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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS DETERMINATION OF ENROFLOXACIN AND ITS PRIMARY METABOLITE CIPROFLOXACIN IN CANINE SERUM AND PROSTATIC TISSUE

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SUMMARY

A simple and sensitive high-performance liquid chromatographic method was developed for the determination of enrofloxacin and ciprofloxacin in canine serum and prostatic tissue. Sample preparation consisted of mixing canine serum with a 1:1 dilution of acetonitrile and 0.1 *M* sodium hydroxide followed by ultrafiltration through a 10 000 molecular mass cut-off filter. Prostatic tissue was sonicated with the same solution prior to ultrafiltration. Separation of these two quinolones in the ultrafiltrate was accomplished by ion-paired liquid chromatography using a reversed-phase analytical column eluted with an acetonitrile–methanol–water solution. Enrofloxacin and ciprofloxacin were detected by a photometric ultraviolet–visible detector set at 278.6 nm and confirmed by a photodiode array detector operating from 230 to 360 nm. The limits of detection for enrofloxacin and ciprofloxacin were 4 and 2 ng/ml, respectively.

INTRODUCTION

Enrofloxacin and ciprofloxacin belong to the new quinolone carboxylic acid group of antibiotics with an extended antibacterial spectrum (Fig. 1). In the dog (as well as other species), enrofloxacin is de-ethylated to ciprofloxacin which is detectable in both urine and serum. Enrofloxacin is under investigation as to its usefulness in the treatment of chronic bacterial prostatitis in dogs,

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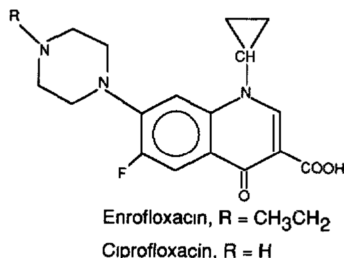

Fig. 1. Chemical structures of enrofloxacin and ciprofloxacin.

and an assay designed to measure both enrofloxacin and its primary metabolite, ciprofloxacin, in serum and prostatic tissue is needed.

Recently, several high-performance liquid chromatographic (HPLC) methods concerning determination of ciprofloxacin in body fluids have been described [1–6]. These methods employ multiple steps for sample preparation involving solvent extraction followed by reversed-phased separation and detection by UV–VIS or fluorometric methods. The analysis of enrofloxacin in canine serum and prostatic tissue by HPLC has not been reported.

The methodology to be described in this paper involves simultaneous determination of enrofloxacin and ciprofloxacin in serum and prostatic tissue from normal healthy dogs. Sample preparation was simplified by using ultrafiltration (rather than solvent extraction) and reversed-phase ion-paired chromatography for enhanced separation of enrofloxacin and ciprofloxacin.

Detection was achieved using photometric UV–VIS and photodiode array (PDA) detectors. The PDA detector was used to develop the assay since it produced multiple chromatograms allowing the evaluation of chromatographic peak homogeneity while the photometric detector was used for quantitative analysis and improved sensitivity.

EXPERIMENTAL

Reagents and chemicals

Enrofloxacin and ciprofloxacin standards were kindly supplied by Mobay (Shawnee Mission, KN, U.S.A.) and Miles Labs. (Elkhart, IN, U.S.A.), respectively. Acetonitrile and methanol were glass-distilled and supplied by American Burdick & Jackson (Muskegon, MI, U.S.A.). HPLC-grade phosphoric acid and triethylamine were obtained from Fisher Scientific (Raleigh, NC, USA). The ion-pairing reagents, octanesulfonate (S_8) and dodecanesulfonate (S_{12}) were supplied by Regis (Morton Grove, IL, U.S.A.). All water was distilled and deionized with the Model 1000 Hydro Ultrapure Water System from Hydro Services and Supplies (Research Triangle Park, NC, U.S.A.).

Stock solutions of 1 mg/ml enrofloxacin and ciprofloxacin were prepared in acetonitrile–0.1 *M* sodium hydroxide (1:1). Working concentrations (250 and

50 ng/ml) were prepared daily with the same diluent. All standards were protected from the light with aluminum foil and amber vials.

