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SPECTROPHOTOMETRIC METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF ENROFLOXACIN IN PURE AND DOSAGE FORMS

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ABSTRACT

Objective: A new, simple, sensitive, precise and linear two spectrophotometric methods was developed and validated for the estimation of enrofloxacin in bulk and dosage forms. **Method:** The developed spectrophotometric methods based on the determination of enrofloxacin by using bromothymol blue (Method-A) and Gibb's reagent (Method-B). By using bromothymol blue, the drug forms ion-pair complex with bromothymol blue (BTB) in acidic medium. These colored complexes were extracted with chloroform and maximum absorbance at 418 nm. By using Gibb's reagent, the drug forms a colored product with Gibb's reagent in alkaline medium (pH 9.4) at 473 nm. **Results:** Linearity calibration curve were obtained in a concentration range of 1.0-10 µg/mL in method A and 1.0 - 7.0 mg/mL in method-B. The results of analysis shows that the amount of drugs present in the formulation has a very good correlation with the label claim of the formulation and % RSD will be less than 2 for all the validation parameters. Recoveries studies revealed that results within the specified limits. **Conclusion:** The simplicity of the method was found to be satisfactory and could be used for the routine analysis of enrofloxacin in their marketed formulation.

KEYWORDS: Bromothymol blue, Enrofloxacin, Gibb's reagent and Method development.

INTRODUCTION

Enrofloxacin is a synthetic chemotherapeutic agent from the class of the fluoroquinolone carboxylic acid derivative which was developed exclusively for use in animals.[1] Chemically it is a 1-cyclopropyl-7-(4-ethyl-1-piperazinyl)-6-fluoro-1,4-dihydro-4-oxo-3-quinoline

carboxylic acid. It acts by inhibiting bacterial DNA gyrase (a type-II topoisomerase), thereby preventing DNA super coiling and DNA synthesis.[2,3] It exhibits a wide spectrum of antimicrobial activity against Gram-negative bacteria and Gram-positive bacteria.[4,5,6]

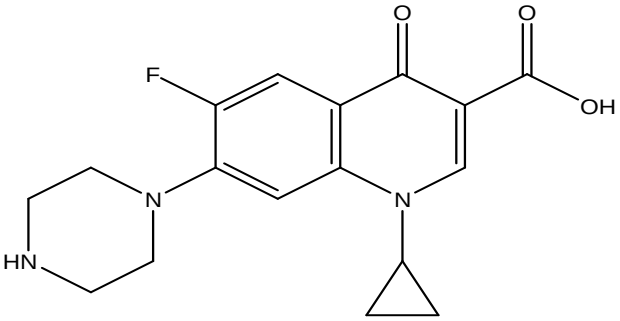


Fig 1: Chemical Structure of Enrofloxacin

International trade in aquaculture has been growing continuously in the recent years, and is expected to persist in the future. The variety of farmed aquatic species is now diversifying and fish farming is intensifying. At the same time, increased incidence of disease also due to intensive aquaculture requires a more intense use of veterinary drugs and chemicals. During the last few years enrofloxacin has received growing attention because of its potential efficiency for the treatment of diseases in fish.[7,8]

The extensive literature survey's revealed that few methods have been reported for the estimation of enrofloxacin in pure and dosage forms. There is a wide scope for the development of new analytical methods for estimation of enrofloxacin. This research work presents simple, sensitive, accurate and reproducible two spectrophotometric methods were developed for the estimation of enrofloxacin in bulk and dosage forms.

EXPERIMENTAL

MATERIALS AND METHODS

Instruments

An Elico model SL 164 UV-Visible double beam spectrophotometer connected to computer loaded with spectra treats software with spectral bandwidth of 1nm with a pair of 10 mm matched quartz cells. All weights were taken on electronic balance (Denver, Germany). All the experiments were performed at room temperature (25±1) °C.

Reagents

All chemicals and reagents were used as analytical grad. Enrofloxacin standard was obtained as a gift sample from shasun Pharmaceuticals (puducherry, India). Enrofloxacin is available commercially with brand names of BAYTRIL and GYROFLOX (50 mg) and were procured from the local market.

a) Preparation of Standard Solution:

Method A: Standard stock solutions of enrofloxacin were prepared in 0.1N HCL at the concentration of 1mg/mL. (Stock - A solution)

Method B: Standard stock solutions of enrofloxacin were prepared in 0.1N NaOH at the concentration of 1mg/mL. (Stock - B solution)

