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Development and Validation of Stability Indicating RP-LC Method for Estimation of Related Substances of Enrofloxacin in Bulk and Its Pharmaceutical Formulations.

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ABSTRACT

An isocratic reverse phase liquid chromatography (RP-LC) method has been developed and subsequently validated for the determination of Enrofloxacine and its related substances in Bulk and its pharmaceutical tablet formulation. Separation was achieved with a Inertsil ODS 3V (150 mmx4.6 mm I.D; particle size 3 µm)) Column and 1% orthophosphoric acid buffer (pH adjusted to 2.5 with triethyl amine): acetonitrile (800:200) v/v as eluent at a flow rate of 1.0 mL/min. UV detection was performed at 270nm. The method is simple, rapid, and selective. The described method was validated as per ICH guidelines for specificity, forced degradation, linearity and ruggedness. Known and unknown impurities are well resolved from each other and also degradation products. all the known impurities shown a linear responses with very good LOD and LOQ values below 0.05%. The % Recoveries for all the impurities lies within the ICH limits. The results showed that the proposed method is suitable for the precise, accurate and rapid determination of related substances of enrofloxacin in bulk, its dosage forms.

Keywords: Enrofloxacin, Related Substances, RP-LC, Validation, Dosage form.

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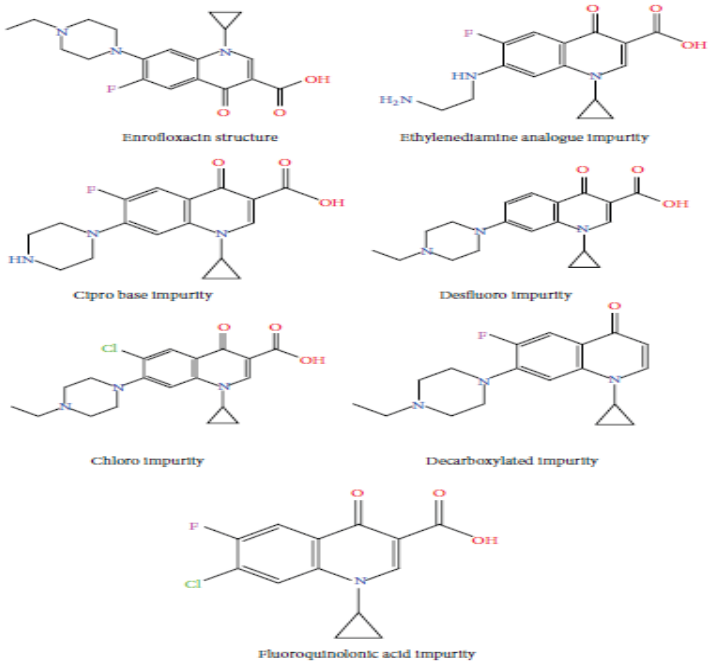
INTRODUCTION

Ciprofloxacin (CFX) and enrofloxacin (EFX) are second generation fluoroquinolones with a broad spectrum of antibacterial activity. Both have good bioavailability after oral administration and good to excellent tissue distribution (Papich, 1998). The interest of the medical community in fluoroquinolones has not decreased during the last 10 years and many new ones have been developed and are under investigation. Enrofloxacin (EFX) (fig.:-1) is a (1-cyclopropyl-7-(4-ethylpiperazin-1-yl)-6-fluoro-4-oxo-1,4-dihydro quinoline-3-carboxylic acid) belongs to the group of synthetic 6-fluoroquinolones or 4-quinolones. these fluoroquinolones shows great theraputic effects against gram positive and gram negative bacteria and mainly used for the veterinary use.

Figure 1: Chemical Structure of Enrofloxacin and its related substances



EFX is intensively used in our country in poultry and pig production for preventive and therapeutic purposes in India. EFX has been used for the first time into human medicine, being effective in several infections. Because of its broad and intense activity against Gram negative bacteria and the fact that no cross-resistance with beta- lactams or aminoglycosides occurs, it was also suggested for veterinary medicine use (Nouws et al., 1988). It is proposed to be used in dogs and cats (Brown, 1996) but not in food producing animals. Due to the antibacterial advantages and pharmacokinetic properties reported, its clinical application in veterinary medicine could be of considerable usefulness. There are reports of pharmacokinetics of CFX in domestic animals (Aramayona et al., 1996; Dowling et al., 1995; García Ovando et al., 2000a; García Ovando et al., 1999) showing good pharmacokinetic properties and therapeutic possibilites.

Following the introduction of fluoroquinolones for use in poultry, there has been a dramatic emergence of Salmonella with reduced susceptibility to fluoroquinolones in humans (WHO, 1997; WHO, 1998). This fact marks the importance to develop new methods for a fast, simple and accurate quantification of related substances of these antibacterial in food producing animals [1-3].

The authors have developed a new, simple and fast analytical method by RP-LC to quantify Enrofloxacin and its related substances in bulk and its dosage forms. This validation study is carried out as per ICH guidelines.

EXPERIMENTAL [4-6]

Instrumentation

The analysis of the drug was carried out on a waters LC system equipped with 2695pump and

2996 photodiode array detector was used and a Reverse phase HPLC column Inertsil ODS-3V ((Make: GL Sciencies, Ireland); 150 mmx4.6 mm I.D; particle size 3 µm)) was used. The output of signal was monitored and integrated using waters Empower 2 software.

