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STABILITY INDICATING RP-HPLC METHOD FOR ESTIMATION OF TWO SYNTHETIC ANTIBIOTICS, AMOXICILLIN AND ENROFLOXACIN, SIMULTANEOUSLY

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Keywords:

Amoxicillin, Enrofloxacin, Veterinary drug, Stability indicating, HPLC

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ABSTRACT: This work proposes a precise, accurate, sensitive and selective stability indicating RP-HPLC method for the simultaneous quantification of amoxicillin and enrofloxacin in bulk powder and oral suspension formulations. Chromatographic separation was performed on the reverse phase C18 analytical column using 0.1M potassium dihydrogen orthophosphate–methanol (65:35, *v/v*) as mobile phase and with detection at 235 nm. The retention times of amoxicillin and enrofloxacin were 3.364 min and 6.604 min, respectively. The linearity ranges were found to be 38.5-115.5-10 µg/ml (for amoxicillin) and 17.5-52.50 µg/ml (for enrofloxacin). The developed method was validated following International Conference on Harmonization guidelines. All validation parameters are fulfilled by the proposed method. Amoxicillin and enrofloxacin was subjected to different stress conditions like acid, alkali, oxidation, thermal, photo, and hydrolytic degradation. Both the analytes undergo degradation in all stress conditions. Since, the method effectively separated the analytes from their degradation products, it can be used as a stability-indicating method.

INTRODUCTION: Amoxicillin is a semi-synthetic antibiotic belonging to penicillin class of antibacterial agents [1]. Chemically, it is described as (2S,5R,6R)-6-[[(2R)-2-amino-2-(4-hydroxyphenyl) acetyl] amino] -3,3- dimethyl- 7-oxo-4-thia-1-azabicyclo [3.2.0] heptane-2-carboxylic acid. It shows antibacterial activity against several Gram-negative and Gram-positive organisms. In animals, amoxicillin is useful in the treatment of wounds, skin infections, infections of lower respiratory tract, genitourinary tract and urinary bladder [2, 3].

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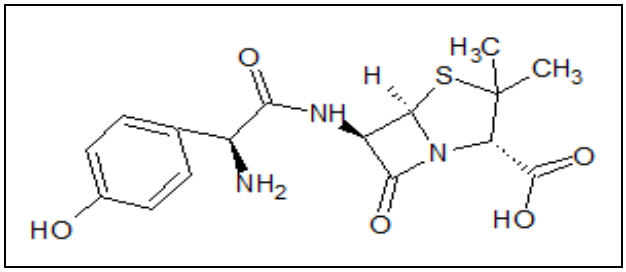
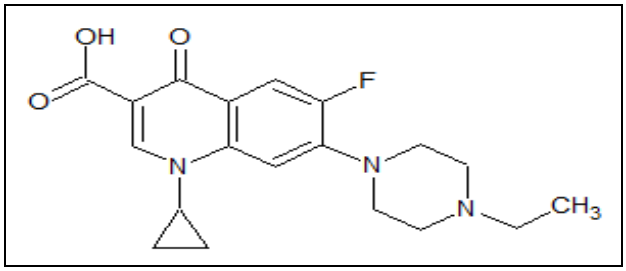


Amoxicillin



Enrofloxacin

FIG. 1: CHEMICAL STRUCTURES OF THE ANTIBIOTICS SELECTED

Amoxicillin hinders bacterial growth by preventing the formation of peptidoglycan chains which is essential for the formation of bacterial cell wall [4].

Enrofloxacin is a synthetic antibiotic that belongs to the class of fluoroquinolones [5]. Chemically, enrofloxacin is described as 1-cyclopropyl-7-(4-ethylpiperazin-1-yl)- 6- fluoro- 4- oxoquinoline- 3-carboxylic acid. Enrofloxacin is used to treat individual pets and domestic animals with bacterial infections of the skin, urinary tract, respiratory system and infections, which result from wounds [6].

Enrofloxacin exerts its bactericidal activity through inhibiting bacterial enzyme DNA gyrase, which is needed for DNA supercoiling and synthesis in the bacteria [7].