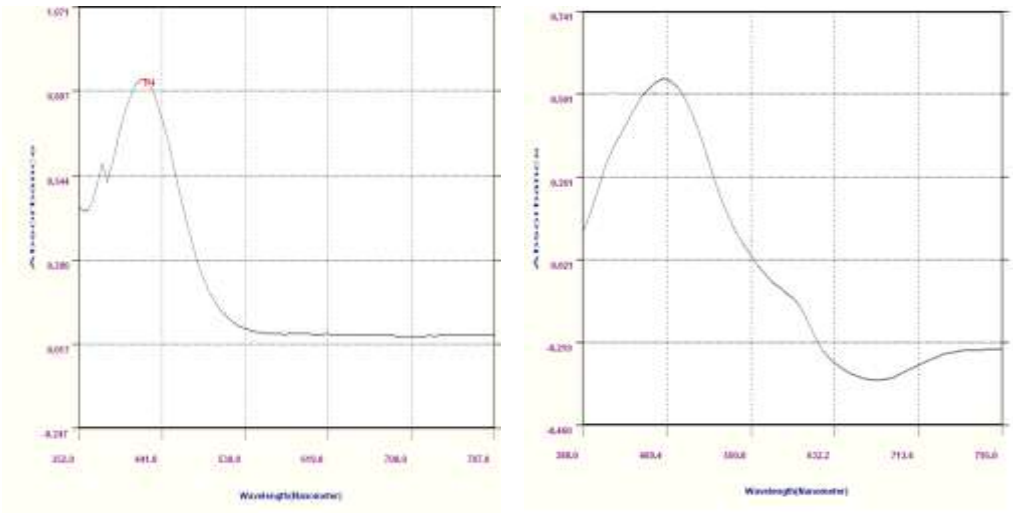
b) Determination of Maximum absorbance (ʎ max) and Calibration curve

Method-A

From the standard stock-A solution, Prepare 10 µg/mL with 0.1N HCL. The solution was scanned in the visible range 400-800 nm. Absorbance was recorded at 418 nm [Figure 2(a)]. Prepare a series of solutions having the concentration ranging from 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 µg/mL of enrofloxacin with 0.1N HCL and each solution has shake well for 10 min with chloroform (5 mL x 3 times), separate the chloroform layer and measure the absorbance at 418 nm, and a calibration curve of absorbance against concentration was plotted were shown in Figure 3(a).

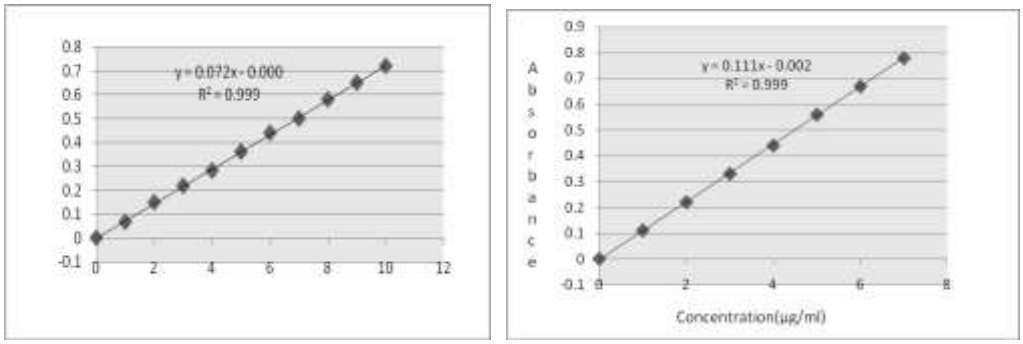
Method-B

From the standard stock - B solution, prepare 10 µg/mL with 0.1N NaOH. The solution was scanned in the visible range 400 - 800 nm. The absorbance was recorded at 473 nm [Figure 2 (b)]. Prepare a series of solutions having the concentration ranging from 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 µg/mL of enrofloxacin with 0.1N NaOH. Absorbances of the above solutions were measured at 473 nm, and a calibration curve of absorbance against concentration was plotted were shown in Figure 3(b).



(a) **(b)**

Figure 2: λ max of Enrofloxacin (a) using BTB reagent and (b) Gibb's reagent.



(a) **(b)**

Figure 3: Calibration curve of Enrofloxacin (Method-A) and (Method-B)

Procedure for the assay

Method-A

Twenty tablets of enrofloxacin were powdered and accurately weighed equivalent quantity of 250 mg and make up to 250 mL with 0.1N HCl (1000 µg/mL). From this prepare 100 µg/mL then take 1.0 mL of sample solution, add 2 mL of 0.05% w/v Bromothymol blue (BTB) and 1 mL of phthalate buffer (pH - 2.8) make up to 10 mL by using 0.1N HCl (10 µg/ml). The above solution was transferred in to a separating funnel, add 5 mL x 3 times chloroform and shake for 10 min. Take chloroform layer and measure the absorbance of yellow colored products at 418 nm against blank.