Chemicals and solvents

Milli-Q Water, Acetonitrile (HPLC Grade), Orthophosphoric acid (GR Grade), were obtained from Qualigens Ltd., Mumbai.

Buffer preparation

1% ortho phosphoric acid was prepared by 10mL ortho phosphoric acid into a 1000 mL of purified water and mixed. Adjusted pH to 2.5 (±0.05) with tri ethyl amine solution. Filter the solution through 0.45µm membrane filter.

Mobile phase preparation

Prepare a filtered and degassed mixture of Buffer and Acetonitrile in the ratio of 800:200 v/v respectively.

Diluent preparation

Mobile phase is used as diluent.

Standard preparation: (Enrofloxacin)

Accurately weigh and transfer about 50.0mg of enrofloxacin into a 200 mL volumetric flask, add 160 mL of mobile phase and sonicate to dissolve. Cool the solution to room temperature and dilute to volume with diluent. Transfer 5.0 mL of the above solution into a 200 mL volumetric flask and dilute to volume with diluent (Mobile Phase).

Sample preparation

Weigh and finely powder not fewer than 20 Tablets. Accurately weigh and transfer equivalent to 136 mg of enrofloxacin into a 100 mL volumetric flask add about 60 mL of mobile phase, and sonicate for 30minutes with intermittent shaking at controlled temperature and dilute to volume with mobile phase and mix. Filter the solution through 0.45 µm membrane Filter.

Chromatographic conditions

An Inertsil ODS-3V ((Make: GL Sciencies; 150 mmx4.6 mm I.D; particle size 3µm)) Column was used for analysis at ambient column temperature. The mobile phase was pumped through the column at a flow rate of 1.0mL/min. The sample injection volume was 20 µL. The photodiode array detector was set to a wavelength of 278nm for the detection and Chromatographic runtime was 60minutes.

RESULTS AND DISCUSSION

Method development [4-6]

To develop a suitable and robust LC method for the determination of related substances of enrofloxacin, different mobile phases were employed to achieve the best separation and resolution. The method development was started with Symmetry RP-18 ((Make: Waters Corporation (Ireland); 150 mmx4.6 mm I.D; particle size 3 µm)) with the following mobile phase. Accurately weigh and transfer about 2.72 grams of Potassium di-hydrogen phosphate monohydrate in 1000 mL of purified water and mix. Adjust pH to 3.0 (±0.05) with dilute orthophosphoric acid solution. Filter the solution through 0.45µm membrane filter. Prepare a filtered and degassed mixture of Buffer and methanol in the ratio of 500:500 v/v respectively.

All impurities are not separated and enrofloxacin peak was eluted at void volume. For next trial the mobile phase composition was changed slightly. The mobile phase composition was Buffer and methanol in the ratio of 600:400 v/v. above trail the peak shape was little broad and ciprofloxacin impurity was not separated from main peak.

Again the mobile phase was changed to 1% orthophosphoric acid with pH 2.5 (adjusted by using TEA): Acetonitrile in the ratio of 600:400:v/v respectively as eluent at flow rate 1.0 mL/min. UV detection was performed at 270nm. The retention time of enrofloxacin was about 5 min an d ciproflocain was separated from main peak. however the resolution between enroflocain and cipofloxacin was less than 2.0. further the mobile phase composition was slightly modified with buffer; acetonitrile 800:200, all the peaks are well resolved. good resolution between ciprofloxacin and enrofloxacin was achieved. (refer Fig-4.) and the peak shape was good.

The chromatogram of Enrofloxacin with all impurities using the proposed method is shown in Fig-4. System suitability results of the method are presented in Table-1. enrofloxacin and its impurities shows significant UV absorbance at Wavelength 270nm. Hence this wavelength has been chosen for detection in analysis of related substances in enrofloacin.

Method validation [4-6]

The developed RP-LC method extensively validated for assay of Enrofloxacin using the following parameters.