Amoxicillin and enrofloxacin is a commonly used combination of veterinary drugs. The synergistic effect has been shown *in-vitro* between amoxicillin and enrofloxacin. This combination is used in the treatment of infections in the digestive tract, respiratory tract, intestinal tract, urinary tract, and skin infections in dogs and cattle caused by Gram-positive and Gram-negative bacteria [8-10].

To the best of our knowledge, till now, only one HPLC with UV detection method had been reported for the simultaneous measurement of amoxicillin and enrofloxacin [11]. The reported method is based on the HPLC separation and analysis of amoxicillin and enrofloxacin using a reversed-phase C18 column at room temperature, with a gradient mobile phase of acetonitrile and phosphate buffer (pH 5.0), a flow rate of 0.8 ml/min and ultraviolet detection at 267 nm.

The aim of the present work was to develop and validate a stability-indicating RP-HPLC coupled with photodiode array detector method for the analysis of amoxicillin and enrofloxacin simultaneously in bulk and oral suspension dosage form.

MATERIALS AND METHODS:

Instrumentation: The samples were separated and analyzed using Waters Alliance 2695 Module equipped with a 2998 PDA detector, a degasser, an auto sample injector, and a column oven. The control of HPLC system and data acquisition was done with Empower 2 software.

Chemicals and Solvents: The HPLC grade methanol was obtained from Merck India Ltd (Mumbai, India). Analytical reagent potassium dihydrogen orthophosphate, orthophosphoric acid, hydrogen peroxide, hydrochloric acid, and sodium hydroxide were obtained from Sd. Fine Chemicals Ltd (Mumbai, India). Water used in the present investigation was obtained using a Milli-Q system (Millipore, USA).

Chromatographic Conditions: The separation and analysis were performed on Waters's symmetry C18 analytical column (250 mm × 4.6 mm, 5.0 µm id). The mobile phase was 0.1M potassium dihydrogen orthophosphate/methanol (65/35, *v/v*) (pH 4.5). The mobile phase was filtered using a 0.45 µm membrane filter before use. The flow rate was 1.0 ml/min. The column temperature was 27 °C. The injection volume was 10 µl. The photodiode array detector was set a wavelength of 235 nm for detection and analysis.

Standard Solutions: Standard reference amoxicillin and enrofloxacin drugs were provided kindly by Lara Drugs Private Limited (Telangana, India). A stock solution of amoxicillin (770 µg/ml) and enrofloxacin (350 µg/ml) was prepared in mobile phase. Working standard solutions (amoxicillin – 38.5, 57.75, 77.0, 96.25, and 115.5 µg/ml; enrofloxacin – 17.5, 26.25, 35.0, 43.75 and 52.50 µg/ml) were prepared by proper dilution of the stock solution with the mobile phase.

Sample Solution: Wedgewood Pharmacy's oral suspension (Swedesboro, USA) with strength - amoxicillin 77 mg/ml and enrofloxacin 35 mg/ml were used in the present investigation. An accurately measured volume of oral suspension equivalent to 77 mg and 35 mg of amoxicillin and enrofloxacin, respectively was transferred into a 100 ml volumetric flask. The flask was sonicated for 20 min with 30 ml of mobile phase. This solution was filtered *via* 0.45 µm membrane filter and then completed to the mark with mobile phase. Suitable dilution (amoxicillin 77.0 µg/ml and enrofloxacin 35.0 µg/ml) was made for the analysis using the same solvent.

Construction of Calibration Curve: For the construction of calibration curve, 5 standard concentrations of amoxicillin and enrofloxacin in

the range of 38.5-115.5 µg/ml and 17.5-52.50 µg/ml, respectively were prepared using stock standard solution and mobile phase. The solutions (10 µl) were injected into the HPLC system. The chromatograms and peak area at each concentration level were determined using the described chromatographic conditions. The linearity was determined by linear regression.

