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Note

Microbiological assay for enrofloxacin injection

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Abstract

A simple, sensitive and specific agar diffusion bioassay for the antibacterial enrofloxacin was developed. Using a strain of *Staphylococcus aureus* ATCC 6538P as the test organism, enrofloxacin at concentrations ranging from 3.2 to 12.8 $\mu g\,ml^{-1}$ could be measured in injection. A prospective validation of the method showed that method was linear ($r = 0.99998$), precise (R.S.D. = 0.27) and accurate (it measured the added quantities). The method shows results that confirm its precision, not differing significantly the other method described in the literature. We conclude that microbiological assay is satisfactory for quantitation of in vitro antibacterial activity of enrofloxacin.

Keywords: Fluoroquinolone; Microbiological assay; Quality control; Enrofloxacin; Cylinder-plate method

1. Introduction

The fluorquinolone antibiotics have a wide range of antibacterial activity and have seen increasing use in the veterinary medicine because of their effectiveness in treating bacteria infections (Bauditz, 1990; Cinquina et al., 2003).

Enrofloxacin (Fig. 1) a fluorquinolone marketed for use in veterinary medicine, has been shown to be rapidly bactericidal against a broad spectrum of aerobic and facultative anaerobic bacteria including strains resistant to many other antimicrobial agents (Bauditz, 1987; Scheer, 1987). Enrofloxacin also has been reported to have activity against some obligate anaerobic bacteria. As with the other fluorquinolones, enrofloxacin inhibits DNA synthesis and is rapidly bactericidal against susceptible proliferating and dormant bacteria (Cozzarelli, 1980; Vancutsem et al., 1990). Chemically enrofloxacin is a 1-cyclopropil-7-(4-ethyl-1-piperazinyl)-6-fluoro-1,4-dihydro-4-oxo-3-quinoline carboxylic acid (Budavari et al., 1996). No official method is available for the assay of pure drug and its formulations. The LC method has been described to determine enrofloxacin injection (Souza et al., 2002). Bioassay for other fluorquinolones has also been described (Marona and Schapoval, 1998; Marona and Schapoval, 2001; Ev and Schapoval, 2002). The vast majority of quinolone assays currently available, however, are designed for the evaluation of samples in biological fluids, such as serum and urine. The microbiological assay for the determination of enrofloxacin in plasma has been described (Elsheikh et al., 2002), but no bioassay of the enrofloxacin drug and its formulations has been reported for laboratory

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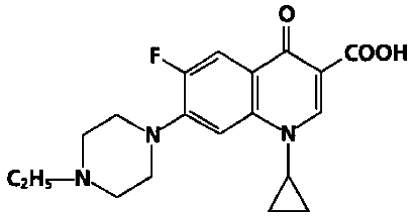

Fig. 1. Chemical structure of enrofloxacin.

quality control. The objective of the present study was to find a sensitive and reproducible agar diffusion method to quantify enrofloxacin in injection dosage form and compare it with high-performance liquid chromatographic (HPLC) method.

2. Materials and methods

The enrofloxacin reference substance (assigned purity 99.28% w/w) were generous gifts from Hoechst Vet (Fortaleza, Brazil). The enrofloxacin 5% (w/v) injection were obtained commercially that claimed to contain 5% (w/v) of enrofloxacin in sterile water. All reagents were analytical grade. Distilled water was used in all experiments.

2.1. Preparation of enrofloxacin reference standard

The standard solution was diluted in 0.1 M potassium phosphate buffer, pH 8.0 (USP 25, 2002) and assayed at concentrations of 3.2, 6.4 and 12.8 $\mu g\,ml^{-1}$.

2.2. Assay of enrofloxacin

A quantify of the enrofloxacin injection containing 500 mg (10 ml) de enrofloxacin were transferred volumetrically into 50 ml volumetric flasks, followed by making up to volume with 0.1 M potassium phosphate buffer, pH 8.0 to give a final concentrations of 3.2, 6.4 and 12.8 $\mu g\,ml^{-1}$.

2.3. Organism and inoculum

The cultures of *Staphylococcus aureus* ATCC 6538P (obtained of INCQS, Rio de Janeiro, Brazil) were cultivated on Grove Randall number 1 agar (Difco) at freezer and pealed to another Grove Randall number 1 agar (24 h before assay) that was kept in stove for 1 day 35 °C. Diluted cultures suspensions of 25% turbidity were obtained at 580 nm, using a suitable spectrophotometer (Bausch & Lomb, USA) and a 13 mm diameter test tube as and absorption cells against 0.9% sodium chloride a blank. Portions of 1.0 ml of the inoculated saline solution 0.9% were added to 100 ml of antibiotic medium 1 at 48 °C and used as inoculated layer.

