

Validated Stability Indicating RP-HPLC Method for Simultaneous Estimation of Ofloxacin and Cefixime in their Combined Dosage Form

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Abstract

The objective of the current study was to develop and validate a simple, accurate, precise and selective stability-indicating gradient reverse phase high performance liquid chromatographic method for simultaneous estimation of Ofloxacin and Cefixime in pharmaceutical formulation in presence of degradation products. The chromatographic separation of Ofloxacin and Cefixime was achieved on Shimadzu LC-20AT series HPLC having C18-ODS bonded column (250 × 4.6 mm, 40 °C, 10 µL) using UV/Visible detector at 276 nm. The optimized mobile phase was consisted of a methanol: phosphate buffer (50:50) at a flow rate of 1.0 ml/m. The retention times were 4.799 and 1.602 m for Ofloxacin and Cefixime respectively. The proposed method provided linear responses within the concentration ranges 5-25 µg/ml for Ofloxacin and Cefixime both. The limit of detection (LOD) and limit of quantification (LOQ) values were found to be 0.0259, 0.078 µg/ml and 0.0206, 0.062 µg/ml for Ofloxacin and Cefixime F respectively. The developed method was validated as per ICH guidelines with respect to specificity, linearity, accuracy, precision, robustness and ruggedness. The studies data revealed that developed method was convenient, fairly reliable, sensitive, less expensive and reproducible.

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INTRODUCTION

Ofloxacin (OFL) are wide spectrum quinolones and Cefixime (CEF) is a third-generation cephalosporin with a wider spectrum of activity against gram-positive and gram-negative bacteria and anaerobics (Ozyüncü *et al.*, 2010). OFL chemically is a fluorinated carboxy quinolone, is a racemate, (±)- 9-fluoro-2, 3-dihydro-3-methyl-10-4-methyl-1-piperazinyl)-7-oxo-7H-pyrido[1,2,3-de]-1,4-benzoxazine- 6-carboxylic acid (Figure 1A) that is active against both Gram-positive and Gram-negative bacteria. It is mainly used for the treatment of urinary tract infection and sexually transmitted diseases and also are good candidates for the development of more powerful treatment of leprosy (Chopra *et al.*, 2001 and Veziris *et al.*, 2013). OFL is a fluoroquinolone antibiotic with activity against various micro-organisms causing Acute exacerbation of chronic obstructive pulmonary disease (AECOPD) (Yoon *et al.*, 2013). It inhibits DNA gyrase, a type II topoisomerase, and topoisomerase IV. CEF is chemically known as (6R, 7R)-7-[[2-(2-amino-1,3-thiazol-4-yl)2(carboxymethoxyimino) acetyl]amino]-3-ethenyl-8-oxo-5-thia-1-azabicyclo[4.2.0] oct-2-ene-2-carboxylic acid (Figure 1B) (Ali Ahmed *et al.*, 2013). CEF is the extended-spectrum cephalosporins, are currently the recommended first-line antimicrobials in most countries worldwide, can be used for the treatment of gonorrhoea as an empirical first-line therapy (Sethi *et al.*, 2013 and Olsen *et al.*, 2013). Synthesis of cell wall inhibit by the bactericidal action of cephalosporin, which inhibits the final

transpeptidation step of the peptidoglycan synthesis in the bacterial cell wall, thus inhibiting biosynthesis of cell wall assembly by arresting, resulting in bacterial cell death.

Elbashir *et al.*, 2012, Khan *et al.*, 2011, Nemutlu *et al.*, 2009, Pulgarín *et al.*, 2013, Hubicka *et al.*, 2013, Lalitha Devi *et al.*, 2009, Patel *et al.*, 2011, Shervington *et al.*, 2005 and Deekonda *et al.*, 2014 reported various analytical methods, like RP-HPLC/UV detection method, Spectrofluorimetric method, Capillary Electrophoresis, UPLC-MS/MS. Validated stability-indicating RP-HPLC methods have been already reported. Fixed dose combination of a fluoroquinolone antibiotic (200 mg) and an antimicrobial agent CEF (200 mg) would take care of both the components of antimicrobial which give greater clinical efficacy. The present article introduces a simple, specific, precise and rapid RP- HPLC method for simultaneous estimation of OFL and CEF in combined dosage form. The developed method is further validated and from the validation study, it was found that the method is specific, accurate, precise, and reproducible.

