 80 %, 100 % and 120 % of the standard concentration. The calculation of % RSD and % recovery was performed for each of the spiked placebos. LOD and LOQ of this developed method were evaluated by the study of the relationship between the standard deviation of the response and the slope observed in the linearity curve according to ICH guidelines. Robustness of the developed method was found out under a number of conditions involving wavelength of the system and pH of the mobile phase. There was no modification in the flow rate of the developed method as it was noticed to be sensitive in this method.

Forced degradation study

To test the stability-indicating qualities and specificity of the new approach, forced degradation studies were carried out. By exposing the combined OFL and RAC standard form and formulation to 5 distinct stress situations, intentional deterioration was carried out. As mentioned in Table I the result of forced degradation samples was analyzed.

Table I: Forced degradation conditions

S. No.	Forced degradation	Conditions
1	Acid hydrolysis	1 mg mL^{-1} in 0.05 N HCl at 60 °C for 1 h
2	Base hydrolysis	1 mg mL^{-1} in 0.01 N NaOH at 60 °C for 1 h
3	Oxidative degradation	1 mg mL^{-1} in 1% H_2O_2 at 60 °C for 1 h
4	Thermal degradation	1 mg mL^{-1} in 60 °C for 1 h
5	Photo degradation	Normal sunlight for 8 h

RESULTS AND DISCUSSION

Method development

The ultimate goal of this study was to establish stability indicating method by HPLC for estimation of OFL and RAC in a short run time (less than 10 minutes). The stationary of the column and the constitution mobile phases play an important role on peak shape, symmetry, theoretical plates and resolution. To get symmetrical peaks having good peak purity and resolution, various chromatographic conditions were checked and optimized for the estimation of OFL and RAC; such as mobile phases with different ratios, pH and different packed stationary phases, etc. The pKa values of both OFL and RAC are strong acidic, for that it may get ionized in a solution of pH below 6.0. As result, OFL and RAC may get ionized as positive ions (+) in the mobile phase (pH below 6). Absorption maximas of OFL and RAC were observed by UV spectrum at 294 and 231 nm respectively. The Waters X Bridge Stainless steel C_{18} column (250 mm ×4.6 mm, 5 µm) packed with ODS chemically bounded porous silica particles was used as stationary phase for analysis. In all of the preceding attempts, broad characteristic peaks were obtained though using different ratios of methanol/acetonitrile and water. No improvement of retention times and in the peak shapes was seen even when the temperature of column was increased to 40 °C. For that acetonitrile was added to methanol and water. Different ratios of methanol, acetonitrile and water were used. The best peaks were obtained in mobile phase consists of acetonitrile: methanol: water (40:40:20 V/V/V). Different pH values were tried to improve the peak shape (2.5, 2.7, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5). Attempts were tried at different wavelengths like 210, 230 and 290 nm. Best peak shapes were obtained at pH 2.7 and at wavelength of 210 nm. Flow rate plays important role in this method. So, 0.8, 0.9 and 1.0 mL min^{-1} were tried. 0.8 mL min^{-1} was investigated to be the most suitable flow rate for this method. So, the optimized mobile phase was of acetonitrile: methanol: water (40:40:20 V/V/V) with pH 2.7 adjusted by orthophosphoric acid and with 0.8 mL min^{-1} flow rate. Based on the optimized mobile phase, a sharp and symmetrical characteristic peaks of OFL and RAC were obtained on X bridge stainless steel C_{18} column (250 mm X 4.6 mm, 5 µm) packed with ODS

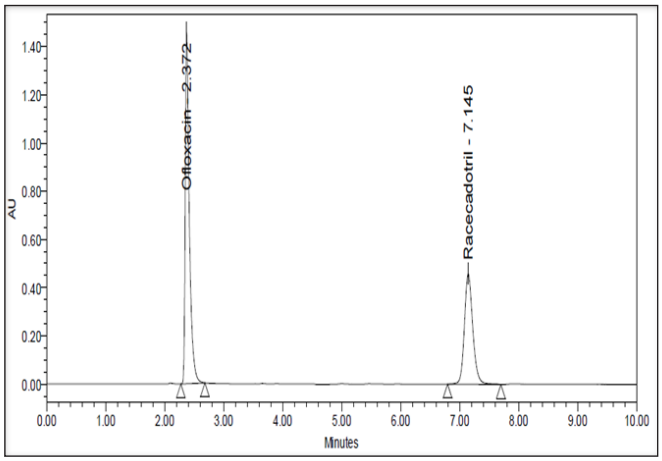

Fig. 3: RP-HPLC chromatogram of OFL and RAC 200 µg mL^{-1} and 100 µg mL^{-1}, respectively

chemically bounded porous silica particles with 0.8 mL min^{-1} flow rate. Chromatogram of typical HPLC obtained through the simultaneous estimation of OFL and RAC is given in Fig. 3.