High-performance liquid chromatography

The HPLC system consisted of a Waters 820 work station, Models W600 and 590 LC solvent delivery systems, U6K injector and 712 WISP automatic injector with a temperature control accessory set at 40°C, Model 990 PDA detector and Model 481 variable-wavelength UV–VIS detector (all Waters, Milford, MA, U.S.A.). The LC separations were performed using a mobile phase consisting of acetonitrile–methanol–water (15:2:83, v/v) containing 3 m*M* dodecanesulfonate, 1.5 m*M* octanesulfonate, 0.4% phosphoric acid (85%; v/v), and 0.4% (v/v) triethylamine. The column was a 3 μm, 100 mm×4.6 mm phenyl Spherisorb from Phenomenex (Ranch Palos Verdes, CA, U.S.A.). The column effluent was analyzed in the wavelength range 230–360 nm using the PDA detector with a sensitivity of 0.005 absorbance units full scale (a.u.f.s.) or monitored by the UV–VIS detector at 278.6 nm using 0.005–0.01 a.u.f.s. Peak-area measurements were computed by the Waters 820 work station.

Sample preparation

Serum. A 0.5-ml aliquot of serum was diluted with an equal volume of a solution consisting of acetonitrile–0.1 *M* sodium hydroxide (1:1) in the microseparation system (Centricon-10), an ultrafiltration device with a molecular mass cut-off filter of 10 000 daltons supplied by Amicon (Danvers, MA, U.S.A.). Samples were vortexed for approximately 10–15 s and centrifuged for 30 min at 2677 *g* in a 45° fixed-angle rotor. A 30–120 μl aliquot of colorless ultrafiltrate was injected into the HPLC system with column effluent monitored by either a photometric or PDA UV–VIS detector.

Prostate. Prostatic tissue samples were minced using No. 10 Band-Parker scalpel blades and weighed. A mixture of acetonitrile–0.1 *M* sodium hydroxide was added to 100–300 mg of the minced prostate to a total volume of 500 μl and ultrasonicated in a Model 450 ultrasonic water-bath from E/MC (Fisher Scientific, Raleigh, NC, U.S.A.) for 30 min. The extract was then transferred to the microseparation device and centrifuged for 30 min at 2677 *g* in a 45° fixed-angle rotor. A 80–120 μl colorless ultrafiltrate was injected into the HPLC system and monitored as above.

RESULTS AND DISCUSSION

Enrofloxacin and ciprofloxacin exhibit high-polarity properties due to the presence of two ionizable groups in these molecules. Therefore, ion-paired reversed-phase chromatography was utilized to enhance separation from other observable serum and prostatic tissue components. The microseparation system was successful in extracting these antibiotics from canine serum and pros-

tatic tissue. This is consistent with our experience in extracting other antibiotics from various biological matrices [7–10]. The quinolone carboxylic acid antibiotics exhibit varying degrees of protein binding (14–30%) to serum proteins [11]. Several solutions were tested for their ability to free enrofloxacin and ciprofloxacin from protein binding, and due to the amphoteric properties of free ciprofloxacin, 0.1 *M* sodium hydroxide was considered. Based on the recoveries of these drugs from canine serum, a mixture of acetonitrile–0.1 *M* sodium hydroxide (1:1) was deemed optimal and chosen for the extraction–ultrafiltration step.

Several stationary phases and elution systems were investigated to produce optimal separation of these drugs and to assure a required sensitivity of less than 10 ng/ml of each antibiotic. The photometric detector response was linear from 5 to 1000 ng/ml for both enrofloxacin and ciprofloxacin standards, and ciprofloxacin solutions were also linear with correlation coefficients (r) of 0.9999 ($n=10$ each).