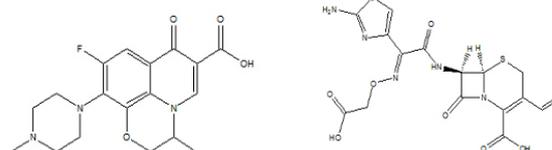


Figure 1: Chemical structure of (A) OFL and (B) CEF.

MATERIALS AND METHODS

Instrumentation

The HPLC system consisted of a model LC-20AT (Prominence) Shimadzu, Japan was used having UV (SPD-20A) Prominence UV/Visible detector. Column Phenomenex Gemini C18 –ODS bonded column of length 250 mm and an inner diameter 4.6 mm was selected for analysis. The particle size of the stationary phase was 5 μ . The Mobile phase was a degassed and filtered (0.45 μ m, Millipore, Watford, UK) methanol: phosphate buffer (50:50) Flow rate 1 ml/m. Frontline Ultra Sonic Cleaner Fs-10 Sonicator was used for sonication. The column temperature was maintained at 40°C using a model 7716 HPLC column block heater CTO-20AC.

Material

The gift samples of drugs i.e. OFL and CEF was provided by Anupam biotech, Manpur, Bhand, India and was confirmed by the official methods. Methanol, phosphate buffer and all other chemicals were purchased from Himedia labs Mumbai. All the chemicals of HPLC and analytical grades and used without any further purification.

Standard Solution Preparation

Methanol: Phosphate buffer (50:50) was used as diluent. The stock solutions of OFL and CEF (1000 μ g/ml of both) were prepared by dissolving an appropriate amount of analyte in diluent, separately. Working standard solution was prepared in diluent from mixing above stock solutions of OFL and CEF with final concentration of 100 μ g/ml.

Sample Solution Preparation

Tablet powder equivalent to 100 mg of OFL and CEF was transferred into 100 ml volumetric flask, added 35 ml of diluent and ultrasonicated for 20 m, and then diluted to volume with diluent. This solution was further diluted with diluent to give a solution containing 100 μ g/ml of OFL and CEF each.

Analysis of Tablet Formulation

Twenty tablets were weighed accurately, and a quantity of tablet powder equivalent to 200 mg OFL and 200 mg CEF was weighed and aliquate of concentration 10 μ g/ml was formed with mobile phase, ultrasonicated for 5 m. The solution was filtered through Whatman (Florham Park, NJ) No. 41 paper. The tablet sample solution was injected, the chromatogram was obtained, and the peak areas were recorded.

Validation of the Method

System Suitability

System suitability was determined before sample analysis from six replicate injections of the standard solution containing 10 μ g/ml of OFL and CEF each in methanol: phosphate buffer (50:50) into the chromatograph to determine peak areas, retention times (*t_R*) and chromatographic relative standard deviation (%RSD). The retention factor (*k*), theoretical plates (*N*), tailing (*T_f*) and peak asymmetry (*A_s*) chromatographic parameters were also evaluated.

Selectivity and Specificity

The specificity is performed to check ability of method to measure the analyte accurately and specifically in presence of other components or drugs. The other

compound should not interfere with drug and should be estimated separately.

Linearity, Concentration Range, Limits of Detection and Quantitation

Linearity test solutions were prepared by diluting the stock solutions to the required concentrations. The solutions were prepared in mobile phase at six concentration levels from 5-25 μ g/ml for OFL and CEF both. Calibration curves were plotted between the responses of peak versus analyte concentrations. The coefficient correlation, slope, y-intercept of the calibration curve and % bias at 100% response are reported. For LOD and LOQ, 1 μ g/ml of solution of drugs was prepared from standard stock solution of 100 μ g/ml by diluting appropriate volume with mobile phase. Five standard solutions for CEF 0.02, 0.04, 0.06, 0.08 and 0.10 μ g/ml and for OFL 0.05, 0.06, 0.07, 0.08, 0.09 and 0.10 μ g/ml were prepared in mobile phase from 1 μ g/ml of solution, area was noted. The LOD and LOQ of OFL and CEF by proposed methods were determined using calibration standards.

Precision and Accuracy

The intra-day precision and accuracy for the proposed method were studied at five concentration levels for each compound using three replicate determinations for each concentration within one day. Similarly, the inter-day precision and accuracy were tested by analyzing the same three concentrations for each compound using three replicate determinations repeated on five days. The percentage recoveries for both components were calculated using the corresponding regression equations and they were satisfactory. The percentage relative standard deviation (% RSD) and percentage relative error (% Er) did not exceed 2.0% proving the high repeatability and accuracy of the developed method % indicating that the developed method was accurate for the determination of OFL and CEF in pharmaceutical formulation.