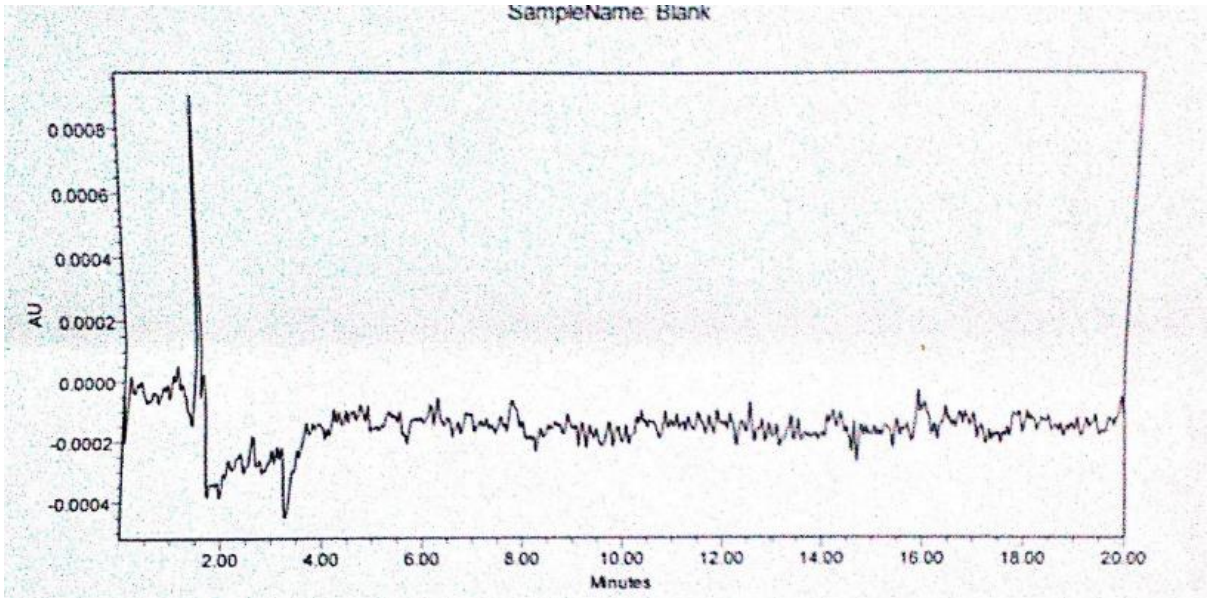
Specificity

Blank and Placebo interference

A study to establish the interference of blank and placebo were conducted. Diluent and placebo was injected into the chromatograph in the defined above chromatographic conditions and the blank and placebo chromatograms were recorded. Chromatogram of Blank solutions showed no peaks at the retention time of enrofloxacin and its impurities. This indicates that the diluent solution used in sample preparation do not interfere in estimation of impurities in enrofloxacin in enrofloxacin tablets.

The chromatogram of enrofloxacin related substances Blank using the proposed method is shown in Fig- 2.

Figure 2: A typical HPLC Chromatogram showing the no interference of diluent



The chromatogram of Enrofloxacin Placebo using the proposed method is shown in Fig-3.

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Figure 3: A typical HPLC Chromatogram showing the no interference of placebo

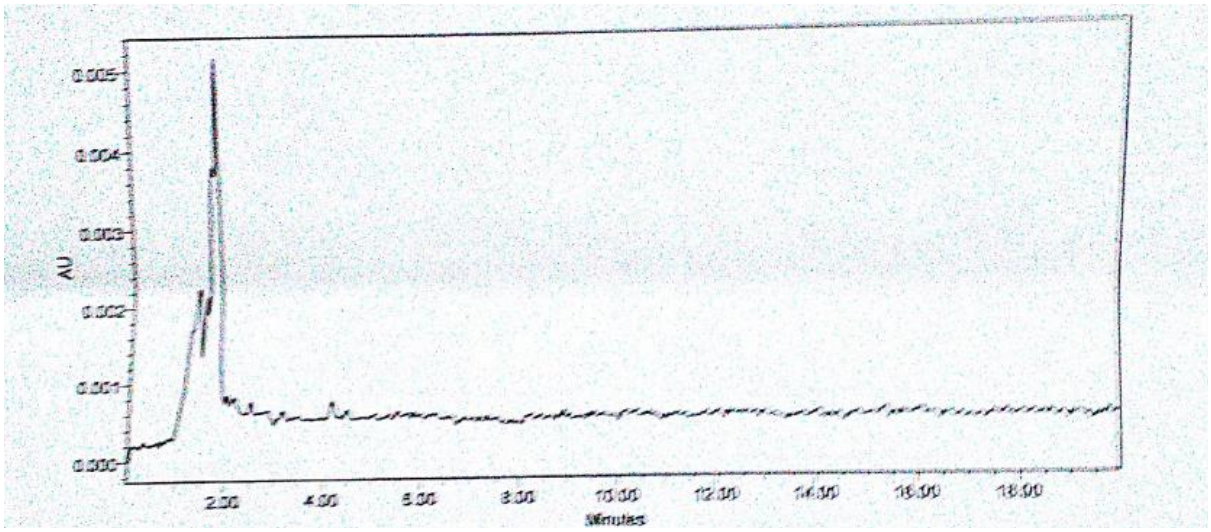

Figure 4: A typical HPLC Chromatogram showing the peak of Enrofloxacin

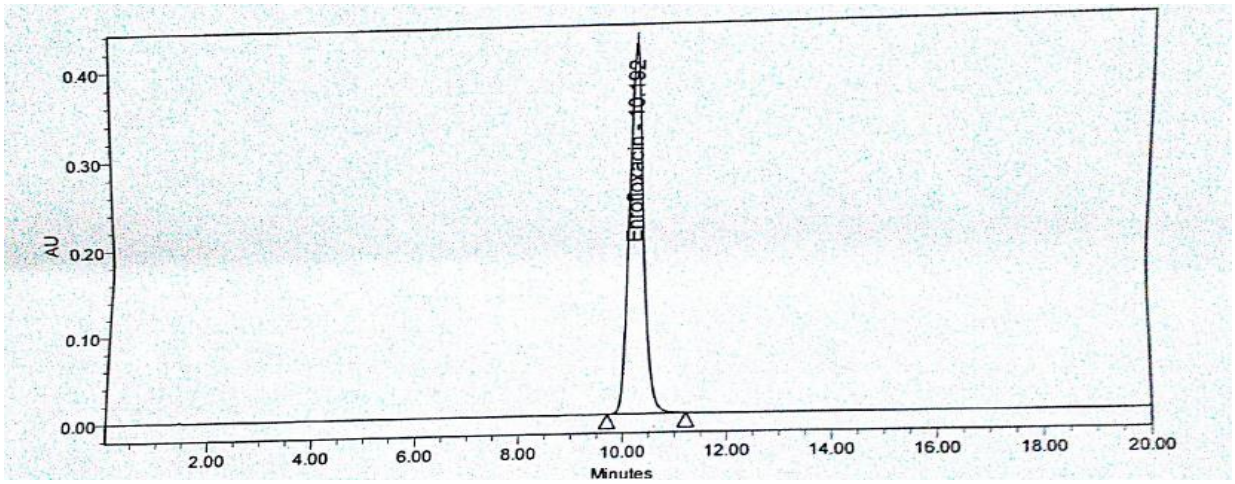

Figure 5: A typical HPLC Chromatogram showing the peaks of Enrofloxacin and its impurities

