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Short communication

LC determination of enrofloxacin

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Abstract

A simple, rapid and sensitive high-performance liquid chromatographic (HPLC) method was developed for the assay of enrofloxacin in raw material and injection. The validation method yielded good results and included the range, linearity, precision, accuracy, specificity, recovery, limit of detection (LOD) and limit quantification (LOQ) values. The HPLC separation was carried out by reversed phase chromatography on a C-18 absorbosphere column (150×4.6 mm i.d. 5 μm particle size) with a phase composed of sodium acetate (pH 4.7; 0.1 M): acetonitrile (60:40, v/v; pH 5.0), pumped isocratically at a flow rate of 1.5 ml min^{-1}. The effluent was monitored at 278 nm with the eluting solvent. The calibration graph for enrofloxacin was linear from 10.0 to 80.0 μg ml^{-1}.

Keywords: Fluoroquinolone; HPLC; Quality control; Enrofloxacin

1. Introduction

Infectious diseases are a serious problem for the livestock and fish-farm industries, therefore, various kinds of antibiotics and synthetic antibacterials are widely used for prevention and treatment [1]. Enrofloxacin is a synthetic antibacterial agent of the fluoroquinolone class (Fig. 1). In human as well as in veterinary medicine, fluorquinolones have proved to be highly valuable antimicrobial and pharmacokinetic properties in a unique way [2,3]. Enrofloxacin, the first fluoroquinolone developed for veterinary application, is available in several oral and parental formulations for the treatment of some infectious diseases in pets and livestock. It is approved in dogs and cats for the treatment of infections, for example, urinary tract, respiratory tract, and skin [4]. In dogs and other species, enrofloxacin is de-ethylated to ciprofloxacin, a fluoroquinolone used in human medicine [5]. The fluoroquinolones are bactericidal and act principally by inhibition of bacterial DNA-girase [6]. Enrofloxacin is an antimicrobial with a broad spectrum of activity, against a wide

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range of Gram-negative and Gram-positive bacteria, including those resistant to β-lactam antibiotics and sulfonamides [3]. Enrofloxacin is potentially valuable for the treatment of bacterial infections in cattle, pigs, poultry, dogs and cats [1,7].

Chemically enrofloxacin is a 1 cyclopropil-7-(4-ethyl-1-piperazinyl)-6-fluoro-1,4-dihydro-4-oxo-3-quinoline carboxylic acid [8] and is not official in any pharmacopoeia.

Enrofloxacin was determined in raw material and dosage form only by extractive spectrophotometric method [11]. High-performance liquid chromatographic (HPLC) is the most frequently applied technique in the determination of drugs in biological fluids and dosage forms. We believe that the availability of this new method, with increased simplicity, sensitivity and selectivity, will be very useful for the determination of enrofloxacin in raw material and pharmaceutical preparations. This isocratic HPLC method uses a simple mobile phase, UV–vis detection and does not require complicated sample preparation. The aim of this study was to develop a simple, rapid and reproducible reversed-phase HPLC method for the quantification of enrofloxacin in raw material and injection. This method can also be used to identify product degradation of enrofloxacin in stability studies. The validation of the proposed method was also carried out [9].

2. Experimental

2.1. Samples

The enrofloxacin reference substance (assigned purity 99.28%) were generous gifts from Hoechst Vet. The enrofloxacin 5% injection were obtained commercially and claimed to contain 5% of enrofloxacin in sterile water for injection.

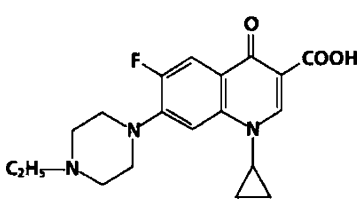


Fig. 1. The chemical structure of enrofloxacin.

2.2. Reagents and solvents

All other chemicals used were of pharmaceutical or special analytical grade. The water was bidistilled in a glass for all analytical purposes.

2.3. Apparatus and chromatographic conditions

The HPLC system consisted of a Shimadzu SCL-10A-VP, equipped with a model LC-10AD-VP pump; and SPD 10A-VP, variable- wavelength UV–vis detector (set at 278 nm), an SCL-10A system controller; a class VP software package integrator and Rheodine injection valve with a 20 μl loop (Shimadzu, Kyoto, Japan). A shim-pack CLC-ODS (M) column (150 mm × 4.6 mm i.d. 5 μm particle size). The mobile phase was a mixture of sodium acetate (pH 4.7; 0.1 M), acetonitrile (60:40, v/v), (pH 5.0) adjusted by addition of acetic glacial acid, isocratic as the mobile phase, was flow rate of 1.5 ml min^{-1}. The sensitivity was 1.0 AUFS. The HPLC system was operated at ambient temperature (20 ± 1 °C). The mobile phase was filtered through a 0.45 μm membrane filter (Supelco) and degassed with a helium sparge for 15 min.