Analysis of the Studied Drug Combination in Oral Suspension Preparation: The sample solution with a concentration 77.0 µg/ml of amoxicillin and 35.0 µg/ml of enrofloxacin was prepared in mobile phase. The method described under "Construction of calibration graph" was applied. The nominal contents were calculated either from corresponding calibration curve or using the corresponding regression equation.

Stress Degradation Study: The stress degradation study was performed according to ICH guidelines [12]. This study was done by subjecting a stock sample solution (amoxicillin - 770 µg/ml and enrofloxacin - 350 µg/ml) to degradation by acidic, basic, oxidative, thermal, photolytic and hydrolytic conditions.

Alkali Hydrolysis: Ten ml of stock sample solution was mixed in a volumetric flask (100 ml) with 10 ml of 0.1 N sodium hydroxide. The solution was sonicated for 30 min at room temperature. The solution was cooled and neutralized with 0.1 N hydrochloric acid. The solution was completed with the mobile phase to reach a concentration 77.0 µg/ml of amoxicillin and 35.0 µg/ml of enrofloxacin in the volumetric flask.

Acid Hydrolysis: Stock sample solution (10 ml) was mixed in a volumetric flask (100 ml) with 0.1 N hydrochloric acids (10 ml). The solution was sonicated for 30 min at room temperature, cooled and neutralized with 0.1 N sodium hydroxide. The solution was completed with mobile phase to reach a concentration 77.0 µg/ml of amoxicillin and 35.0 µg/ml of enrofloxacin in the volumetric flask.

Oxidative Degradation: Ten ml of the stock sample solution was mixed with 10 ml of 30% hydrogen peroxide and sonicated at room temperature for 30 min. The solution was cooled and completed with the mobile phase until the volumetric flask (100 ml) mark to reach a targeted concentration of 77.0 µg/ml amoxicillin and 35.0 µg/ml enrofloxacin.

Photo Degradation: Ten ml of the stock sample solution was transferred into a 100 ml volumetric flask and exposed to direct sunlight for 24 h. The solution was completed to the flask mark with the mobile phase (concentration: 77.0 µg/ml - amoxicillin and 35.0 µg/ml – enrofloxacin).

Thermal Degradation: Ten ml of stock sample solution was transferred into the volumetric flask (100 ml) and kept in oven at 105 °C for 30 min. Then, the solution was cooled and diluted to the flask mark with mobile phase (concentration: 77.0 µg/ml - amoxicillin and 35.0 µg/ml – enrofloxacin).

Hydrolytic Degradation: Ten ml of stock sample solution was transferred into a volumetric flask and mixed with deionized water (10 ml). The solution was kept at room temperature for 30 min. Then, the solution was completed until the 100 ml flask mark with mobile phase to reach a target concentration of 77.0 µg/ml amoxicillin and 35.0 µg/ml enrofloxacin.

After the described treatments, all the stress degraded samples were filtered through a 0.45 µm pore size membrane filter and analyzed using the proposed method.

RESULTS AND DISCUSSION:

Method Development: The main aim of the present investigation is to develop a stability-indicating RP-HPLC method that could be able to separate and analyze amoxicillin and enrofloxacin simultaneous in the presence of all stress degradation products. Also, the developed method should be adequate for a routine quality control laboratory. In order to achieve this target, 0.1M potassium dihydrogen phosphate and methanol in different ratios, with different temperatures, pH values, and flow rates have been examined. The retention time, tailing factor, number of theoretical plates, and resolution were determined. Using Waters symmetry C18 analytical column (250 mm × 4.6 mm, 5.0 µm id), the optimal results were obtained with a mobile phase of 0.1M potassium dihydrogen phosphate in combination with methanol (65:35, v/v) pH adjusted to 4.5 with orthophosphoric acid, at a flow rate of 1 ml/min and 27 ºC of column temperature.

In optimized conditions, the amoxicillin and enrofloxacin retention times were about 3.364 min and 6.604 min, respectively. A typical chromatogram obtained through optimized conditions is shown in **Fig. 2**.

