Analytical Method Development and Validation for Enrofloxacin in Bulk and Formulation by RP-HPLC Method

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ABSTRACT

Enrofloxacin (EFX) is a third generation Fluoroquinolone with a broad spectrum antibacterial activity. Enrofloxacin hydrochloride is 1-cyclopropyl-7-(4-ethylpiperazin-1-yl)-6-fluoro-4-oxo-1, 4-dihydroquinoline-3-carboxylic acid. A Sensitive, simple and rapid reverse phase high performance liquid chromatographic method was developed for the determination of Enrofloxacin (EFX) in tablet dosage form. The chromatographic separation was performed on a Kromasil C-18 column (250mm x 4.6 mm x 5μ) in isocratic mode using phosphate buffer pH 3:Methanol (40:60 v/v), pH adjusted to 3.0 using orthophosphoric acid as mobile phase at a flow rate of 1.0 ml/min with column temperature 30 ^{O}C. The quantification was performed at 280 nm. The method showed good linearity over the concentration range of 5-25 μg/ml with correlation coefficient r^2 0.9996. LOD and LOQ was found to be 1.0 and 3.0. The developed RP HPLC method was applied to EFX in tablet dosage form and results were found to be in agreement with the label claim.

Keywords: Enrofloxacin, RP-HPLC, Fluoroquinolone, ICH guideline (Q2R1)

INTRODUCTION

Enrofloxacin (EFX) is a third generation Fluoroquinolone with a broad spectrum antibacterial activity[1]. Enrofloxacin hydrochloride is 1-cyclopropyl-7-(4-ethylpiperazin-1-yl)-6-fluoro-4-oxo-1, 4-dihydroquinoline-3-carboxylic acid. (Figure-1) The empirical formula of the monohydrate is $C_{19}H_{22}FN_3O_3$ and its molecular weight is 359.39. Enrofloxacin is a synthetic antibacterial agent from the class of the fluoroquinolone carboxylic acid derivatives[2]. It has antibacterial activity against a broad spectrum of Gram-negative and Gram-positive bacteria. It act by inhibiting bacterial DNA gyrase, thereby preventing DNA super coiling and DNA synthesis. In veterinary medicine it is administered by subcutaneous injection to cattle and intramuscular injection to pigs and orally to cattle, pigs, turkeys and chickens for the treatment of infections of the respiratory and alimentary tract[3]. It is evident that few methods were available for the de-termination of EFX and its pharmaceutical dosage forms by using HPLC. Garcia et al developed a method for the simultaneous determination of EFX and its primary metabolite ciprofloxacin in plasma by HPLC with fluorescence detection[4]. Souza et al developed a HPLC method for determination of EFX[5]. Tyczkowska et al developed HPLC method for the simultaneous determination of EFX and its primary metabolite ciprofloxacin in canine serum and prostatic tissue[6]. Horie et al developed simultaneous determination of Benofloxacin, Danofloxacin, Enrofloxacin and Oflaxacin in chicken tissue by HPLC[7]. Reviewing the literature revealed that, few methods have been reported for the determination of Enrofloxacin in raw material, pharmaceutical formulation and/or human plasma. These methods include spectrophotometric methods[8], spectroflurimetric methods[9], electrochemical method[10], capillary electrophoresis[11], HPLC methods[12,13] etc.
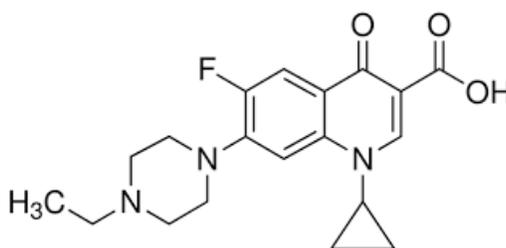

Figure 1: Chemical structure of Enrofloxacin

The present study was focused to develop and validate a sensitive, simple, rapid, reproducible and reliable RP-HPLC method for estimation of Enrofloxacin in bulk drug and its formulation.

MATERIALS AND METHOD

The Enrofloxacin was obtained from reputed firm with the certificate of analysis. Enrofloxacin tablets (Baytril, 15 mg per tablet; BAYER) were purchased from local market. All the chemicals

and reagents used in the present study were of AR grade and solvents were of HPLC grade.