System suitability

For the evaluation system suitability parameters, six replicates of mixed standard solution of OFL and RAC were injected. It was done compared with USP limits as give in Table II.

Table II: System suitability studies for OFL and RAC standard solution (n=6) in the optimized RP-HPLC method

System suitability parameter	Limit	OFL	RAC
Retention time	-	2.391	7.265
% R.S.D. of R_t	-	2.072	0.176
Mean peak area	-	6978500	4510478
% R.S.D. of peak area	≤ 2.0	0.978	0.090
Peak asymmetry	≤1.5	1.11	1.21
Resolution	2 to 20	-	4.874
Mean number of theoretical plates	≥ 2000	4458	2568

Method validation

Before being used, any optimised technique has to be validated. System suitability testing was done in accordance with ICH's Q2 criteria for the validation of analytical methods (R1). The validation investigations were carried out in accordance with the aforementioned points.

Specificity

The studies of specificity for the developed method proved that the interference with the main peaks is absent, as no another peak is seen at the specific retention time (RT) (2.37 and 7.145 min) of OFL and RAC. In addition to that, specificity studies indicated that there is no interaction between the drug with each other and the acceptance level was below 2.0 % of RSD (Fig. 4).

Range and linearity

Five different concentrations (160, 180, 200, 220, 240 µg mL^{-1}) and (80, 90, 100, 120, 140 µg mL^{-1}) of the mixed solution of OFL and RAC respectively were prepared for the determination of linearity parameters. The standard plot was studied to obtain the calibration curve. The linear regression equations for OFL and RAC were found to be y=39416x - 795812, and y= 23423x-179053, respectively. The regression coefficient (R2) values for OFL and RAC were shown to be 0.9966 and 0.9991, respectively. The findings demonstrated that within the chosen concentration range, there was a strong association between peak area and drug concentration.

Table III: Summary of resulting linearity and LOD and LOQ of HPLC

Validation parameter	OFL	RAC
Absorption maxima (nm)	210	210
Linearity range	160-240 µg mL^{-1}	80-120 µg mL^{-1}
Coefficient of regression (R^2)	0.9966	0.9991
Regression equation	39416x - 795812	23423x-179053
Slope (b)	39416	23423
Intercept (a)	795812	179053
Limit of detection	4.223 µg mL^{-1}	5.740 µg mL^{-1}
Limit of quantification	12.667 µg mL^{-1}	17.221 µg mL^{-1}

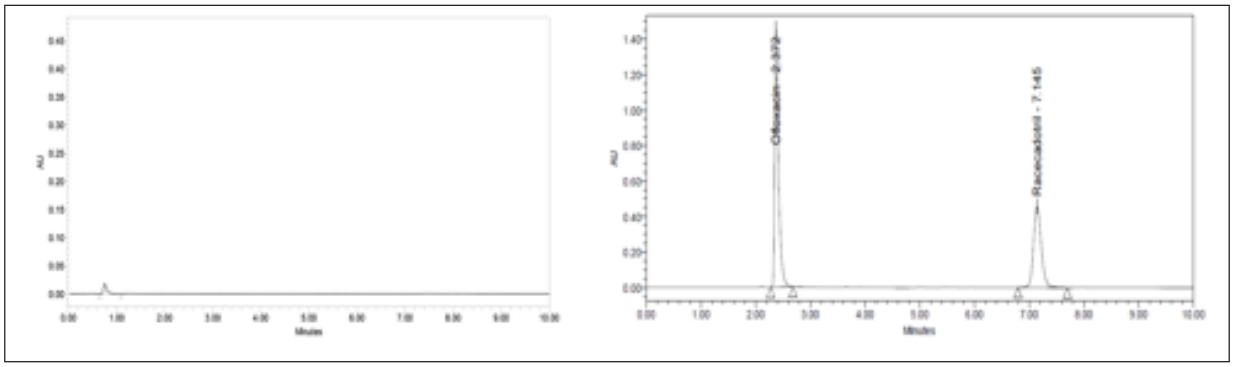

Fig. 4: Blank chromatogram Vs OFL and RAC chromatogram in sample showing no interferences

The assay method's linearity and repeatability were supported by the results. Regression characteristics of this developed method are given in Table III.