A chromatogram of 10 ng of ciprofloxacin and 10 ng of enrofloxacin standards (0–10 min run time) and canine serum spiked with 250 ng/ml of these analytes (10–20 min run time) is shown in Fig. 2A. The chromatogram depicts the maximum wavelength absorbance for all existing peaks found in the 230–

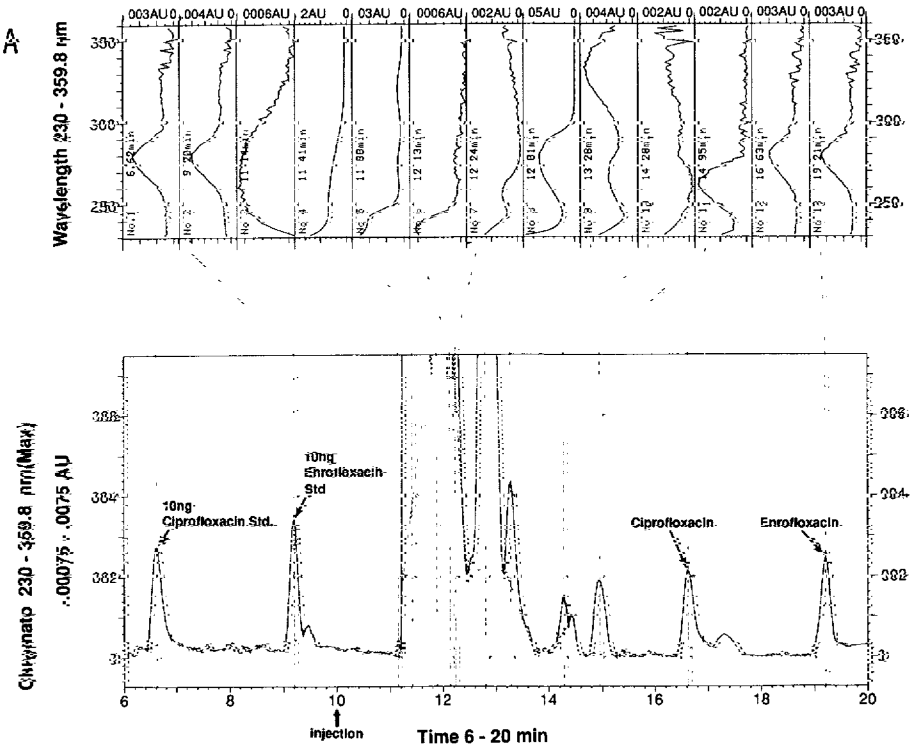

Fig. 2.

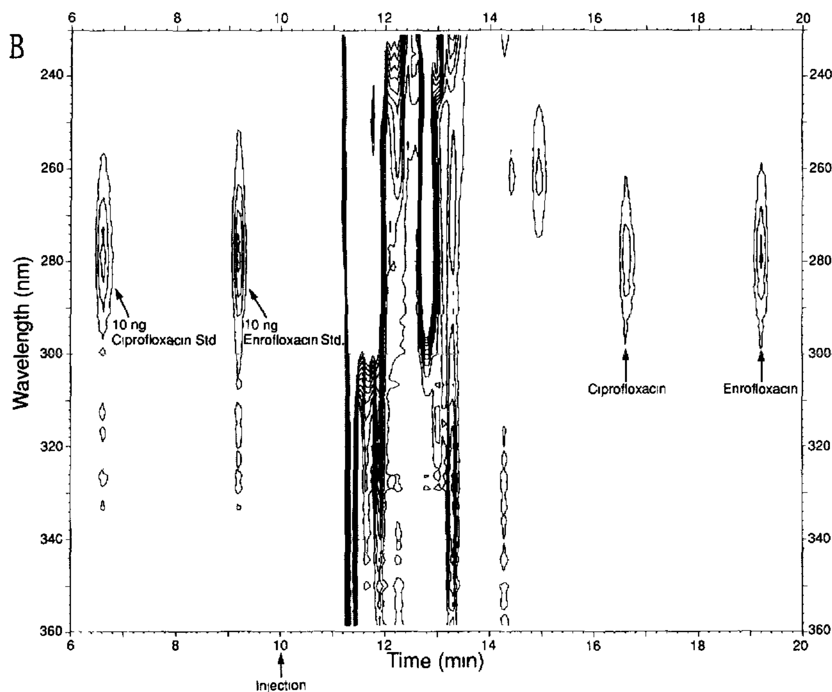

Fig. 2. (A) LC-UV-VIS photodiode array detection fragment of chromatogram for 10 ng of ciprofloxacin and 10 ng of enrofloxacin standards (0–10 min, injected at 0 min, not shown) and chromatogram of canine serum spiked with 250 ng/ml of both analytes (10–20 min, injected at 10 min). Injection volume was 40 μl. (B) Contour plot of the chromatogram introduced in (A).