Instrumentation

The HPLC system used was HPLC binary gradient system equipped with HPLC3000 series, UV detector and Kromasil C-18 column (250mm x 4.6 mm x 5µ). Analytical balance of SHIMADZU (ATY 64) and SciTech (SE-366) Sonicator were used in study.

Preparation of phosphate buffer pH 3.0:

Phosphate buffer (10 mM) was prepared by dissolving 12.112 g KH_2PO_4 in 890 mL of HPLC grade water. To this, 110 mL of 0.1M H_3PO_4 was added. The pH was adjusted 3.0 with triethyl amine.

Preparation of mobile phase

Phosphate buffer pH 3.0 (40 ml) and methanol (60 ml) were mixed, filtered through 0.45 µm nylon membrane filter and degassed by sonication. The prepared solution was used as mobile phase.

Diluents Preparation: Mobile phase used as diluent

Stock solution of Enrofloxacin

EFX (100 mg) was dissolved in and diluted upto 100 ml with diluent (1000 µg/ml). The solution (10 ml) was diluted further to 100 ml with diluent (100µg/ml).

Working standard solution of Enrofloxacin

Stock solution (0.5ml, 1.0ml, 1.5ml, 2.0ml, 2.5ml) was pipette accurately in a series of 10 ml volumetric flasks and diluted upto the mark with diluents to get 5,10, 15, 20, 25 µg/ml EFX.

Preparation of sample solution.

Twenty tablets (Baytril tablet; BAYER) were weighed accurately and finely powdered. The amount of tablet powder equivalent to 100 mg of drug EFX was transferred to 100 ml of volumetric flask, diluent (60 ml) was added and sonicated for 15 min. The resulting solution was filtered through 0.45 µm nylon membrane filter. The solution (10 ml) was transferred to 100 ml of volumetric flask and diluted upto the mark with diluent (100µg/ml). This solution was further suitably diluted for chromatography.

METHOD VALIDATION

The method was validated according to ICH guideline (Q2R1) for specificity, linearity, range, accuracy, precision, limit of detection (LOD)/limit of quantification (LOQ), ruggedness, and robustness

Specificity:

The specificity study was carried out to verify different parameters like column efficiency, resolution, retention time and peak tailing of the chromatographic system.

LOD and LOQ

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices.

LOD and LOQ were determined by the calibration curve method based on the standard deviation of the response and the slope. The detection limit is expressed as 3.3 σ/S and quantification limit is expressed as 10 σ/S where σ is the standard deviation of the response and S is the slope of the calibration curve.

Linearity

The linearity of responses for EFX was determined in the range of 5-25 μg/ ml for the proposed method. The regression equation and correlation coefficient were calculated for the calibration data.

Precision

The instrument precision was evaluated by determining the peak area of the standard solution six times repeatedly. The results are reported in terms of % RSD. The intra-day precision was evaluated through replicate analysis of three concentrations of EFX at three successive times on a same day. The inter-day precision was also evaluated through replicate analysis of three concentrations on three different successive days. The precision of the methods was expressed in terms of %RSD.

Accuracy

The accuracy of the analytical method was assessed by spiking of standard EFX to the pre-analyzed test sample of EFX of the method at three different concentration levels (50%, 100% and 150% concentration). For each concentration level three sets were prepared and analyzed and the results were calculated as mean % recovery of EFX.