2.4. Cylinder-plate assay

The agar was composed of two separate layers. The antibiotic medium 1 (21 ml) was poured into 100 m × 20 mm Petri dish for the base layer. After solidification this layers portions of 4 ml of inoculated antibiotic medium 2 agar was poured to the base layer. Six stainless steel cylinders of uniform size (8 mm × 6 mm × 10 mm) were placed on the surface of inoculated medium. Three alternated cylinders were filled with 100 μl of reference concentrations solutions on the other three cylinders with the concentrations samples solutions. Six plates were performed for each sample. The bioassay plates were incubated at 35 °C aerobically for 18 h. The zone sizes (in mm) were carefully measured with electronic caliper (Starrett®).

2.5. Calculation

To calculate the activity of enrofloxacin the Hewitt equation was used (Hewitt, 1977). The assays were statistically calculated by the linear parallel model and by means of regression analysis and verified using analysis of variance (ANOVA) (Hewitt, 1977; ICH, 1996).

2.6. Validation of the method

The method was validated by determination of the following operational characteristics: linearity, precision, accuracy (ICH, 1996; USP 25, 2002).

2.6.1. Linearity

In order to assess the validity of the assay three doses of the reference substance were used. The linearity of the calibration curves was determined for intra- and inter-day precision in 3 different days. The

calculation of regression line by the method of least squares was employed.

2.6.2. Precision

The precision of analytical procedure was evaluated through the repeatability (intra-assay) and intermediate precision (inter-assay). Method repeatability was studied by assaying samples of enrofloxacin injection, of the same concentration, on the same day and under same the experimental conditions. The intermediate precision was evaluated by comparing the assays on different days.

2.6.3. Accuracy studies

The recoveries were determined at three-concentration levels, by adding known amounts of enrofloxacin reference substance to the samples at the beginning of the process. Aliquots (2 ml) of the enrofloxacin injection were transferred volumetrically into 100 ml volumetric flasks, followed by making up to volume with potassium phosphate buffer, pH 8.0 to give a stock solution with concentration of 100 $\mu g\,ml^{-1}$. Portions of 2.5 ml of this stock solution were transferred to 25 ml volumetric flasks where 3.0, 6.0 and 9.0 ml of enrofloxacin reference solutions (6.4 $\mu g\,ml^{-1}$), equivalent to 0.76; 1.53; 2.30 $\mu g\,ml^{-1}$ enrofloxacin were added. Potassium phosphate buffer, pH 8.0 was added to make up the volume in order to give a concentration of 10.76, 11.53, 12.30 $\mu g\,ml^{-1}$, respectively, of the sample concentrations used in the assay. The percentage recovery of enrofloxacin reference added was calculated using the equation proposed by AOAC (1990).

2.6.4. Specificity

The previous tests proposed with the samples in comparison to the standard should detect probable impurities and in this case the contents would propositional to the microbiological inhibition.

2.6.5. Methods comparison

It was analysed six samples using the proposed proceeding and by HPLC. The method used in determination by HPLC was described by (Souza et al., 2002).

3. Results and discussion

The activity of antimicrobial agents may be demonstrated under suitable conditions by their inhibitory effect on microorganisms (USP 25, 2002). The activity of enrofloxacin in vitro has been tested against various microorganisms. These studies have shown that enrofloxacin has potent in vitro activity against *S. aureus* (Scheer, 1987; Vancutsem et al., 1990). There are no official microbiological cylinder-plate assays described in the official codes to determine enrofloxacin in pharmaceutical formulations. In this work experi-

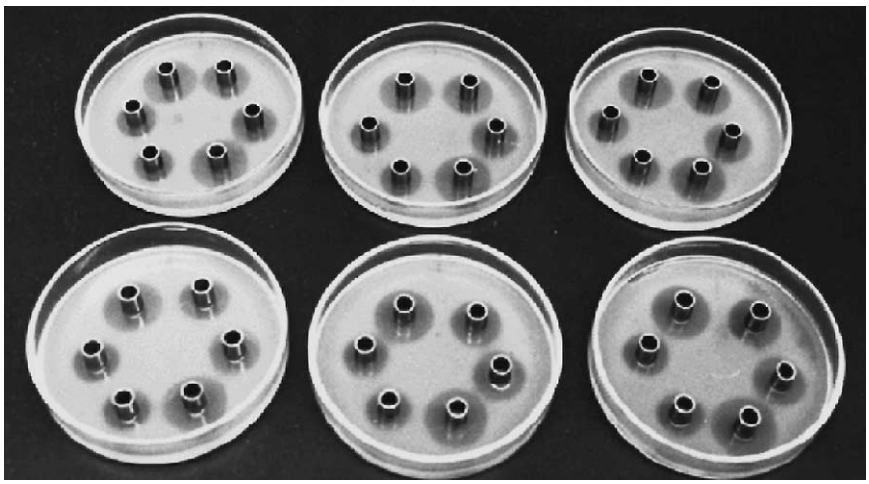

Fig. 2. Microbiological assay (cylinder-plate method), using a strain *S. aureus* ATCC 6538P as the test organisms, enrofloxacin at concentrations 3.2; 6.4 and 12.8 $\mu g\,ml^{-1}$.