Method-B

Twenty tablets of enrofloxacin were powdered and accurately weighed equivalent quantity of 100 mg and make up to 100 mL with 0.1N NaOH (1000 µg/mL). From this prepare 100

µg/mL then take 1.0 mL of sample solution, add 2 mL of 0.05% w/v of Gibb's reagent and 2 mL of borate buffer (pH - 9.4) make up to 10 mL with 0.1N NaOH, stands for 10 min, the absorbance of wine red colored products was measured at 473 nm against blank.

Table l: Estimation of Enrofloxacin

Tablet (brand)	Labelled amount (g)	Estimated amount (Mean ± SD, g)		% Recovery		% RSD	
		Method A	Method B	Method A	Method B	Method A	MethodB
Brand A	0.05	0.0503±0.00021	0.049±0.0076	100.06%	99.0%	0.417	0.95
Brand B	0.05	0.0496±0.00048	0.05016±0.00049	99.2%	100.3%	0.967	0.976

Validation of the Method

The optimized spectrophotometric methods were validated according to the procedures described in ICH guidelines Q2 (R1).

Linearity

The linearity of a method is its ability to obtain test results that are directly proportional to the sample concentration over a given range. A concentration of 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 µg/mL for Method-A and 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10µg/mL for Method-B have been investigated. The relationship between absorbance and concentration was used to make this determination. Calibration curves for each method were plotted and the obtained data were subjected to regression analysis. The result was showed in table 6.

Precision

The reproducibility of the proposed method was determined by performing the assay for the same day (intra-day assay precision) and on three different days (inter-day assay precision). Precision studies in spectrophotometric methods were performed by preparing nine determinations covering the specified range for the procedure (3 x 3 replicates for each concentration). The results were showed in table 4 & 5.

Table 4: Precision studies for Enrofloxacin [Method-A]

CONC.	Ab.(Intra-day) (mean±SD)	% RSD	CONC.	Ab.(Inter-day) (mean±SD)	% RSD
3µg/mL	0.2813±0.00055	0.196	3µg/mL	0.2863±0.0015	0.52
6µg/mL	0.4513±0.00059	0.174	6µg/mL	0.4553±0.0015	0.33
9µg/mL	0.6513±0.00011	0.25	9µg/mL	0.6553±0.00023	0.035

Table 5: Precision studies for Enrofloxacin [Method-B]

CONC.	Ab.(Intra-day) (mean±SD)	% RSD	CONC.	Ab.(Inter-day) (mean ± SD)	% RSD
3µg/mL	0.221±0.00039	0.176	3µg/mL	0.2316±0.0001	0.043
5µg/mL	0.5516±0.00091	0.164	5µg/mL	0.5592±0.0043	0.768
7µg/mL	0.7813±0.00011	0.014	7µg/mL	0.7856±0.0021	0.267

Accuracy

In order to ensure the suitability and reliability of proposed method, recovery studies were carried out. The accuracy study was carried out by the analysis of standard additions at three levels that is multi-level recovery studies. To a fixed equivalent quantity of formulation powder as well as synthetic mixture, a known quantity of standard enrofloxacin added at 50%, 100% and 150% level and the contents were re-analyzed by Spectrophotometricaly. The % recovery and % RSD were calculated. The results were showed in table 2 & 3.

Table 2: Recovery Studies for Enrofloxacin (Method A & B).

Level of addition (%) Pure drug	Con. of drug in formulations (µg/ml)		Conc. Drug of Pure (µg/ml)	
	Method-A	Method-B	Method-A	Method-B
50%	6	5	3	2.5
100%	5	6	6	5
150%	5	6	9	7.5

Table 3: Statistical validation data for accuracy determination of Enrofloxacin

Level of % recovery	Mean		Standard Deviation		% RSD		% Analytical recovery	
	A	B	A	B	A	B	A	B
50%	9.027	8.976	0.0057	0.000583	0.617	0.085	100.3	99.71
100%	10.17	12.08	0.412	0.000234	0.578	0.245	101.4	100.72
150%	12.44	14.9633	0.0016	0.00146	0.012	0.123	99.94	99.73

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ were separately determined based on the standard calibration curve. The residual standard deviation of y- intercept of regression lines may be used to calculate LOD and LOQ. LOD = 3.3*D/S and LOQ = 10*D/S where , D is the standard deviation of the intercept of regression line and S is the slope of the calibration curve. The result was shown in table 6.