Robustness and Ruggedness

As per ICH guidelines, small, but deliberate variations in concentration of the mobile phase were made to check the accuracy of the method. These variations did not cause any significant difference in the resolution of HPLC method.

Analysis of the Pharmaceutical Dosage Form

The optimized RP-HPLC procedure was applied for the assay of this drug combination in the pharmaceutical formulation available in the local market (Xiomix-O, Azine healthcare Pvt. Ltd.) The active ingredients were extracted with the same solvent used for the preparation of the standard stock solutions, then dilution was made to reach concentration levels within the specified ranges. The active ingredients eluted at their specific retention times and no interfering peaks were observed from any of the inactive ingredients. The photodiode array detection enabled peak purity verification where no signs of coelution from any of the inactive components were detected. Recoveries were calculated using both external standard and standard addition methods. The assay results of OFL-CEF laboratory-prepared mixtures and pharmaceutical preparation revealed satisfactory accuracy and precision as indicated from % recovery, SD, and RSD% values.

Stability Studies

Stability study and Force degradation

The purpose of the stability or degradation study was to ensure the peak purity of the OFL and CEF in the presence of degradation products and establish the stability indicating ability of the method. Under basic, acidic, peroxide and thermal degradation conditions. Studies were performed at 20 µg/ml concentration of OFL and CEF on tablets to provide an indication of the stability-indicating property and specificity of proposed method. By using UV/Visible detector, peak purity test was carried out for the OFL and CEF peaks on samples. All the solutions were prepared by dissolving the drug product in small volume of stressing agents. After degradation, these solutions were diluted with diluent to yield stated OFL and CEF concentration of about 20 µg/ml. Conditions employed for performing the stress studies were as follows (Samanthula *et al.*, 2013; Roy *et al.*, 2013 and Awzalewski *et al.*, 2014)

Base Degradation

Tablet powder equivalent to 20 mg of OFL and CEF was accurately weighed and dissolved in 20 ml of diluent, added 5 ml 0.05 N NaOH and the mixture was kept at 70°C for 1 h. The solution was brought to ambient temperature, neutralized by addition of 5 ml 0.1 N HCl and diluted to 100 ml with diluent. 5 ml of this solution was diluted to 50 ml with diluent.

Acid Degradation

Tablet powder equivalent to 20 mg of OFL and CEF was accurately weighed and dissolved in 20 ml of diluent, added 5 ml 0.1 N HCl and the mixture was kept at 70°C for 1 h. The solution was brought to ambient temperature, neutralized by addition of 5 ml 0.05 N NaOH and diluted to 100 ml with diluent. 5 ml of this solution was diluted to 50 ml with diluent.

Oxidative Degradation

Tablet powder equivalent to 20 mg of OFL and CEF was accurately weighed and dissolved in 20 ml of diluent, added 5 ml of 1% hydrogen peroxide. The mixture was kept at room temperature for 1 h and diluted to 100 ml with diluent. 5 ml of this solution was diluted to 50 ml with diluent.

Hydrolytic Degradation

Tablet powder equivalent to 20 mg of OFL and CEF was accurately weighed and dissolved in 15 ml of diluent, added 15 ml of water and the mixture was kept at 70°C for 24 h. The solution was brought to ambient temperature and diluted to 100 ml with diluent. 5 ml of this solution was diluted to 50 ml with diluent.

Thermal Degradation

Tablet powder equivalent to 20 mg of OFL and CEF was stored at 100°C for 12 h, dissolved and diluted to 100 ml with diluent. 5 ml of this solution was diluted to 50 ml with diluent.

RESULTS AND DISCUSSION

Method Development and Optimization

The objective of the chromatographic method was to separate and quantitate OFL and CEF in presence of degradation products. Two component formulations have gained a lot of importance as there is greater patient acceptability, increased potency and decreased side effect. A calibration curve was plotted for the OFL and CEF range of 5-25 µg/ml for both to allow an assessment of the assay and the plot equation was used for quantization. On the optimization of gradient programme, OFL and CEF peaks were well resolved from degradation products. Based on these experiments, the final optimized conditions are described below. Detection wavelength was selected by overlain spectra at 276 nm. The injection volume was 20 µl. The typical retention time of OFL and CEF 4.658 and 2.692 m (Figure 2). The regression value, intercept values, determination coefficient (R^2) and the %RSD were evaluated. The limits of detection (LODs) and quantitation (LOQs) were determined (n=6) by successive dilutions of the lowest calibration plot point. Repeatability and intermediate precision for OFL and CEF solutions (n=6) were performed in six days and by a single analyst. Recovery was determined through the addition of OFL and CEF at 50, 75, 100, 125 and 150 % to a tablet formulation using methanol: phosphate buffer (50:50). The robustness was verified through variation in the mobile phase and flow rate for OFL and CEF standard and tablet solutions at 10 µg/ml (n=6) (Figure 2).