RESULTS AND DISCUSSION

Selection of wavelength for detection

The working standard solution of EFX was scanned between 200 - 400 nm in the UV spectrophotometer and spectrum was obtained (Figure-2) which showed maximum absorbance at 280 nm. So it was selected as the detection wave length of EFX.
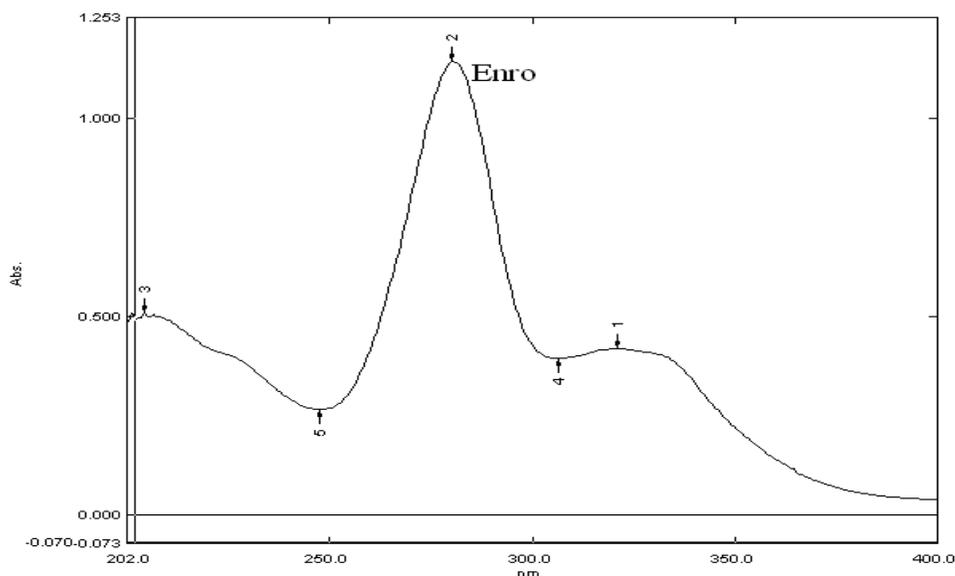
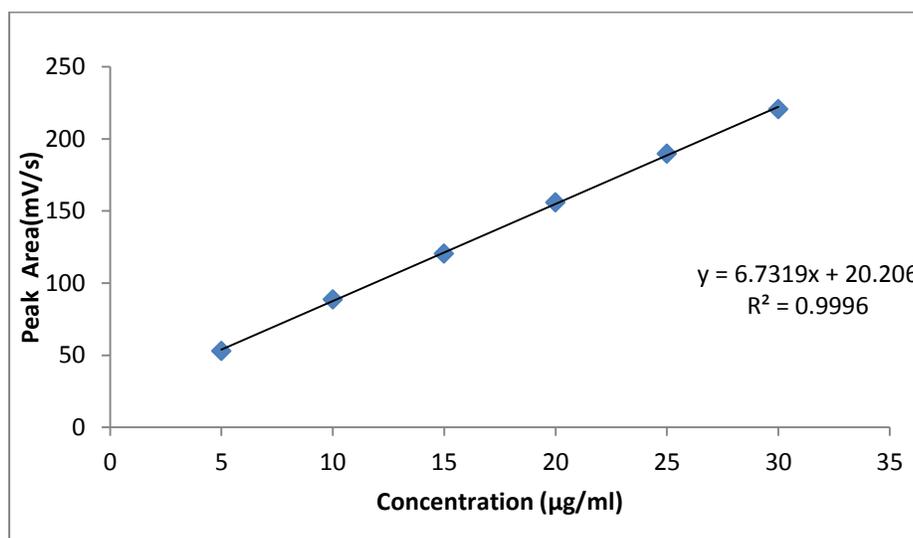**Figure 2: UV spectra of Enrofloxacin (Absorption maxima : 280nm)**

$y = 6.7319x + 20.206$
$R^2 = 0.9996$

**Figure 3: Calibration curve of Enrofloxacin (5-25 μg/ml)**

The developed RP-HPLC method was applied for the determination of Enrofloxacin in bulk and marketed tablet formulation. To optimize the chromatographic system, different mobile phase compositions including methanol, water and phosphate buffers with different pH were tried. Among them methanol: phosphate buffer pH 3 (60:40 v/v) with a flow rate of 1 ml/min showed best response. So it was selected for the analysis of EFX.