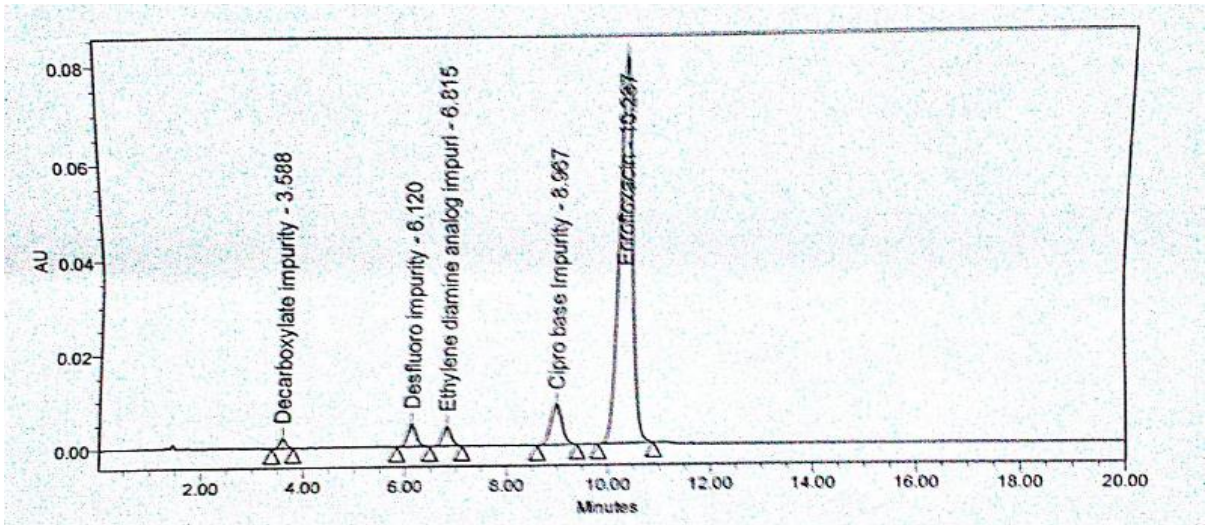

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Figure 6: A typical HPLC Chromatogram showing the peak of decarboxylated impurity