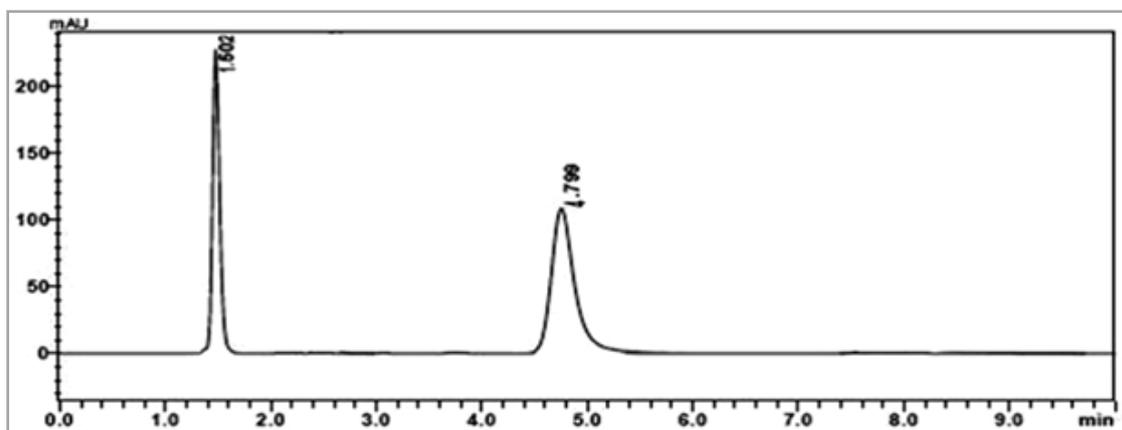


Figure 2: Preliminary chromatograms of OFL and CEF solution prepared in methanol: phosphate buffer (50:50) as the mobile phase (1.0 ml/min, C18 –ODS column (250×4.6 mm, 40°C, 20 µL).

Validation of the Method

Result shows that an excellent correlation existed between peak area and concentration of OFL and CEF. The linearity range was found to be 5-25 µg/ml for both

drug and data was shown in Table 1. The LOD values were 0.0259 and 0.0206 µg/ml and LOQ values were 0.0787 and 0.0625 µg/ml for OFL and CEF, respectively and shown in table 2. For the determination of accuracy,

the sample was spiked with standard and % recovery was calculated. The method was found to be accurate and precise and data was shown in Table 3. Table 4 shows the robustness with respect to change in ratio of mobile phase. The assay results of OFL-CEF laboratory-prepared mixtures and pharmaceutical preparation revealed satisfactory accuracy and precision as indicated from % recovery, SD, and RSD% values and data was shown in Table 5 and 6. It is evident from these results that the proposed method is applicable to the assay of this drug combination with a satisfactory level of selectivity, accuracy, and precision.

Table 1: System suitability parameters

Parameters	OFL	CEF
Calibration range ($\mu\text{g/ml}$)	5-25	5-25
Retention time (min)	4.799	1.602
K	4.09	1.32
T _f	1.57	1.83
A _s	1.18	1.27
Resolution	4.85	2.73

Table 2: Evaluation of linearity data, LOD and LOQ

Parameters	OFL	CEF
Detection wavelength (nm)	276	276
Linearity range ($\mu\text{g/ml}$)	5-25	5-25
Correlation coefficient	0.99706	0.9970
Intercept	150577	250577
Slope	99101	99101
Detection limit ($\mu\text{g/ml}$)	0.0259	0.0206
Quantitation limit ($\mu\text{g/ml}$)	0.078	0.062

CEF and OFL were found unstable under acidic, basic, peroxide, aqueous and thermal condition and degraded slightly under thermal degradation. Peak purity test was carried out for the OFL and CEF peak by using UV/Visible detector in stress samples analysis. CEF was found stable but OFL degrades significantly. The purity angle was within the purity threshold limit in all of the stressed samples, demonstrating the homogeneity of the peaks. A summary data of stress study is shown in Table 7 and chromatogram shown in Figure 3a-3e.