Table 6: Calibration Data of Enrofloxacin (Method A & B)

Parameters	Method-A	Method-B
λmax	418.5	473nm
Linearity(µg/mL)	1-10	1-7
Correlation coefficient(r^2)	0.9997	0.9999
Slope(m)	0.0722	0.1118
Intercept(c)	-0.0006	-0.0025
LOD (µg/mL)	0.02	0.06
LOQ (µg/mL)	0.28	0.87

Ruggedness

The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions, such as different laboratories, analysts, instruments. Ruggedness of the proposed methods was determined by analysis of aliquots from homogenous slot by two analyst using same operational and environmental conditions. The results were shown in table 7.

Table 7: Ruggedness for Enrofloxacin

SL NO:	Conc. (µg/mL)		Method-A		Method-B	
			Analyst-1	Analyst-2	Analyst-1	Analyst-2
	A	B	Absorbance	Absorbance	Absorbance	Absorbance
1	6	5	0.441	0.440	0.56	0.56
2			0.441	0.441	0.56	0.56
3			0.440	0.443	0.57	0.56
4			0.441	0.442	0.56	0.56
5			0.441	0.441	0.56	0.56
6			0.441	0.441	0.57	0.57
Ab.Mean			0.4406	0.4413	0.5633	0.5616
S.D			0.00049	0.00059	0.0051	0.0041
%RSD			0.111	0.135	0.6080	0.730

Robustness

The robustness of an analytical procedure is the measure of its capacity to remain unaffected by small, but deliberate, variations in method parameters and provides an indication of its reliability during normal usage. The proposed methods parameters were change of volumetric flasks (10 mL, 50 mL and 100 mL) were performed. Three replicates were made for the same conc. (10 µg/mL) in 10 mL, 50 mL and 100 mL volumetric flasks in spectrophotometric methods. The result is expressed in % RSD. The results were shown in table 8.

Table 8: Robustness for Enrofloxacin

Vol. flask	Conc. (µg/mL)		Absorbance (mean)		Label claim (g)	Estimated Amount (g) mean		Data	
	Method		Method			Method		Method	
	A	B	A	B		A	B	A	B
10mL	3	6	0.1573	0.4256	0.0500	0.054	0.0506	%Label Claim=100.8 SD=0.0004 %RSD=0.25	%Label Claim=101.34 SD=0.0015 %RSD=0.35
25mL	3	6	0.1576	0.4096	0.0500	0.050	0.0499	%Label Claim=100.4 SD=0.00023 %RSD=0.148	%Label Claim=99.98 SD=0.0011 %RSD=0.279
50mL	3	6	0.1583	0.4164	0.0500	0.049	0.0500	%Label Claim=99.34 SD=0.0040 %RSD=0.259	%Label Claim=100.16 SD=0.0028 %RSD=0.679

RESULTS AND DISCUSSION

In this proposed method, based on spectrophotometric determination of Enrofloxacin in pure and dosage forms were developed in two methods. One was extractive spectrometric method by using bromothymolblue and other method by using Gibb's reagent.

Method-A

Enrofloxacin react with Bromothymolblue in acidic medium and extract with chloroform to get a yellow colored ion-pair complex and measured the maximum absorbance at 418.5nm. The ion-pair formation is due to Enrofloxacin contains secondary amino group which is protonated in acidic medium, while sulphonic acid group is present in BTB, which is the only group undergoing dissociation in the pH range 1-5. The color of such dye is due to the opening of lactiod ring and subsequent formation of quinoid group. Finally the protonated Enrofloxacin forms ion-pairs with the dye and extracted into chloroform.

Method-B

Enrofloxacin react with Gibb's reagent in alkaline medium at a pH (9.4) to form a pinkish red colour product. The maximum absorbance is measured at 473nm. Enrofloxacin having amino group coupled with imide portion of 2, 6-dichloroquinone chloramide to get a coupled product in alkaline medium at a pH of 9-10. The method was validated as per ICH guidelines. Accurate results were obtained by utilizing the proposed methods for the estimation of enrofloxacin and excellent concurrence has occurred with the results by the reported methods was found, linearity was obtained in the concentration range of 1-10 µg/ml at 418 nm in

method-A and 1-7 µg/ml in method-B at 473 nm. The % recovery is higher than 98 % has found it revealed that the method is safe and free from interference of excipients which is used in the formulation. The value of standard deviation and % R.S.D. were found to be < 2 %, showed the high precision of the method. High % recovery and low % RSD suggests that the method can be used for the routine analysis of commercial formulations.

CONCLUSIONS

The objective of the proposed work was to develop spectrophotometric methods for the determination of enrofloxacin, and validate the methods according to ICH guidelines and applying the same for its estimation in marketed formulations. The proposed spectrophotometric methods developed were found to be rapid, simple, precise, accurate and economic for routine estimation of enrofloxacin in commercial dosage forms.

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