Precision

The intra-day precision of the optimized method was investigated by sample preparation of tablets of the same batch three concentrations (triplicate). The inter-day precision was also investigated by analyzing the tablets for three consecutive days in triplicate every day. The RSD (%) value explained that the method is précised (RSD less than 2%). The inter and intra day precision data are given in Table IV.

Table IV: Intermediate precision of OFL and RAC at 200 µg mL^{-1} and 100 µg mL^{-1} conc.

Parameter	OFL	RAC
Intra-day (%RSD)	1.168	0.91
Inter-day (%RSD)	0.89	1.29

Accuracy

The accuracy studies were carried out by the addition of known amounts of the APIs in the placebo at three concentrations: 80%, 100% and 120% of the label claim of the dosage form. The preparation of the samples was carried out for each recovery level. After the analysis of the solutions, their % recoveries were calculated by the help of the calibration curve. The mean values obtained for the recovery of OFL and RAC were calculated to be 99.15 %, and 100.59 %. According to these results, it is concluded that the excipients does not show any interference. The accuracy results of this method are shown in Table V.

Table V: Results of recovery studies of OFL and RAC by HPLC

Drug	% Simulated dosage nominal	Amount of spiked (µg mL^{-1})	% Recovery
OFL	80	200	98.891
	100	200	99.1
	120	200	100.9
RAC	80	100	100.106
	100	100	100.03
	120	100	99.04

Limit of detection and limit of quantitation

The LOD and LOQ give prediction about the method sensitivity. The LOD and LOQ for OFL and RAC were calculated at a signal-to-noise ratio of 3:1 and 10:1, respectively. It was performed by a series of injection for the diluted solutions having known concentrations. The LOD for OFL and RAC were 4.223 µg mL^{-1} and 0. 5.740 µg mL^{-1}, respectively, whereas LOQ were 12.667 µg mL^{-1} and 17.221 µg mL^{-1}, respectively. The values obtained indicate that the method has good sensitivity. The LOD and LOQ values are summarized in Table III.

Robustness

The robustness of the developed method was determined by studying its ability to remain valid even by applying small changes in the method development parameters including pH of the mobile phase, percent organic content, injection volume, flow rate, temperature, and buffer concentration. A change of ± 2nm in the detection wavelength and ± 0.2 units of the pH of the mobile phase was applied. The results are given in Table VI.

Table VI: Results of robustness determination of OFL and RAC by HPLC

Parameter		% RSD	
		OFL	RAC
Wavelength ± 2	208 nm	1.91	1.417
	212 nm	1.163	1.84
pH ± 0.2	2.5	0.94	1.336
	2.9	0.66	1.8

Analysis of tablets formulations

This optimized method was applied for analyzing OFL and RAC in marketed formulation (Tablet). The recovered amounts were given as % of the label claim of the marketed formulation. Analysis of marketed tablets (Racigyl-O, Mankind) was carried out using an optimized method. The average percentage of drug contents of tablets obtained by the developed method for OFL and RAC were noted to be 100.1% and 100.5%, respectively. The results are given in the Table VII. These values comply with the official specifications.

Table VII: Analysis of marketed tablets

Drug	Label claim (mg tab^{-1})	Amount found (mg tab^{-1})	%Assay
OFL	200	100.012	100.1
RAC	100	100.07	100.5

Forced degradation study

All the stress conditions applied in this method were enough to cause degradation of OFL and RAC in the bulk or in the pharmaceutical formulation. The results of such

Table VIII: Percentage degradation in OFL+RAC (Pure form)

Degradation condition	OFL				RAC			
	R_t (min)	Purity angle	Purity threshold	% drug degraded	R_t (min)	Purity angle	Purity threshold	% drug degraded
Acid	2.37	1.651	2.180	20	7.17	0.180	0.192	15.6
Base	2.37	1.112	1.890	12.7	7.17	0.158	0.186	8.6
Oxidation	2.37	1.063	1.751	7.2	7.17	0.205	0.318	2.0
Thermal	2.37	1.21	1.602	4.3	7.17	0.256	0.276	1.1
Photolytic	2.37	0.99	1.699	3.2	7.17	0.234	0.325	2.5

Table IX: Percentage degradation in OFL+RAC (Test)