2.4. Procedure

2.4.1. Enrofloxacin reference standard

Solutions of the enrofloxacin reference standard (1 mg ml^{-1}) were in mobile phase prepared by accurately weighing 25 mg enrofloxacin standard transferred to a 25 ml volumetric flask, followed by adding mobile phase to make up the volume. Aliquots (4 ml) of the enrofloxacin standard solution were transferred volumetrically into 100 ml volumetric flasks and mobile phase added to make up to volume to give a final concentration of 40 μg ml^{-1}.

2.4.2. Assay of enrofloxacin

Aliquots (2 ml) of the enrofloxacin injection were transferred volumetrically into 100 ml volumetric flasks, followed by making up to volume

Table 1
Experimental values obtained in the recovery test for enrofloxacin injection by HPLC

Amount of standard (μg ml^{-1})			Recovery[a](%)	RSD (%)
	Added	Recovered		
R_1	20	99.72	19.94	0.21
R_2	40	101.80	40.72	0.28
R_3	60	98.30	58.98	0.30

[a] Mean of three replicate analysis.

with mobile phase. This sample solution had a concentration of 100 μg ml^{-1}. From this solution, the dilutions were made with mobile phase to give a final concentration of 40 μg ml^{-1}. All determinations were conducted in triplicate.

2.4.3. Calculations

Having established the quantitative relationships between the parameters studied, and knowing the predictive performance of their association model, a linear simple regression by the least squares method was applied.

2.4.3.1. Recovery of enrofloxacin. The recoveries were determined by adding known amounts of the enrofloxacin reference substance (20.0, 40.0, 60.0 μg ml^{-1}) to the samples at the beginning of the process (Table 1). A recovery exercise was then performed.

2.5. Method validation

The method was validated by determination of the following operational characteristics, linearity; range; precision; specificity; accuracy intra and inter day, limit of detection (LOD) and limit of quantification (LOQ).

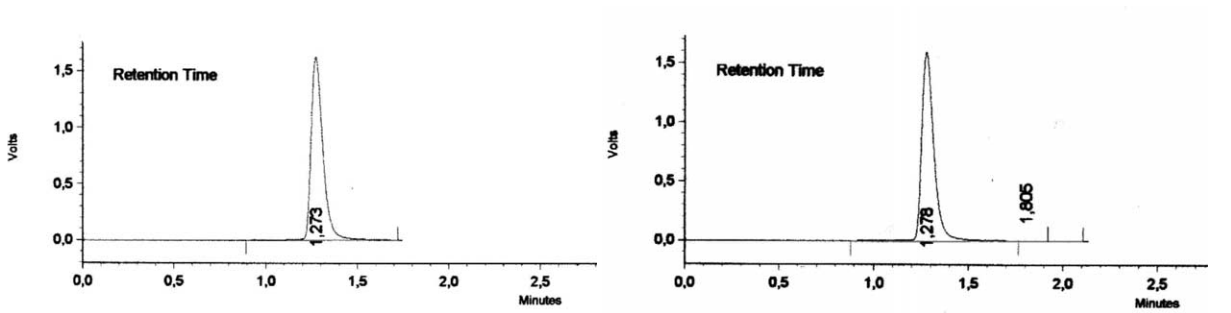
The precision was expressed as the percent coefficient of variation of each curve. The statistical data were calculated by analysis of variance (ANOVA). The specificity test involved forced degradation of enrofloxacin, to determine whether the degradation products are resolved from the enrofloxacin peak. Solutions of the enrofloxacin reference standard (1 mg ml^{-1}) were in mobile phase prepared by accurately weighing 25 mg enrofloxacin standard transferred to 25.0 ml volumetric flask, followed by adding mobile phase to make up the volume. Aliquots (5 ml) of the enrofloxacin standard solution were transferred volumetrically into 50 ml volumetric flasks and 1 N hydrochloric acid added to make up to volume to give a concentration of 100 μg ml^{-1}. This sample was refluxed for 5 h and diluted the nominal assay concentration with mobile phase. A similar experiment was conducted with 1 N sodium hydroxide.

Stability of the analytes, mobile phase, standard and sample solutions were subjected to long term (5 days) stability studies. The stability of these solutions was studied by performing the experiment and looking for the change in the chromatographic pattern compared with freshly prepared solutions.