Table 3: Precision and accuracy for the determination of OFL and CEF in bulk form using the RP-HPLC method

Analyte	Nominal Value ($\mu\text{g/ml}$)	Intra day			Inter day		
		% Estimated \pm % SD ^a	%RSD	%Er	%Estimated \pm % SD	% RSD	%Er
OFL	50	49 \pm 0.42	0.86	-2.0	49.3 \pm 0.51	1.03	-1.40
	75	76.1 \pm 0.90	1.18	1.46	74.2 \pm 0.89	1.19	-1.07
	100	100.18 \pm 1.63	1.63	0.18	99.1 \pm 1.1	1.11	-0.9
	125	125.4 \pm 0.81	0.65	0.32	124.2 \pm 1.3	1.05	-0.81
	150	149.4 \pm 0.32	0.21	-0.4	148.1 \pm 0.42	0.28	-1.27
CEF	50	49.63 \pm 0.43	0.87	0.74	49.7 \pm 0.81	1.63	-0.6
	75	76.38 \pm 1.16	1.52	1.84	74.1 \pm 1.1	1.48	-1.2
	100	100.19 \pm 0.24	0.24	0.19	100.1 \pm 0.71	0.72	0.1
	125	124.07 \pm 0.61	0.49	-0.81	124.3 \pm 0.81	0.65	-0.56
	150	149.6 \pm 0.42	0.28	-0.27	148.2 \pm 0.32	0.22	-1.2

Table 4: Robustness result of HPLC method

Variation in Chromatographic Condition	OFL			CEF		
	t \pm SD ^a	RSD (%)	Er (%)	t \pm SD ^a	RSD (%)	Er (%)
Methanol:phosphate buffer (60:40)	4.498 \pm 0.21	0.47	-6.27	1.580 \pm 0.03	1.89	-1.37
Methanol:phosphate buffer (70:30)	4.575 \pm 0.01	0.22	-4.66	1.432 \pm 0.02	1.39	-10.6
Methanol:phosphate buffer (40:60)	4.698 \pm 0.08	1.70	-2.10	1.421 \pm 0.01	0.70	-11.3
Flow rate 1.5ml/min	4.632 \pm 0.10	2.26	-3.47	1.692 \pm 0.01	0.59	5.61
Flow rate 1.1ml/min	4.610 \pm 0.03	0.68	-3.93	1.542 \pm 0.02	1.29	-3.74
Flow rate 1.8ml/min	4.532 \pm 0.09	2.07	-5.56	1.367 \pm 0.03	2.19	-14.7

Table 5: Determination of OFL-CEF laboratory-prepared mixtures using the proposed RP-HPLC method

Nominal value ($\mu\text{g/ml}$)		Found \pm SD ^a ($\mu\text{g/ml}$)		RSD(%)		Er (%)	
OFL	CEF	OFL	CEF	OFL	CEF	OFL	CEF
25	25	24.78 \pm 0.41	25.08 \pm 0.44	1.65	1.75	-0.88	0.32
25	75	24.89 \pm 0.06	74.89 \pm 0.36	0.24	0.48	-0.44	-0.15
50	25	49.34 \pm 0.76	24.78 \pm 0.25	1.54	1.00	-1.32	-0.88
50	50	48.87 \pm 0.45	49.54 \pm 0.38	0.92	0.76	-2.26	-0.92
75	25	76.02 \pm 0.43	24.81 \pm 0.31	0.56	1.24	1.36	0.76

Table 6: Analysis of OFL-CEF mixture in its pharmaceutical preparation by the proposed RP-HPLC method

	External standard		Standard addition	
	OFL	CEF	OFL	CEF
% Recovery	100.02	99.87	98	99.26
\pm SD	0.41	0.10	0.42	0.81
%RSD	0.01	0.20	0.86	1.63

Table 7: Summary of forced degradation results

Stress condition	OFL		CEF	
	% Degradation	Purity Threshold	% Degradation	Purity Threshold
Base hydrolysis 0.05 N NaOH at 70°C, 1 h	10.4	0.257	3.8	0.098
Acid hydrolysis 0.1 N HCl at 70°C, 1 h	18.4	0.209	6.5	0.089
Oxidation 1% H ₂ O ₂ at RT, 1 h	17.9	0.202	3.3	0.059
Hydrolytic Water at 70°C, 1 h	5.6	0.212	8.2	0.089
Thermal 100°C, 12 h	11.5	0.209	0.9	0.026