Degradation condition	OFL				RAC			
	R_t (min)	Purity angle	Purity threshold	% drug degraded	R_t (min)	Purity angle	Purity threshold	% drug degraded
Acid	2.37	1.643	2.221	19.9	7.17	0.172	0.209	15.6
Base	2.37	1.221	1.198	12.7	7.17	0.185	0.231	8.5
Oxidation	2.37	1.187	1.823	7.2	7.17	0.198	0.412	2.0
Thermal	2.37	1.25	1.689	4.3	7.17	0.287	0.245	1.1
Photolytic	2.37	1.13	1.716	3.1	7.17	0.254	0.311	2.5

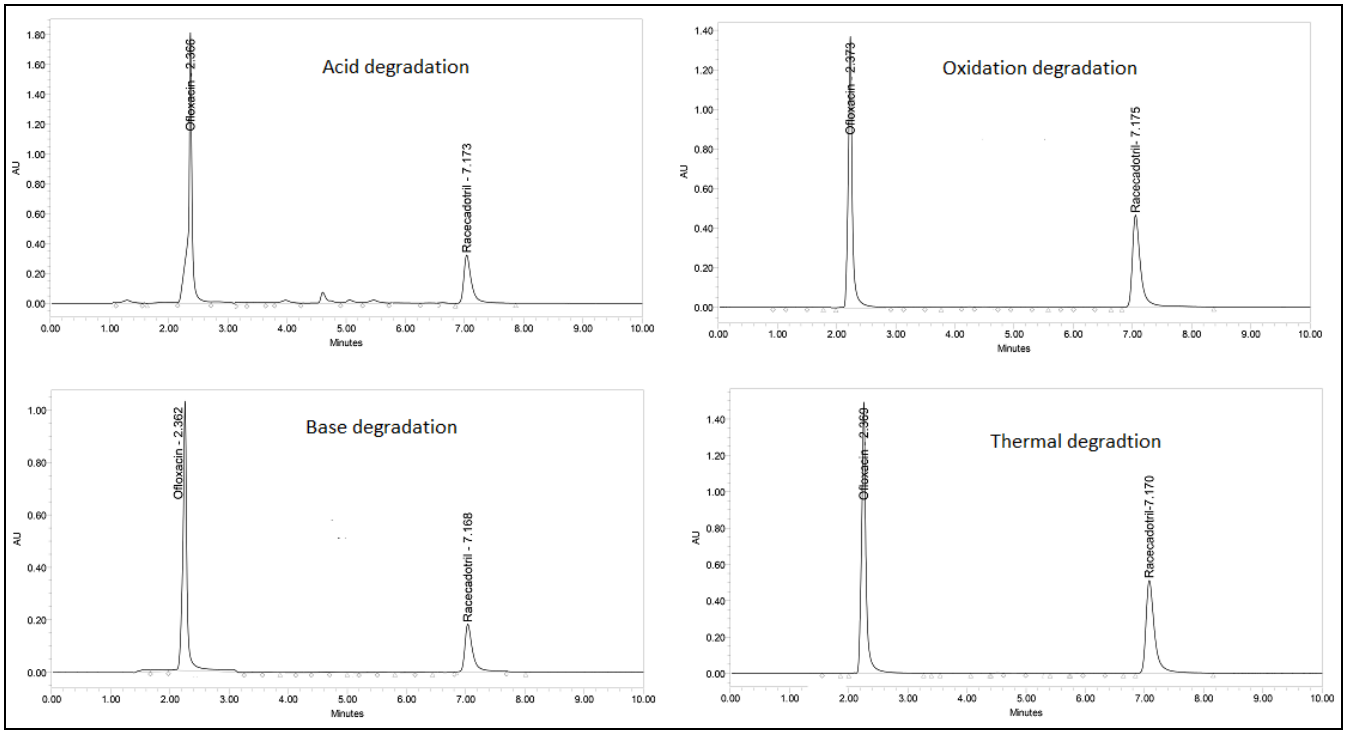

Fig. 5: Degradation studies for OFL+RAC (Pure Form)