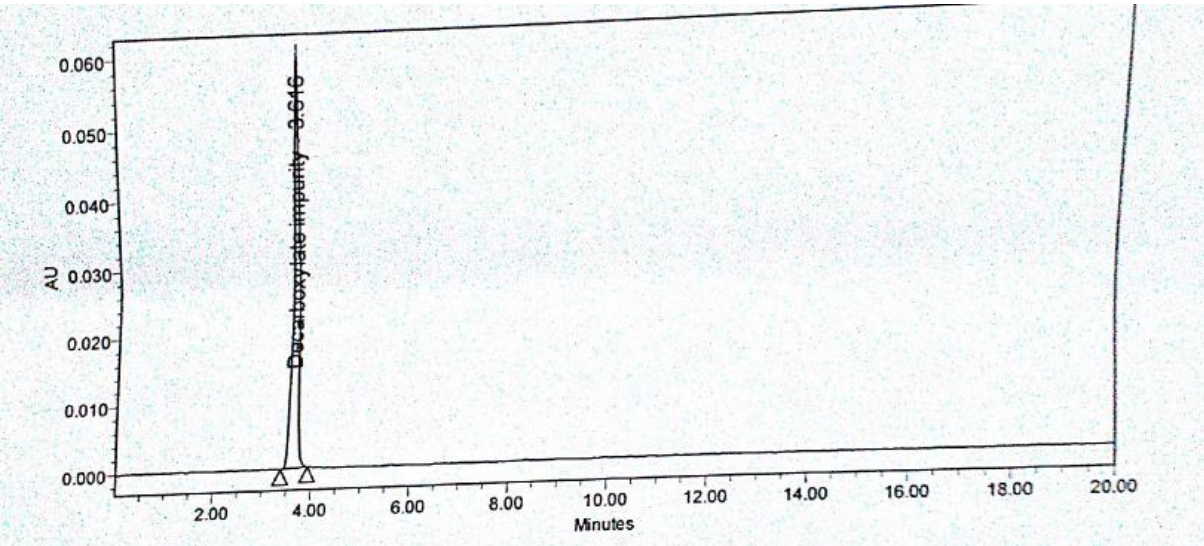

Figure 7: A typical HPLC Chromatogram showing the peak of desfluoro impurity

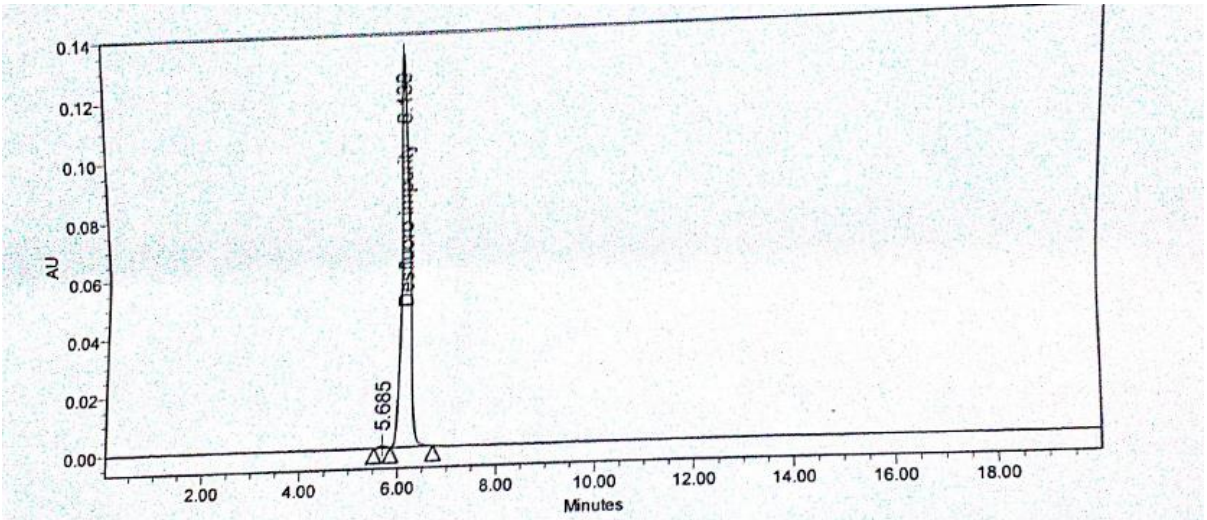

Figure 8: A typical HPLC Chromatogram showing the peak of ethylenediamine impurity

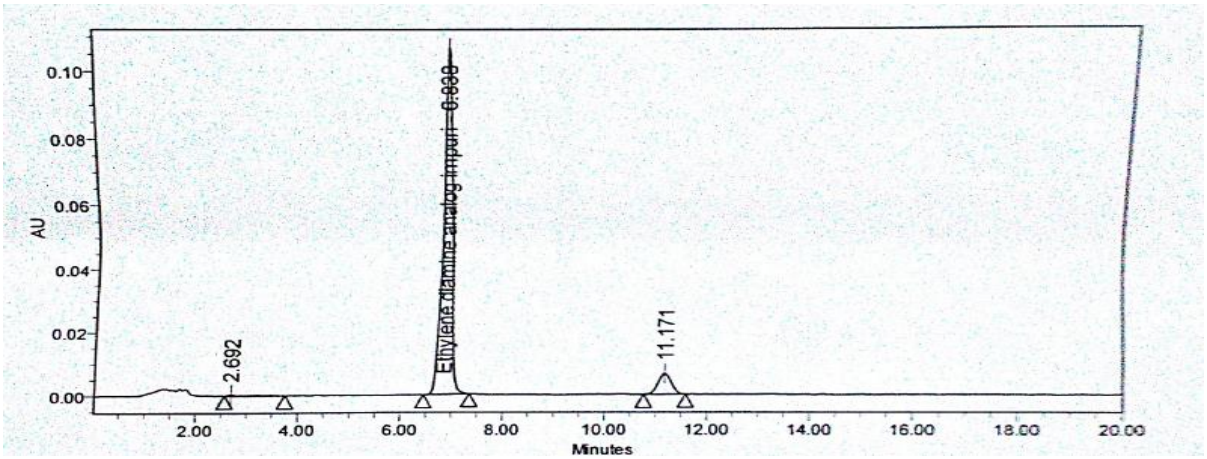

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Figure 8: A typical HPLC Chromatogram showing the peak of Ciprofloxacin impurity