The HPLC analysis was carried out at ambient temperature. The mobile phase was prepared and filtered by passing through a 0.45μm membrane filter (Millipore, Bedford, MA, USA). EFX samples (20μl) were run in the isocratic mode on a reverse phase Kromasil C-18 column (250mm x 4.6 mm x 5μ) with mobile phase containing methanol (HPLC grade) and phosphate buffer pH 3
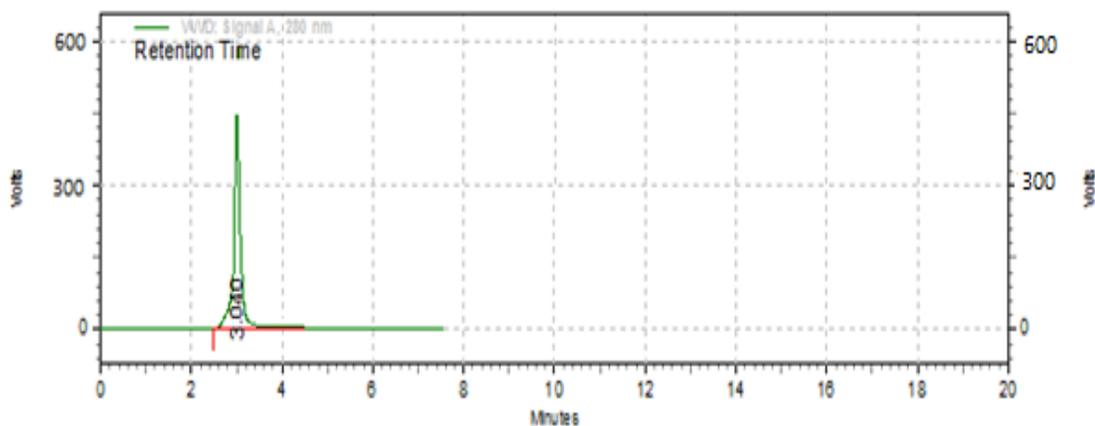
(60:40 v/v) at flow rate was 1.0ml/min. The effluent was monitored at 280nm. Figure 3 shows the chromatograms of EFX. The average retention time for 3 replicate samples of EFX were found to be 3.147±0.222, 3.220±0.053 and 3.040± 0.043 for 5, 15 and 25 µg/ml EFX respectively.
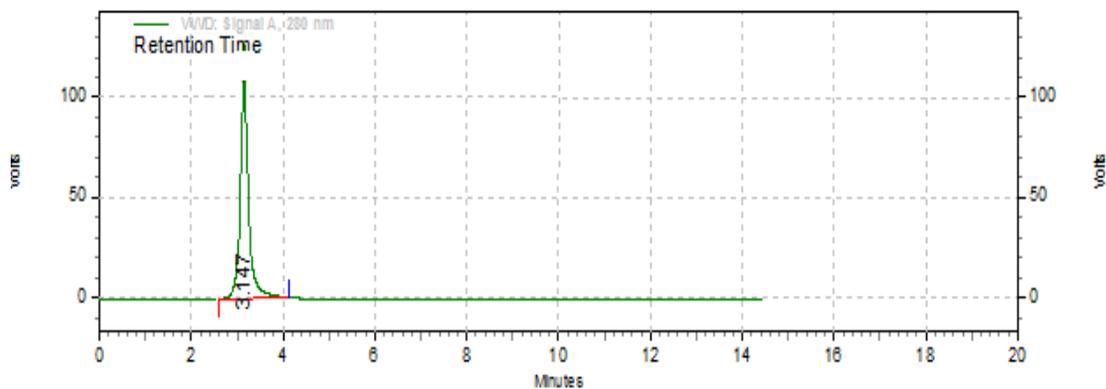
(A)
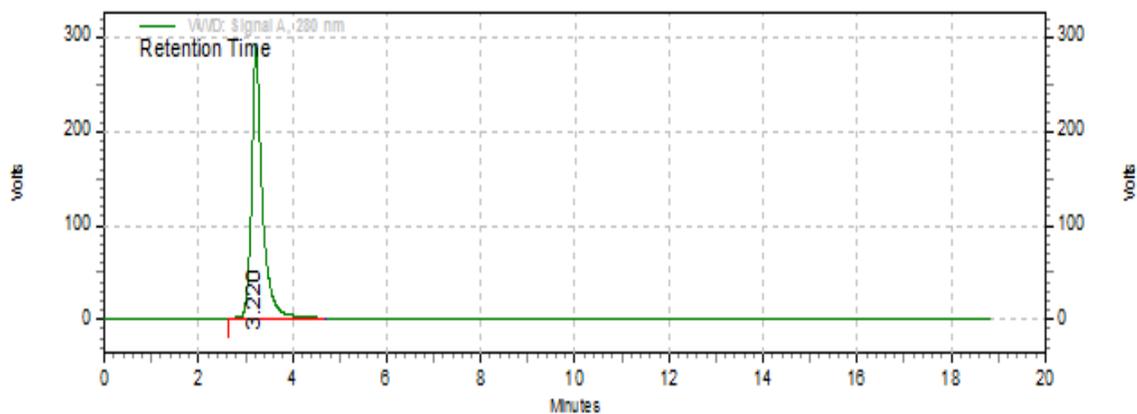
(B)