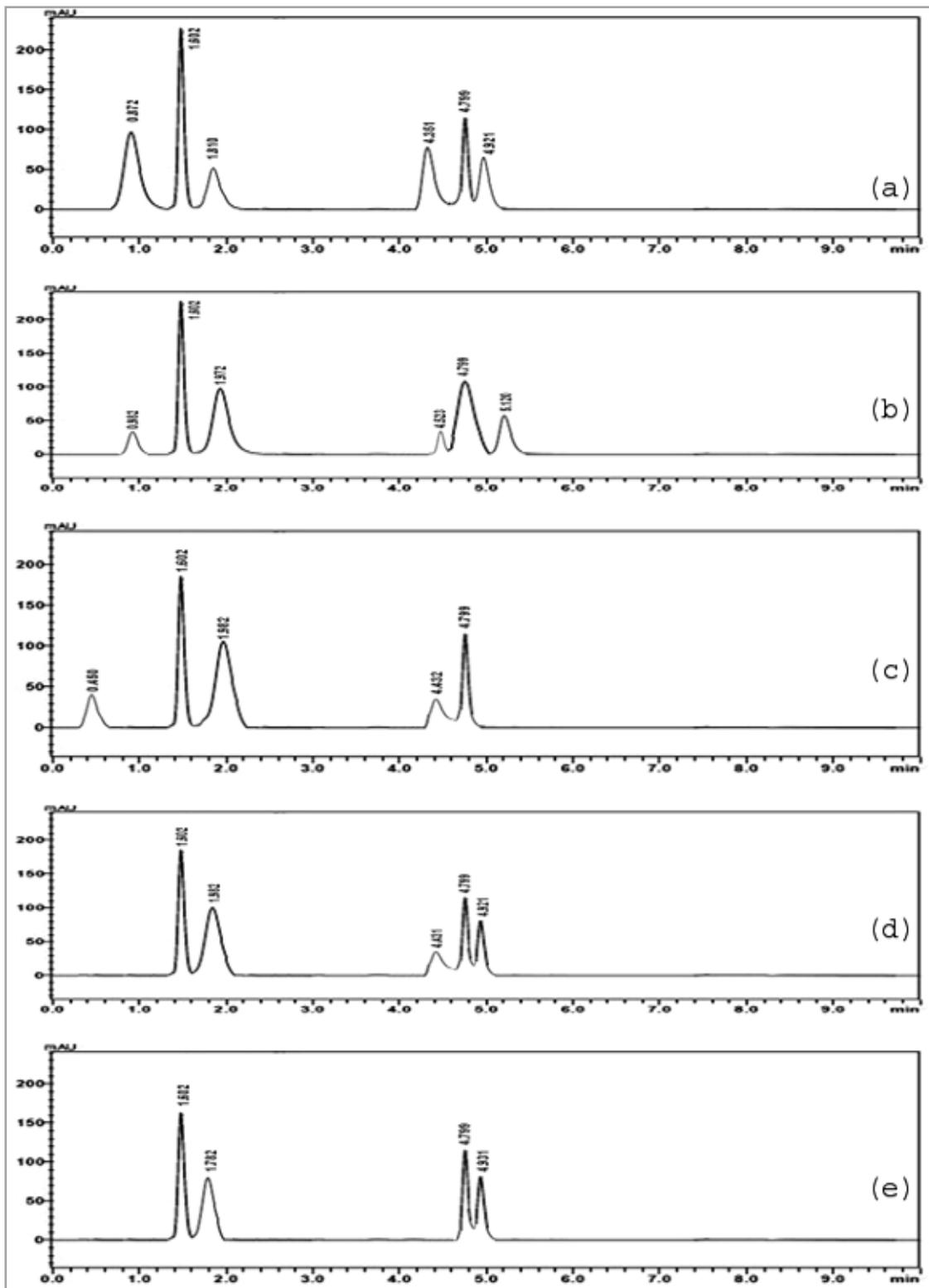


Figure 3: Degradation chromatogram, Alkaline Hydrolytic (3a), Acid Hydrolytic (3b), oxidative (3c), hydrolytic (3d), Thermal (3e) of OFL-CEF

CONCLUSIONS

A simple and efficient reverse-phase HPLC method was developed and validated for quantitative determination of OFL and CEF in pharmaceutical dosage forms. The method found to be precise, accurate, linear, robust and rugged during validation. Satisfactory results were obtained from the validation of the method. The purity of both analytes was unaffected by the presence of degradation products and thus confirms the stability-indicating power of the developed method. The method is stability indicating and can be used for routine analysis of production samples.

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