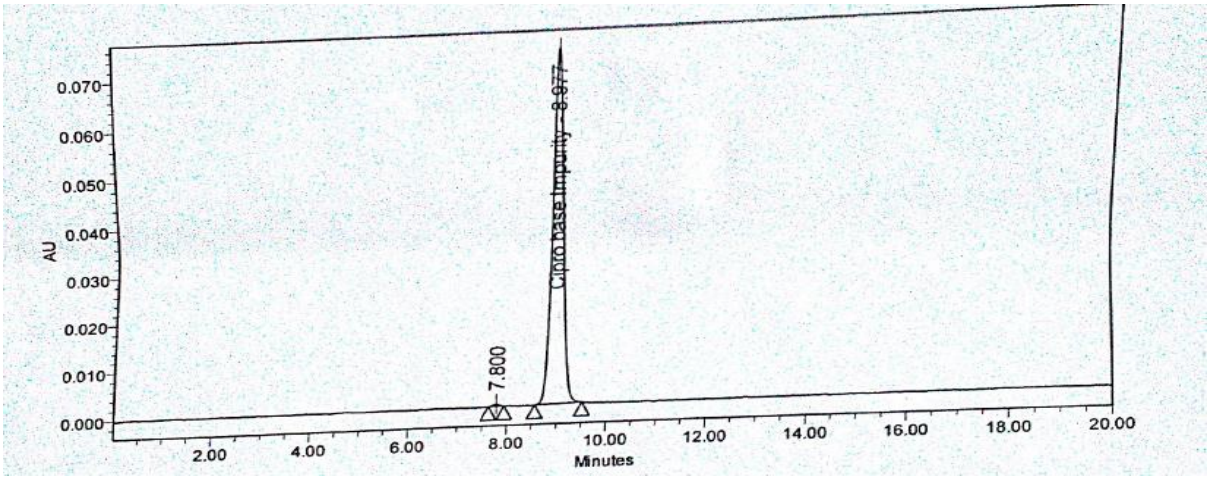

Figure 9: A typical HPLC Chromatogram showing the peak of chloro impurity

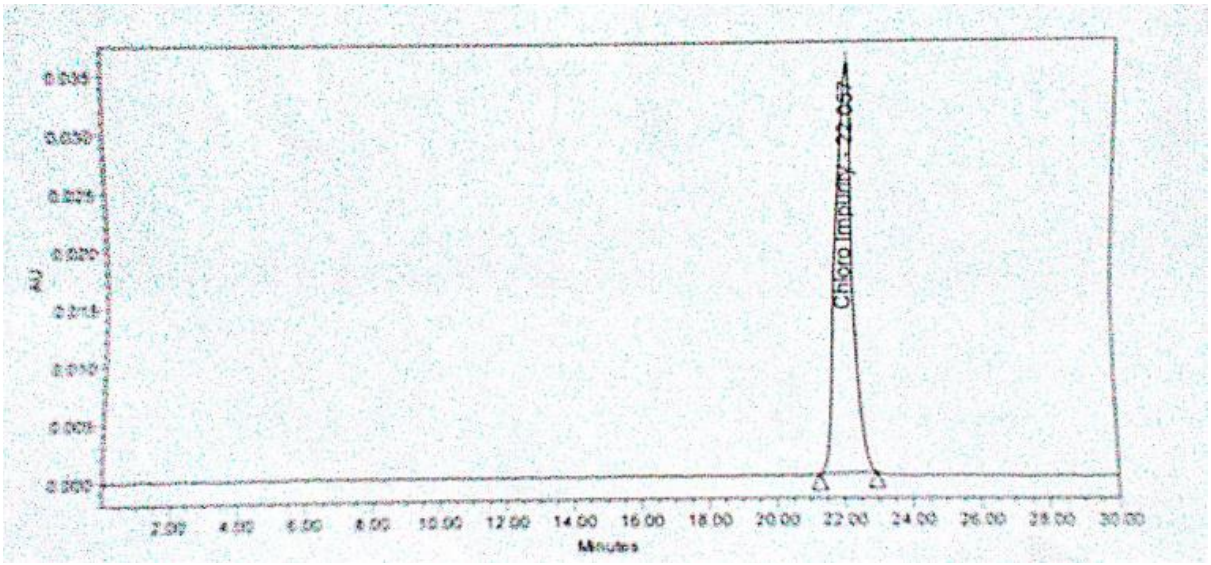

Table 1: System suitability parameters for Enrofloxacin by proposed method

Name of the Compound	Theoretical plate	Tailing factor
Enrofloxacin	6821	1.35

Forced Degradation

Control Sample: Weigh and finely powder not fewer than 20 Tablets. Accurately weigh and transfer equivalent to 136 mg of enrofloxacin into a 100 mL volumetric flask add about 60 mL of diluent, and sonicate for 30minutes with intermittent shaking at controlled temperature and dilute to volume with diluent and mix. Filter the solution through 0.45 µm membrane Filter.

Acid Degradation Sample: Weigh and finely powder not fewer than 20 Tablets. Accurately weigh and transfer equivalent to 136 mg of enrofloxacin into a 100 mL volumetric flask add about 160 mL of diluent , and sonicate for 30minutes with intermittent shaking at controlled temperature. Then add 5mL of 1N acid, refluxed for 30min at 60°C, then cooled to room temperature, neutralize with 1N NaOH and dilute to volume with diluent and mix. Filter the solution through 0.45 µm membrane Filter.

Base Degradation Sample: Weigh and finely powder not fewer than 20 Tablets. Accurately weigh and transfer equivalent to 136 mg of enrofloxacin into a 100 mL volumetric flask add about 60 mL of diluent , and sonicate

for 30minutes with intermittent shaking at controlled temperature. Then add 5mL of 1N Base (NaOH), refluxed for 30min at 60°C, then cooled to room temperature, neutralize with 1N Acid (HCl) and dilute to volume with diluent and mix. Filter the solution through 0.45 µm membrane Filter.

Peroxide Degradation Sample : Weigh and finely powder not fewer than 20 Tablets. Accurately weigh and transfer equivalent to 136 mg of Enrofloxacin into a 100 mL volumetric flask add about 60 mL of diluent , and sonicate for 30minutes with intermittent shaking at controlled temperature. Then add 5mL of 30% Peroxide, refluxed for 30min at 60°C, then cooled to room temperature and dilute to volume with diluent and mix. Filter the solution through 0.45 µm membrane Filter.

Thermal Degradation Sample: Powder collected from 20 tablets are exposed to heat at 105°C for about 5days. Then Weigh and finely powder not fewer than 20 Tablets. Accurately weigh and transfer equivalent to 136 mg of enrofloxacin into a 100 mL volumetric flask add about 60 mL of diluent, and sonicate for 30minutes with intermittent shaking at controlled temperature and dilute to volume with diluent and mix. Filter the solution through 0.45 µm membrane Filter.