(C)

Figure 4: Typical chromatogram of Enrofloxacin (A) 5µg/ml (B) 15µg/ml (C) 25µg/ml

The average retention times for 3 replicate tablet samples of EFX was found to be 3.136±0.106. The concentration estimated by proposed method for marketed formulation of EFX was found to be 99.86% ±0.158 with % RSD of 1.5 that indicates that the developed method could be used for estimation of EFX in tablet dosage form.

Method validation

Linearity

The linear regression data for the calibration curves (n=5) showed good linear relationship over the concentration range of 5-25 µg/ml for standard drug for HPLC methods. The results for regression analysis are given in Table 1. The results for linearity of the calibration curve were validated by value of correlation coefficient of the regression line.

Table 1: Results of Linearity of Enrofloxacin (5-25µg/ml)

Concentrations (µg/ml)	Mean of Area(mV/s)*	Mean of concentration* ± SD	%RSD
5	52.794	4.93±0.222	1.0
10	88.573	9.50±0.310	1.7
15	120.396	15.98±0.220	1.5
20	150.961	20.96±0.253	0.95
25	189.744	24.99±0.202	1.5

*n=3 readings

Accuracy and precision

The results of accuracy and precision study found were within the acceptable limits. The % recovery was found in the range of 98% - 102%. The % RSD for the precision study was found to be less than 2.0% (Table 2 and 3).

Table 2: Results of recovery study of Enrofloxacin

Concentration of Std. Solution used (µg/mL)	Concentration of Sample Solution added (µg/mL)	Amount recovered* (µg/mL)	% Recovery ± SD	%RSD
10	5	5.02	100.50±0146	1.4
10	10	10.07	100.75±0.15	0.65
10	15	15.02	100.16±0.19	0.90

Table 3: Results of precision and repeatability of Enrofloxacin

Concentration (µg/ml)		Area		Mean conc. ±SD (µg/ml)		%RSD (NMT 2)	
Intra	Inter	Intra	Inter	Intra	Inter	Intra	Inter
5	5	52.670	52.550				
5	5	52.794	52.445	4.94±012	4.92±0.21	0.96	0.57
5	5	52.918	52.780				
Mean area		**52.794**	**52.49**				

15	15	120.400	120.220				
15	15	120.270	120.167	14.89±0.12	14.94±0.16	1.1	1.0
15	15	120.518	120.318				
Mean area		**120.396**	**120.235**				
25	25	220.558	220.308				
25	25	220.434	220.124	29.95±0.21	29.91±0.14	0.52	0.38
25	25	220.682	220.412				
Mean area		**220.396**	**220.412**				

Limit of detection and limit of quantitation

LOD and LOQ of EFX were found to be 1.0 µg/ml and 3.0 µg/ml respectively for the proposed method which indicates the adequate sensitivity of the method.

The proposed method was applied for the quantitative analysis of EFX in marketed tablet formulation (Baytril, 15 mg per tablet; BAYER). The assay results found were within acceptable limits (99.86% ±0.158; % RSD 1.5). The method was found to be most suitable for estimation of EFX in bulk and tablet dosage form.