Table 1
Diameters of growth inhibition zones to obtain standard curve

Concentration ($\mu g\,ml^{-1}$)	Diameters of growth inhibition zones (mm)[a]	Mean diameters of growth inhibition zones ± S.E.M.	R.S. D. (%)
3.2	15.78 15.80 16.36 15.81 15.83 16.35	15.98 ± 0.116	1.78
6.4	18.78 18.91 18.81 18.91 18.86 18.80	18.84 ± 0.023	0.30
12.8	21.61 21.63 21.58 21.63 21.73 21.70	21.64 ± 0.023	0.26

S.E.M., standard error mean, R.S.D., relative standard deviation.
[a] Each value is the mean of six analyses.

mental 3 × 3 (Fig. 2) design using three dose levels for each standard and sample were used following the procedure described in Brazilian (1988) and European Pharmacopoeia (2002). The calculation procedures normally assume a direct relationship between the observed zone diameter and logarithm of applied dose. The results of growth inhibition zone diameter of enrofloxacin reference substance are presented (Table 1).

The calibration curves for enrofloxacin were constructed by plotting zone diameter (mm) versus log of concentrations ($\mu g\,ml^{-1}$) and showed good linearity in the 3.2–12.8 $\mu g\,ml^{-1}$ range (Fig. 3). The representative linear equation was $y = 11.24178 + 9.40043 \ln x$ ($n = 3$, $r = 0.99998$, $r^2 = 0.99996$). The inter-day precision was evaluated by comparing the linear regressions of the three standards plots on the three different days in a 2 months period, the coefficient of correlation was 0.99998 and the coefficient of variation of the slope of the three lines was 1.14%. The experimental values obtained for the determination of enrofloxacin in samples are present in Table 2. According to Brazilian (1988) and European Pharmacopoeia (2002), if a parallel-line model is chosen, the two log dose–response lines of the preparation

Table 2
Experimental values obtained in the recovery test for enrofloxacin injection by microbiological cylinder-plate assay

	Amount of standard added ($\mu g\,ml^{-1}$)	Amount of standard recovered[a] ($\mu g\,ml^{-1}$)	Recovery (%)	R.S.D. (%)
R1	0.76	0.750	98.7	0.64
R2	1.53	1.519	99.3	0.48
R3	2.30	2.293	99.7	0.30

R.S.D., relative standard deviation.
[a] Mean of three replicate analyses.

to be examined and the reference preparation must be parallel and they must be linear over the range of doses used in the calculation. These conditions were verified by validity test for a given probability, usually $P = 0.05$. The assays were validated by means of the analysis of variance, as described in these official codes already cited in this paragraph. There are no deviations from parallelism and linearity with results obtained here ($P < 0.05$).

The precision and accuracy of the assay were demonstrated. The precision is usually expressed as the R.S.D. of a series of measurements (ICH, 1996). The repeatability shows means R.S.D. of 0.27% (Table 3), indicating good intra-day precision of the method. The results obtained in different days shows coefficient of variation of 0.64%.

The accuracy expresses the agreement between the accepted and the found value (ICH, 1996). The mean recovery was found to be 99.23% (Table 3).

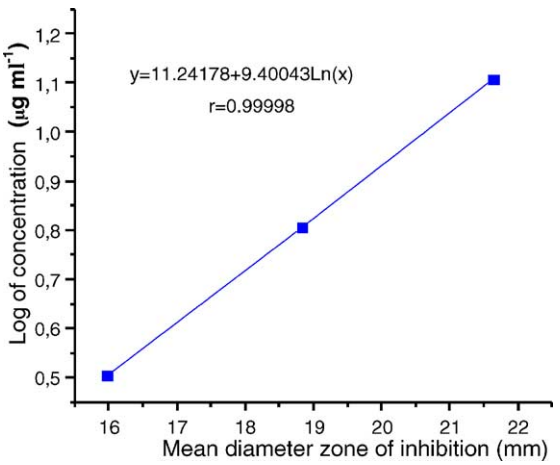

Fig. 3. Calibration curve for enrofloxacin, obtained by the microbiological cylinder-plate assay.