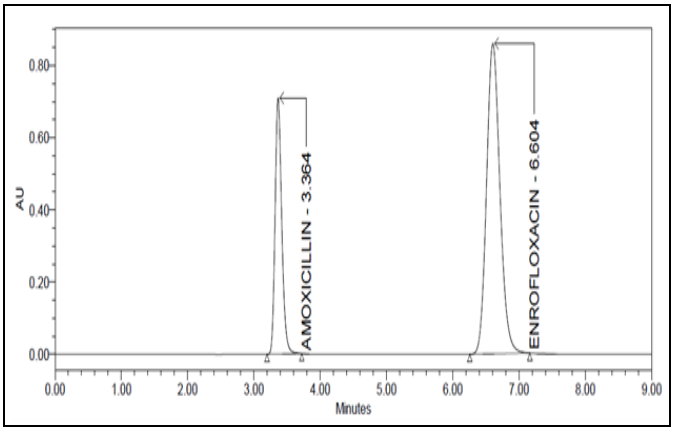


FIG. 2: TYPICAL CHROMATOGRAM OF A MIXTURE OF 77.0 µg/ml AMOXICILLIN (3.364 min) AND 35.0 µg/ml ENROFLOXACIN (6.604 min)

Validation of the Method: The developed method was validated for system suitability, selectivity, specificity, linearity, sensitivity, accuracy, precision, and robustness according to ICH guidelines [13] and US Pharmacopoeia [14].

System Suitability: The system suitability was established by injecting five replicates of the working standard solutions and analyzing amoxicillin and enrofloxacin for its retention time, peak area, tailing factor, resolution, and plate count. The system suitability results are summarized in **Table 1,** and it was concluded that the developed method meets the accepted requirements.

Selectivity: To assess selectivity, the chromatogram of a standard solution of amoxicillin and enrofloxacin was compared to chromatograms of placebo solution, mobile phase blank and sample solution verifying that no interference exists between the excipients and components of mobile phase in the analysis method.

A comparison of the chromatograms obtained revealed no significant interference of excipients and components of mobile phase using the same chromatographic conditions. The placebo and mobile phase blank chromatograms did not show any peaks. **Fig. 3** depicts the chromatograms showing that the method is selective for the analytes concerned.

TABLE 1: SYSTEM SUITABILITY TEST PARAMETERS FOR AMOXICILLIN AND ENROFLOXACIN DETERMINED BY THE PROPOSED METHOD

Injection no.	Retention time	Peak area	Plate count	Tailing factor	Resolution
Amoxicillin (77 µg/ml)					
1	3.372	4899557	5557	1.23	-
2	3.367	4909982	5468	1.22	-
3	3.371	4911395	5509	1.23	-
4	3.376	4912207	5469	1.22	-
5	3.372	4920005	5437	1.23	-
Mean	3.372	4910629	5488.000	1.226	-
RSD	0.095	0.149	0.843	0.447	-
Recommended limit	RSD ≤ 2	RSD ≤ 2	> 2000	≤ 2	-
Enrofloxacin (35 µg/ml)					
1	6.618	11984826	5227	1.12	11.60
2	6.615	12041992	5148	1.13	11.53
3	6.631	12019071	5222	1.12	11.62
4	6.633	11985440	5129	1.13	11.54
5	6.634	12016010	5160	1.13	11.54
Mean	6.626	12009468	5177	1.13	11.57
RSD	0.136	0.203	0.862	0.486	0.354
Recommended limit	RSD ≤ 2	RSD ≤ 2	> 2000	≤ 2	> 1.5

Linearity: The calibration curve was constructed by plotting the peak area versus the corresponding concentrations of amoxicillin and enrofloxacin in the range of 38.5-115.5 µg/ml and 17.5-52.5 µg/ml, respectively. The concentration of amoxicillin and enrofloxacin was calculated from the following regression equation:

$A = 63706\,C + 3127$ ($R^2 = 0.9998$) ------- Amoxicillin

$A = 34503\,C - 17505$ ($R^2 = 0.9999$) ------ Enrofloxacin

Where A is peak area, *C* is the concentration in µg/ml and R^2 is the regression coefficient. Good linearity is demonstrated from the high value of the regression coefficient and less value of intercept.