Table 4: Results of LOD and LOQ

Drug	LOD (µg/ml)	LOQ (µg/ml)
Enrofloxacin	1.0	3.0

Table 5: Results for assay of Marketed Formulation

Drug & Brand Name	Label claim mg/tablet	Conc. estimated* (mg)	% Conc. estimated*±SD	% RSD
Enrofloxacin (Baytril, Bayer)	15 mg	14.98	99.86% ±0.158	1.5

CONCLUSION

A simple, rapid, reliable and cost effective RP-HPLC method has been developed for estimation of EFX in bulk and tablet formulation. The developed method was applied for quantitative estimation of EFX in its marketed tablet formulation. Results are in good agreement with label claim. Values of standard deviation were satisfactorily low indicating developed method is highly accurate and reproducible. Results of recovery study were satisfactory and show that there is no interference of excipients in analysis of EFX in marketed tablet. The developed method was found to be simple, rapid, and accurate hence it can be used for routine analysis of EFX in tablet dosage form.

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REFERENCES

1. Lopez, C., Garcie, J. J., Sierra, M., Diez, M. J., Perez, C., Sahagun, A. M., Fernandez, N. (2015). Systemic and mammary gland disposition of Enrofloxacin in healthy sheep following intra mammary administration. *BMC Veterinary Re-search*, (11), 88.

2. Sultan, I. A. (2014). Detection of Enrofloxacin residues in liver of livestock animals obtained from a slaugh-terhouse in Mosul city. *J Veterinary Sci Technolo*, (5), 168.

3. The European agency for the evaluation of medicinal products, Veterinary medicines evaluation unit, EMEA/MRL/388/98-FINAL, 1998.

4. Garcia, M. A., Solans, C., Aramayona, J. J, Ruda, S., Bregante, M. A., & Dejong, A. (1999). Simultaneous determination of Enrofloxacin and its primary metabolite, ciprofloxacin, in plasma by HPLC with fluorescence detection. *Biomedical Chromatography*, 13(5), 350-353.

5. Esouza, M. J, Bittencourt, C.F & Morsch, L.M. (2002). Method Development and Validation for Estimation of Enrofloxacin By RP-LC In Marketed Formulations. *Journal of Pharmaceutical and Biomedical Analysis*, 28(6), 1195-1199.

6. Tyzkowska, K., Hedeen, K. M., Aucoin, D. P. & Aronson, A. L. (1989). High-performance liquid chromatographic method for the simultaneous determination of Enrofloxacin and its primary metabolite ciprofloxacin in canine serum and prostatic tissue. *Journal of Chromatography –Biomedical applications,* 493(2), 337-346.

7. Horie, M., Saito, K., Nose, N. & Nakazawa H. (1994). Simultaneous determination of benofloxacin, danofloxacin, Enrofloxacin and ofloxacin in chicken tissues by high-performance liquid chromatography. *Journal of Chromatography B: Biomedical Science and Application*, 653(1), 69-76.

8. Samia, M., Mohamed, E. S. E. & Awad, A. (2002). Spectrophotometric determination of Enrofloxacin and pefloxacin through ion-pair complex formation. *Journal of Pharmaceutical and Biomedical Analysis*, 28(1), 173-180.

9. Chen, G. & Schneider, M. J. (2003). A rapid spectrofluorometric screening method for Enrofloxacin in chicken muscle. J. Agric. Food Chem., 51(11), 3249–3253.

10. The European agency for the evaluation of medicinal products, Veterinary medicines evaluation unit, EMEA/MRL/388/98-FINAL, 1998.

11. Zhou, X., Xing, D., Zhu, D., Tang, Y. & Jia L. (2008). Development and application of a

capillary electrophoresis–electrochemiluminescent method for the analysis of Enrofloxacin and its metabolite ciprofloxacin in milk. *Talanta*, 75(5), 1300-1306.

12. Nidal, B., Shorouq W. & Fuad, A. R. (2017). A Validated Stability-Indicating HPLC Method for Simultaneous Determination of Amoxicillin and Enrofloxacin Combination in an Injectable Suspension. *Scientia Pharmaceutica*, 85(6), 1-8.

13. Prasad, K. B. & Murthy, T. G. K. (2010). Analytical method development and validation for the estimation of Enrofloxacin in small volume parenteral formulation by HPLC method. *J. Pharm. Res.*, 3, 1128–1130.