3. Results and discussion

The development of methods in HPLC for the determination of drugs has received considerable attention in recent years because of their importance in quality control in pharmaceutical analysis. The goal of this study was to develop an HPLC assay for the analysis of enrofloxacin in raw material and injection. For pharmacokinetic evaluation, HPLC methods have been developed for determination of enrofloxacin and its metabolites in biological fluids [5] in bovine milk and plasma [10,12]. The chromatographic conditions were used to separate the enrofloxacin and its metabolites in a complex media. For quantitative determination of enrofloxacin in raw material and dosage forms, an extractive spectrophotometric method was reported. This method is based on the formation of ion-association complexes with Supracene violet 3B and Tropaeolin 000 and their complexes are quantitatively extracted into chloroform [11] For drug analysis in quality control, the simplest and fastest procedures can be applied. In this study, the chromatographic conditions were influenced by the physic-chemical properties of enrofloxacin, such as solubility, polarity and UV absorption. The chromatogram of

the sample peaks were matching with the corresponding chromatogram of the standard drug peaks, with shows that the peaks of analyte were pure and also formulation excipient and impurities were not interfering with the analyte peaks. The optimum mobile phase was obtained with sodium acetate (pH 4.7; 0.1 M) acetonitrile (60:40 v/v) pH 5.0 adjusted by addition of acetic glacial acid. In general, it can be stated that the internal standard method clearly has the advantage of not being sensitive to complex sample preparation, or even to sample losses during sample cleanup and enrichment [13]. However, we used the external standard method, because it is simpler, fast and accurate for sample preparation. The retention time repeatability during the precision studies was found to be excellent for all the solutions. The retentions times (t_R) of the enrofloxacin reference substance and the injection were 1.273 and 1.278, respectively. The HPLC chromatogram shows t_R (Fig. 2). The calibration curves for enrofloxacin were constructed by plotting concentration versus peak area and showed good linearity in the 10.0–80.0 μg ml^{-1} range. The representative linear equation was $y = 20604.64 + 71300.6x$ ($n = 5$, $r = 0.9998$, $r^2 = 0.9996$). The method was validated by evaluation of intra and inter day precision. In the range of 10–80 μg ml^{-1} the percent relative standard deviation (R.S.D.) on the basis of peak area ratios for three replicate injections were found to be between 0.02 and 0.83%. The inter day precision (3 days, $n = 9$) was expressed as R.S.D. and ranged 0.24–1.4%. The average coefficient of correlation was $r = 0.9998$ and the R.S.D. of the slope and intercept of the four lines were 0.56 and 0.24%, respectively. Analysis of variance of the data indicated no significant difference in slopes of the four calibration curves ($P < 0.01$). The LOD and LOQ were obtained by use of the slope and the standard deviation of the intercept from four calibration graphs determined by linear regression line as defined by ICH [9]. The LOD and LOQ were found to be 0.16 and 0.5 μg ml^{-1}, respectively. The repeatability of the method was studied by assaying six samples of injection, at the same concentration, during the same day under the same experimental conditions. The coefficient of variation was 0.33%. The mean absolute recovery determined by adding known amounts of enrofloxacin reference substance (20, 40, 60 μg ml^{-1}) to the samples at the beginning of the process, were found to be 99.94%. The detailed accuracy and precision are shown in Table 1. Enrofloxacin injections were analyzed and the results obtained can be seen in Table 2. No interference from the sample solvent, impurities and dosage from excipients could be observed at the detection wavelength (278 nm), as shown in Fig. 2(A and B). A major factor in the improvement of pharmaceutical stability testing has been development of analytical methods that are suitable for use in a stability-indicating assay. In particular, the availability of HPLC for determination of this drug has been a real boon to stability testing. This method can also be applied to stability studies, which quantify enrofloxacin in the presence of



(A) **Time retention (min)** (B)

Fig. 2. HPLC chromatograms of the enrofloxacin reference substance (A) and enrofloxacin injection (B).

Table 2
Data obtained from commercial sample analysis by HPLC

	Theoretical amount (mg ml^{-1})	Experimental amount[a](mg ml^{-1})	Purity (%)	R.S.D (%).
1	50	50.92	101.85	0.24
2	50	50.95	101.90	0.33
3	50	50.94	101.88	0.42

[a] Mean of three determinations.

its degradation products. The ruggedness of the method was carried out by analyzing the same samples under a variety of conditions, such as changing the source of reagents and solvents (different manufacturers), analytes, different elapsed assay times and different days. It was observed that there were no significant changes in the chromatographic pattern when slight changes were made in experimental condition showed that the method is rugged. The specificity test was to determine whether new degradation products were produced from the forced degradation of enrofloxacin reference substance, whether these degradation products are resolved from the enrofloxacin chromatographic peak, and to verify the peak purity of the enrofloxacin peak. Degradation of 48% was observed in enrofloxacin reference substance when refluxed in 1 N hydrochloric acid and 1 N sodium hydroxide for 5 h. These degradants were observed at relative retention time of 0.84 and 0.98, respectively. Peak purity showed that the enrofloxacin peak was pure. The stability of the solutions were studied and the data obtained showed that sample and standard solutions were stable during 5 days when these were stored at about 5 °C (in refrigerator).

4. Conclusion

HPLC is the leading method having significant official status in drug analysis and was found to be simple and efficient compared with other methods.

The HPLC method developed in this study has the advantage of simplicity, precision and accuracy when compared with extractive spectrophotometric method. The method uses simple reagents, with minimum sample preparation procedures, encouraging its application in routine analysis.

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