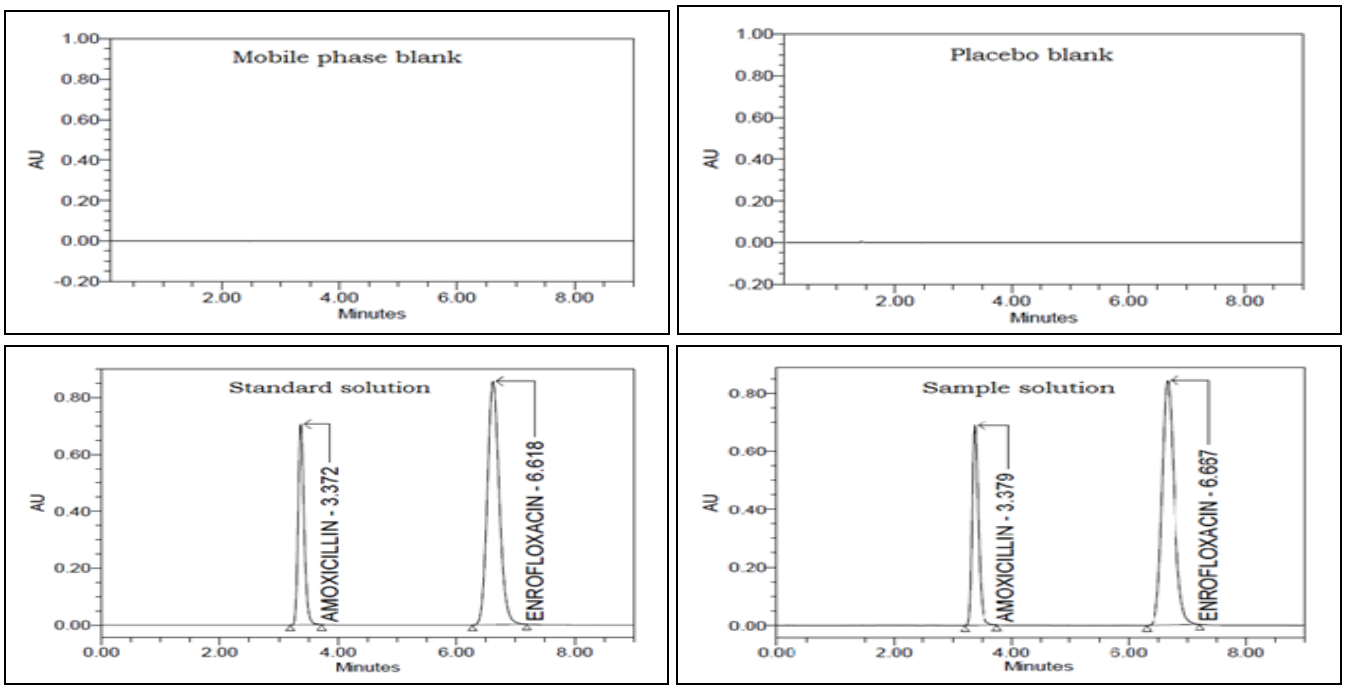


FIG. 3: CHROMATOGRAMS OF SELECTIVITY STUDY

Sensitivity: The limit of detection and limit of quantitation for the selected analytes were determined at a signal-to-noise ratio of 3:1 and 10:1, respectively. The limit of detection was found to be 0.051 µg/ml for amoxicillin and 0.019 µg/ml for enrofloxacin. The corresponding limits of quantification were found to be 0.171 and 0.064 µg/ml, respectively. These values indicate that the proposed method has good sensitivity.

Precision and Accuracy: The method precision and accuracy were assessed on working standard solution with concentration 77 µg/ml amoxicillin and 35 µg/ml enrofloxacin. Precision was expressed by the relative standard deviation of peak area response, whereas accuracy was expressed as percentage assay. The results are shown in **Table 2**. The obtained values indicated that the precision and accuracy of the method were satisfactory.