Similarly Humidity, UV-Light exposure, Sunlight exposure and Water hydrolysis stress samples are prepared and checked for their purity by proposed method.

Table 2 : Summary of the degradation profile of enrofloxacin by proposed method.

Name of the Sample	Condition	Purity angle	Purity threshold	% Degradation of Enrofloxacin
Control Sample	N/A	0.247	0.321	99.4
Acid Degradation Sample	5mL, 5N HCl, 60°C/60min	0.125	0.129	99.1
Base Degradation Sample	5mL,5N NaOH, 60°C/60min	0.347	0.367	98.5
Peroxide Degradation Sample	5mL, H_2O_2, 0°C/30min	0.314	0.363	98.9
Humidity Degradation Sample	@90% RH for 7 days	0.412	0.429	99.3
Thermal Degradation Sample	@105°C for 5 days	0.614	0.687	99.2
Photolytic Degradation Sample	1.2lak Lux units	0.178	0.249	99.4

From the above data of degradation profile it can be conclude that there is no interference found for enrofloxacin and its impurity peaks.

Precision

In the study of the instrumental system precision where, a RSD of 1.5 % was obtained for the standard area obtained corresponding to the first day, being 1.2% for the second day, respectively. The method precision study for six sample preparations from spiked sample in marketed samples showed a less than 2.0% RSD.

Table 3: Method Precision studies for Related substances of Enrofloxacin by proposed method

Method Precision	Decarboxylated Impurity	Desfluoro Impurity	Ethylenediamine impurity	Ciprofloxacin Impurity	Chloro impurity
Sample-1	0.204	0.198	0.204	0.499	0.201
Sample-2	0.201	0.199	0.201	0.497	0.204
Sample-3	0.199	0.201	0.196	0.495	0.199
Sample-4	0.198	0.207	0.198	0.495	0.197
Sample-5	0.204	0.199	0.194	0.498	0.199
Sample-6	0.206	0.204	0.199	0.496	0.199
Mean	0.202	0.201	0.199	0.497	0.200
%RSD	1.57	1.74	1.79	0.33	1.20

For the intermediate precision, a study carried out by the same analyst working on different day. The results calculated as inter-day RSD corresponded to less than 2.0 %(For Standard). The same study was carried out for different analysts (*n* = 6 number of samples per analyst) obtaining a RSD of 1.5 %(Intermediate

Precision) The Overall %RSD for n=12 is less than 2.0. Both results together with the individual results are showing that the proposed analytical technique has a good intermediate precision.

Intermediate Method Precision	Decarboxylated Impurity	Desfluoro Impurity	Ethylenediamine impurity	Ciprofloxacin Impurity	Chloro impurity
Over all %RSD (from 12 replicate Preparations)	1.8	1.6	1.4	1.2	1.4

Accuracy

The accuracy of the method was determined on three concentration levels by recovery experiments. The recovery studies were carried out in triplicate preparations by spiking the impurities on composite blend collected from 20 tablets of enrofloxacin and analyzed as per the proposed method. The percentage recoveries with found in the range of 90.0 to 105.5 with an overall %RSD of less than 2.0 in each level. From the data obtained which given in table-2 the method was found to be accurate.

Table 4: Recovery studies for Related Substances of Enrofloxacin by proposed method

Accuracy	Decarboxylated Impurity		Desfluoro Impurity		Ethylenediamine impurity	
	Recovery Range	%RSD At each level	Recovery Range	%RSD at each level	Recovery Range	%RSD at each level
50% Level	92.8-95.6	1.5	91.5-94.5	1.6	89.2-92.1	1.6
100% Level	98.4-101.2	1.5	92.6-94.6	1.2	90.4-92.7	1.3
150% Level	99.1-100.5	0.7	93.6-94.6	0.7	91.5-94.4	1.6

Accuracy	Ciprofloxacin Impurity		Chloro impurity	
	Recovery Range	%RSD at each level	Recovery Range	%RSD at each level
50% Level	98.5-101.2	1.4	93.7-94.5	0.4
100% Level	98.9-101.2	1.3	94.2-94.9	0.4
150% Level	98.7-99.5	0.4	95.4-96.5	0.6

Linearity

Table 5: Linearity studies for Enrofloxacin and its related substances by proposed method

%Level (Approx.)	Enrofloxacin		Decarboxylated Impurity		Desfluoro Impurity	
	Concentration (µg/ml)	Area	Concentration (µg/ml)	Area	Concentration (µg/ml)	Area
10	0.622	6489	0.0210	1895	0.0196	5489
25	1.560	16897	0.0524	4758	0.0490	15478
50	3.110	35874	0.1048	9557	0.0979	28809
100	6.220	68145	0.2097	19016	0.1959	57618
150	9.330	102851	0.3145	28524	0.2938	86427
Slope		11015		90672		293040
Intercept		136		12		306
% Y-Intercept		1.2		0.1		0.5
STYEX		980		28		583
CC		0.9998		1.0000		0.9999
RSQ		0.9995		1.0000		0.9998
Residual sum of squares		980		28		583
LLD		0.02		0.02		0.03
LLQ		0.08		0.06		0.06