Table 3
Data obtainer from commercial sample analysis by microbiological cylinder-plate assay

Sample	Theoretical amount ($mg\,ml^{-1}$)	Experimental amount[a] ($mg\,ml^{-1}$)	Purity (%)	R.S.D. (%)
1	50	51.12	102.25	0.27
2	50	51.16	102.32	
3	50	51.26	102.52	
4	50	51.07	102.14	
5	50	51.13	102.27	
6	50	50.85	101.70	

R.S.D., relative standard deviation.
[a] Each value is the mean of six analysis independent.

Table 4
Analysis of enrofloxacin injection by two different methods

Methods	Mean (%)	R.S.D. (%)	*n*
HPLC	102.08	0.24	6
Microbiological	102.20	0.27	6

R.S.D., relative standard deviation.

The data obtained by microbiological assay and HPLC method (Table 4) were statistically comparable by ANOVA test, which indicated there is no significant difference between the methods at the ($P < 0.05$). The concordance between the results of both methods was superior a 95%. The R.S.D. of the microbiological method was 0.27% and by HPLC method 0.24.

The microbiological method developed for determination of enrofloxacin injection in this study is linear, precise and accurate. From the results, it can be concluded that the proposed microbiological method was valid and suitable for the quantitative determination of enrofloxacin in the formulation studied.

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References

AOAC, 1990. Official Methods of Analytical Chemists of AOAC, 15th ed. Arlington, 1, p. xvii.

Bauditz, R., 1987. Resultados obtenidos en la comprobación clínica com Baytril em terneros y cerdos. Not. Méd. Rev. 2, 122–129.

Bauditz, R., 1990. Enrofloxacin, clinical evolution in several animal species. Veterinary Pharmacology, Toxicology an Therapy in Food Producing Animals. University of Veterinary Science, Unipharma, Budapest, p. 21.

Budavari, S., O'Neil, M.J., Smith, A., Heckelman, P.E., Kinneary, J.F., 1996. The Merck Index, An Encyclopedia of Chemicals, Drugs, and Biologicals, 12th ed. Merck & Co., Inc., Witehouse Station, NJ, USA, p. 608.

Cinquina, A.L., Roberti, P., Giannetti, L., Longo, F., Draisci, R.A., Fagiolo, R., Brizioli, N.R., 2003. Determination of enrofloxacin and its metabolite ciprofloxacin in goat milk by high-performance liquid chromatography with diode-array detection optimization and validation. J. Chromatogr. A 987, 221–226.

Cozzarelli, N.R., 1980. DNA gyrase and the supercoiling of DNA. Science 73, 3872–3876.

Elsheikh, H.A., Taha, A.A.W., Khalafallah, A.I., Osman, I.A.M., 2002. Disposition kinetics of enrofloxacin (Baytril 5%) in sheep and goats following intravenous and intramuscular injection using a microbiological assay. Res. Vet. Sci. 73, 125–129.

European Pharmacopoeia, 4th ed., 2002. Conseil de l'Europe, Strasbourg, 2.7.2, pp. 160–166.

Ev, L.S., Schapoval, E.E.S., 2002. Microbiological assay for determination of ofloxacin injection. J. Pharm. Biomed. Anal. 27, 91–96.

Farmacopéia Brasileira, 4th ed., Atheneu, 1988. Parte I, v.5.2.17–v.5.2.17-11.

Hewitt, W., 1977. Microbiological Assay: An Introduction to Quantitative Principles and Evolution. Academic Press, New York, pp. 41–42.

ICH, 1996. Harmonised Tripartite Guideline, Validation of Analytical Procedures: Metodology. Geneva, pp. 1–8.

Marona, H.R.N., Schapoval, E.E.S., 1998. Desarrollo de análisis microbiológico para la determinación de esparfloxacino en polvo y en tabletas de 200 mg. Informacion Tecnologica 9, 251–254.

Marona, H.R.N., Schapoval, E.E.S., 2001. Analysis of sparfloxacin and its degradation by bioassay. Acta Pharm. Turcica 43, 7–9.

Scheer, M., 1987. Studies on the antibacterial activity of Baytril. Vet. Med. Rev. 2, 90–99.

Souza, M.J., Bittencourt, C.F., Morsch, L.M., 2002. LC determination of enrofloxacin. J. Pharm. Biomed. Anal. 28, 1195–1199.

US Pharmacopoeia XXV, 2002. US Pharmacopoeial Convention, Rockville, MD, pp. 2256–2258.

Vancutsem, P.M., Babish, J.G., Schwark, W.S., 1990. The fluorquinolone antimicrobials: structure, antimicrobial activity, pharmacokinetics, clinical use in domestic animals and toxicity. Cornell Vet. 80, 173–186.