TABLE 2: PRECISION AND ACCURACY DATA FOR THE SIMULTANEOUS DETERMINATION OF AMOXICILLIN AND ENROFLOXACIN

Injection no.	Amoxicillin		Enrofloxacin	
	Peak area (mAU)	Assay (%)	Peak area (mAU)	Assay (%)
1	4913256	99.85	12057001	100.09
2	4917830	99.95	12013849	99.74
3	4919419	99.98	12012941	99.73
4	4915462	99.9	12061206	100.13
5	4918908	99.97	12082415	100.31
6	4917121	99.93	12017754	99.77
Mean	4916999	99.93	12040861	99.96
RSD	0.047	0.049	0.248	0.247

Recovery Study: Recovery was assessed through standard addition technique. Amoxicillin and enrofloxacin were spiked to the placebo blank solution at three concentration levels (50%, 100% and 150%). Where 100% corresponded to 77 µg/ml and 35 µg/ml of amoxicillin and enrofloxacin, respectively. The resulting mixtures were assayed by the proposed method. The good recoveries with the standard addition method given in **Table 3** demonstrate good accuracy of the method and no interference from commonly used tablet excipients.

TABLE 3: RECOVERY DATA FOR THE SIMULTANEOUS DETERMINATION OF AMOXICILLIN AND ENROFLOXACIN

Spiked level (%)	Concentration of amoxicillin (µg/ml)		Recovery (%)	Mean (%)	Concentration of enrofloxacin (µg/ml)		Recovery (%)	Mean (%)
	Added	Found			Added	Found		
50	38.50	38.40	99.74	99.76	17.50	17.44	99.65	99.72
	38.50	38.35	99.61		17.50	17.45	99.73	
	38.50	38.47	99.92		17.50	17.46	99.77	
100	77.00	76.86	99.81	99.81	35.00	34.93	99.80	99.78
	77.00	76.84	99.80		35.00	34.93	99.79	
	77.00	76.86	99.81		35.00	34.91	99.73	
150	115.50	115.23	99.77	99.79	52.50	52.58	100.16	99.98
	115.50	115.27	99.80		52.50	52.53	100.05	
	115.50	115.26	99.79		52.50	52.36	99.73	

Degradation Study: To prove the specificity and stability-indicating power of the method, oral suspension sample solution was exposed to acid, base, peroxide, heat, water, thermal and photolytic stress conditions. The results of the stress studies have been summarized in **Table 4**. Amoxicillin and enrofloxacin degradation were observed in all cases of stress conditions. The chromatograms of amoxicillin and enrofloxacin sample solution after degradation are shown in **Fig. 4**.

From the chromatograms, it was observed that the proposed method was capable of separating degradants from amoxicillin and enrofloxacin. Peak purity of amoxicillin and enrofloxacin peaks was verified using a photodiode array detector in all the stress samples. As the purity angle is less than the purity threshold value, the peaks of analytes were pure. The results demonstrated the selectivity and stability-indicating properties of the method.