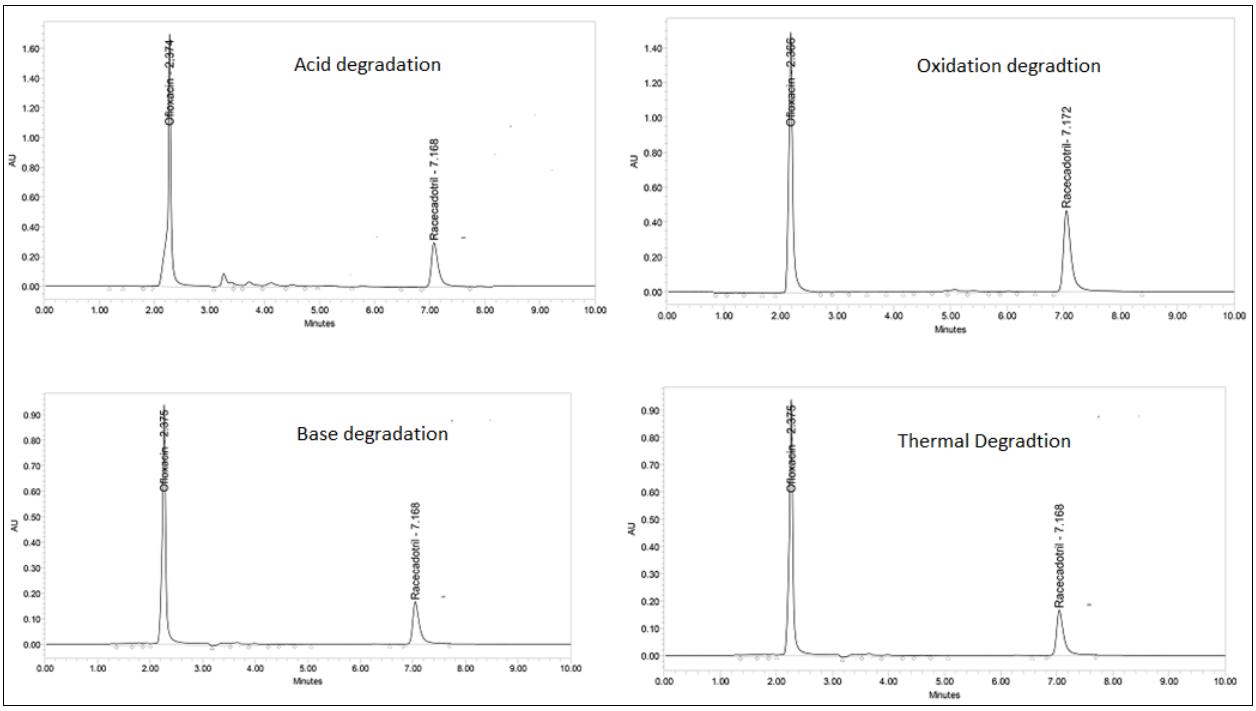

Fig. 6: Degradation studies for OFL+RAC (Test sample)

stress conditions of OFL and RAC are shown in pure form and tablet dosage form in Table VIII and Table IX, respectively. OFL and RAC were degraded and residual concentrations were 80 % and 84.4 % respectively, when exposed to 0.05 N HCl used at room temperature for 1 h. OFL and RAC were degraded and residual concentrations were 87.3 % and 91.4 %, respectively when exposed to 0.01 N NaOH used at room temperature for 1 h. OFL and RAC were degraded and residual concentrations were 92.3 % and 98 % respectively under 1% H_2O_2 at 60 °C for 1 h. OFL and RAC were degraded and 95.7% and 98.9% remained under 60 °C for 1 h. OFL and RAC were degraded and 96.7% and 97.5% remained under the sunlight for 8 h. From these stress studies, it was concluded that OFL and RAC were not stable in strongly basic and strongly acidic condition and OFL was not stable in oxidative condition while RAC is stable in oxidative condition. It was also concluded that OFL and RAC were stable in thermal and photolytic conditions and developed method can be considered highly specific for intended use. The chromatograms of stress studies of OFL and RAC are given in Fig. 5 and Fig. 6 for pure drugs and for the tablet dosage form.

CONCLUSION

A reliable isocratic RP- HPLC method for simultaneous estimation of OFL and RAC was developed and validated as per ICH guidelines. Validation parameters indicate that the HPLC developed method is specific and is also having good linearity in the proposed range of the analysis as well as precise, accurate, and linear. As the developed method exhibits a good recovery percentage of tablet formulations, it tells that there is no interference of the excipients in the estimation. The RSD (%) was also less than 2, which indicates a high degree of precision of the developed method. The method was also found to be robust with respect to pH value and composition of wavelength. In addition, this method has a simple isocratic elution and easy extraction procedure, giving rapid and cost-effective analysis of the drugs. The proposed method can be used for routine analysis of OFL and RAC in combined dosage form and in the quality control in bulk as well. The developed method is also qualified enough and reliable to detect any expected change in the drug product assay during stability studies. Peak purity for OFL and RAC peaks was evaluated and it indicates that they are pure from any other excipients or impurities or derivative materials.

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