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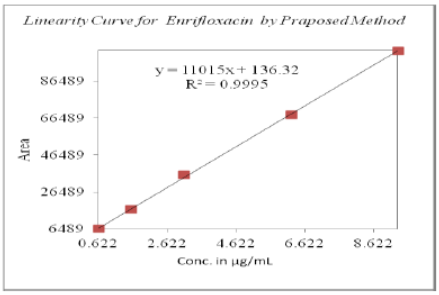
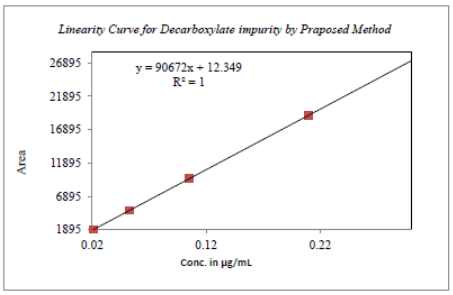
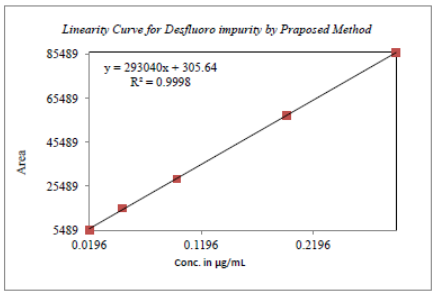
%Level (Approx.)	Ethylenediamine impurity		Ciprofloxacin Impurity		Chloro impurity	
	Concentration (µg/ml)	Area	Concentration (µg/ml)	Area	Concentration (µg/ml)	Area
10	0.0220	4795	0.0548	12468	0.0210	2705
25	0.0549	13647	0.1370	34670	0.0525	6347
50	0.1098	24897	0.2739	68341	0.1049	12478
100	0.2196	49649	0.5478	134681	0.2099	24426
150	0.3293	74587	0.8217	201522	0.3148	36978
Slope		225004		245272		116339
Intercept		412		312		227
% Y-Intercept		0.8		0.2		0.9
STYEX		629		1009		151
CC		0.9998		0.9999		1.0000
RSQ		0.9996		0.9999		0.9999
Residual sum of squares		629		1009		151
LLD		0.03		0.02		0.03
LLQ		0.07		0.04		0.06

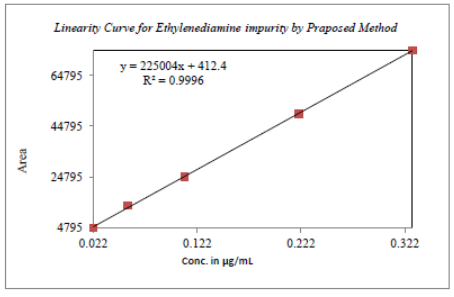
The linearity studies were conducted by spiking the impurity solutions in to diluent from 10% level to 150% of the test concentration. linearity graphs were prepared from the obtained responses and found that the data shows a linear responses for all impurities including enrofloxacin. the co-relation coefficient for all the impurities shows more than 0.95. The statistical data for linearity was shown in table no.5: The statistical data shows that the impurities shows linear responses over the selected range for the proposed analytical method.

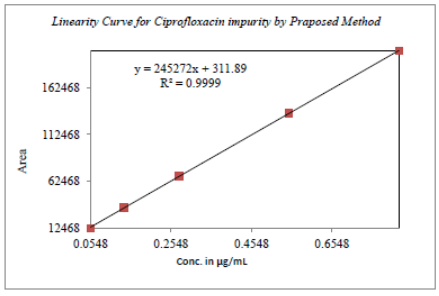
Figure 10: Linearity Graphs for Enrofloxacin and Its Related Substances by Proposed Method

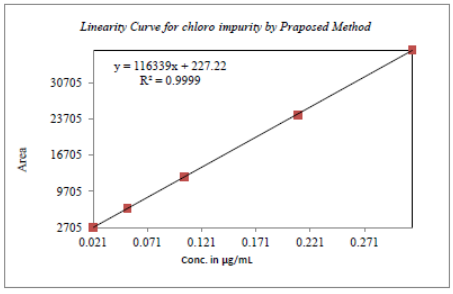












LOD/LOQ/RRF values

LOD and LOQ values are calculated by using the statistical formula from linearity data and the values shows that the developed method has good detection limits.

Robustness

As per ICH guidelines, robustness studies were conducted for flow rate, mobile phase composition, pH variation. In all these conditions, the critical parameter of resolution between ciprofloxacin and Enrofloxacin were monitored. RRT's for all impurities in the robustness study was not much impacted. The peak shape for all the impurities was found to be good. Peak purity for all impurities also tested to observe no placebo peaks interference in all the robust conditions.

Solution Stability

Solution stability was established for Enrofloxacin standard and its impurities up to 24hrs on bench top. The data shows that the standard and sample were stable.

CONCLUSION

We have developed a fast, simple and reliable analytical method for determination of related substances in enrofloxacin in pharmaceutical preparation using RP-LC. As there is no interference of blank and placebo at the retention time of impurities and enrofloxacin, passing of peak purities for all impurities along with Enrofloxacin main peak along with degradation products, the method shows good reproducibility and good response over the selected range of sample concentration. Validation of this method was accomplished, getting results meeting all requirements as per ICH guidelines. The method is simple, reproducible, specific, linear with a good accuracy and precision. It allows reliably the analysis of related substances in enrofloxain in bulk, its pharmaceutical dosage forms.

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