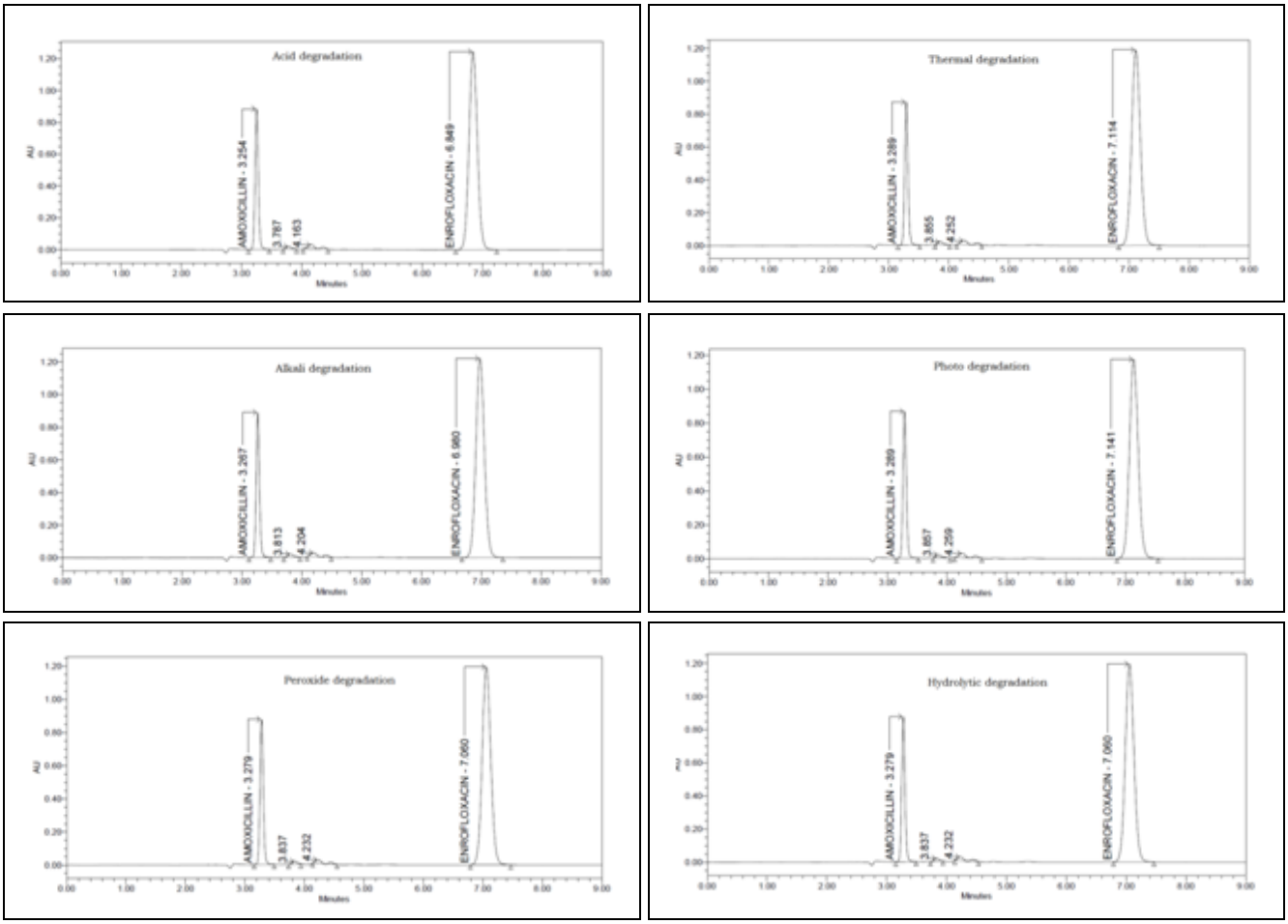


FIG. 4: CHROMATOGRAMS OF SAMPLE SUBJECTED STRESS CONDITIONS

TABLE 4: DEGRADATION AND PEAK PURITY DATA OF AMOXICILLIN AND ENROFLOXACIN

Stress condition	Peak area (mAU)	Assay (%)	Degraded (%)	Purity angle	Purity threshold
Amoxicillin					
Undegraded	4910629	100	-	-	-
Acid	4126041	83.85	16.15	0.149	0.313
Base	4394632	89.31	10.69	0.147	0.415
Peroxide	4279315	86.97	13.03	0.13	0.406
Thermal	4319315	87.78	12.22	0.128	0.403
Photolytic	4140576	84.15	15.85	0.135	0.401
Hydrolytic	4093518	83.19	16.81	0.149	0.415
Enrofloxacin					
Undegraded	12009468	100	-	-	-
Acid	9842262	81.71	18.29	0.229	0.628
Base	9985890	82.9	17.1	0.179	0.608
Peroxide	9437282	78.35	21.65	0.188	0.567
Thermal	10137282	84.16	15.84	0.197	0.564
Photolytic	10047615	83.41	16.59	0.222	0.548
Hydrolytic	9726321	80.75	19.25	0.195	0.617

Robustness: To determine the method robustness, experimental conditions (mobile phase flow rate and column temperature) were deliberately changed, and the system suitability parameters for amoxicillin and enrofloxacin were recorded. In the varied chromatographic conditions (flow rate and column temperature), there were no significant changes in the system suitability parameters **Table 5**. These values indicate the method's robustness.

TABLE 5: ROBUSTNESS DATA OF THE PROPOSED METHOD

Parameter	Amoxicillin (77 µg/ml)			Enrofloxacin (35 µg/ml)		
	USP Tailing	USP plate count	USP resolution	USP Tailing	USP plate count	USP resolution
Flow rate 1.0 + 1 ml/min	1.24	5697	-	1.13	5361	12.00
Flow rate 1.0 - 1 ml/min	1.23	4972	-	1.12	4786	11.55
Temperature 27 + 5 ºC	1.24	5612	-	1.14	5504	11.95
Temperature 27 - 5 ºC	1.23	5050	-	1.13	4641	11.46

Analysis of Sample: The developed and validated method was applied to the simultaneous quantification of amoxicillin and enrofloxacin in a combined oral suspension. The analytical results and recoveries of amoxicillin and enrofloxacin in the oral suspension samples are listed in **Table 6**. The data in **Table 6** prove that the method is accurate and reproducible. The proposed method meets the requirements of quantitative analysis of amoxicillin and enrofloxacin simultaneously.

TABLE 6: ANALYTICAL RESULTS AND RECOVERIES OF THE AMOXICILLIN AND ENROFLOXACIN IN ORAL SUSPENSION SAMPLE

Drug	Labeled claim (mg/ml)	Found (mg)	Recovery (%)	Mean recovery (%)	RSD (%)
Amoxicillin	77	76.80	99.74	99.76	0.157
	77	76.70	99.61		
	77	76.94	99.92		
Enrofloxacin	35	34.88	99.66	99.71	0.057
	35	34.90	99.71		
	35	34.92	99.77		

Comparison Studies: Through reviewing the literature, it was found that only one published article [11] is concerned with the simultaneous estimation of amoxicillin and enrofloxacin using the HPLC method. The performance of the reported and developed method is given **Table 7**.

TABLE 7: SUMMARY OF PROPOSED AND REPORTED RP-HPLC METHODS

Drug	Run time (min)	Linearity (µg/ml)	LOD (µg/ml)	LOQ (µg/ml)	RSD (%)	Recovery (%)	Reference
Amo	13	480-1120	2.0	6.9	0.93	100.42	11
Enr		240-560	0.074	2.4	0.78	101.33	
Amo	9	38.5-115.5	0.051	0.171	0.047	99.76-99.81	Proposed
Enr		17.5-52.50	0.019	0.064	0.248	99.72-99.98	method

Amo - amoxicillin; Enr - Enrofloxacin

From the data shown in the above table, the proposed RP-HPLC method is considered more sensitive, rapid, precise and accurate than the previously reported HPLC method [11].

The proposed method allows the simultaneous quantification of amoxicillin and enrofloxacin in bulk and oral suspension using cheap and simple isocratic elution mode, unlike the reported method that gradient elution mode. Though the reported HPLC method is stability-indicating, peak purity details are not reported.

CONCLUSION: In the present work, a sensitive, cost-effective and selective stability-indicating RP-HPLC method with adequate sensitivity for the determination of amoxicillin and enrofloxacin has been proposed. This method offered good precision and accuracy for analysis of amoxicillin and enrofloxacin in the presence of its stress degradation products using 0.1M potassium dihydrogen orthophosphate and methanol (65:35, *v/v*) as a mobile phase. The developed method can be applied to quality control analyses of amoxicillin and enrofloxacin in the oral suspension dosage forms.

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CONFLICTS OF INTEREST: No conflict of interest exist in the present investigation

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