360 nm range and their respective UV spectral curves. These UV spectral curves for the ciprofloxacin standard (No. 1) and the ultrafiltrate of canine serum (No. 12) have similar shapes. The same applies to the UV spectral curves for the enrofloxacin standard (No. 2) and canine serum ultrafiltrate (No. 13). Contour plots of ciprofloxacin and enrofloxacin are presented in Fig. 2B. The contours for ciprofloxacin and enrofloxacin standards (running time 6.62 and 9.20 min) and ultrafiltrate of spiked canine serum (running time 16.63 and 19.21 min, injected at 10 min) were symmetrical and identical which indicated the absence of interfering compounds.

A typical chromatogram obtained from 1.5 ng of enrofloxacin and ciprofloxacin standard solutions and canine serum spiked with 50 ng/ml of each drug is shown in Fig. 3. These chromatograms were generated on the photometric detector which has greater sensitivity than the PDA detector for these compounds. The comparison of serum spiked with 50 ng/ml enrofloxacin and ciprofloxacin (Fig. 3b) to 1.5 ng of standards of each (Fig. 3a) indicates that these analytes were almost completely separated from other serum components.

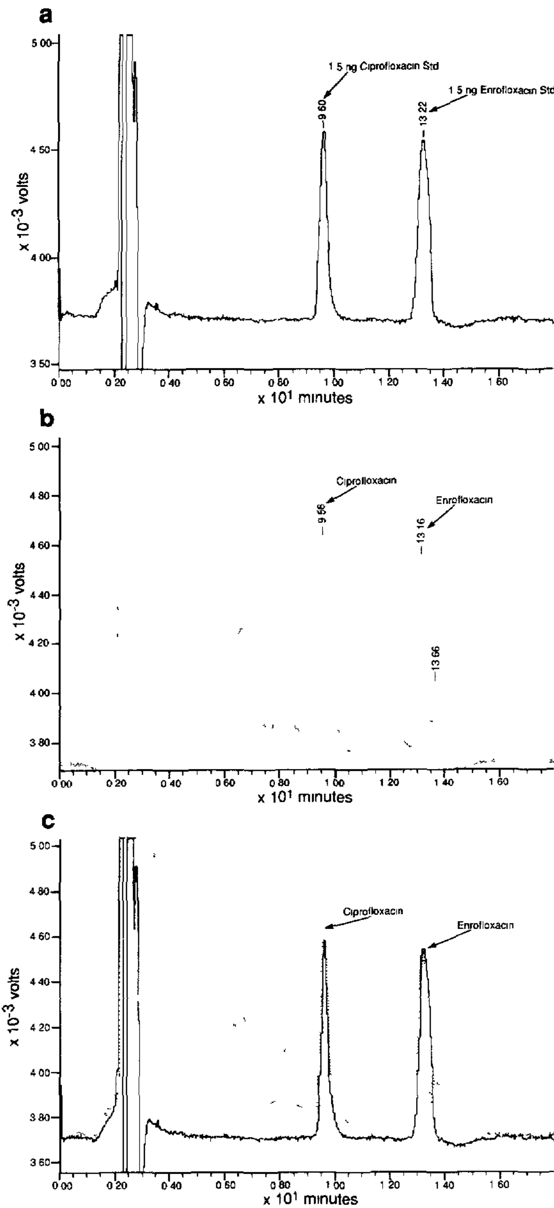

Fig. 3. Representative LC-UV-VIS chromatograms obtained from the photometric detector at 278.6 nm of (a) 1.5 ng of ciprofloxacin and 1.5 ng of enrofloxacin standards, (b) canine serum spiked with 50 ng/ml of each, and (c) the overlaid chromatograms of both the standards and spiked canine samples. Injection volume was 30 μl.

However, a small level of interference was observed on the ciprofloxacin and enrofloxacin downslopes (Fig. 3c).

Fig. 4 shows a chromatogram of canine serum from an anesthetized dog dosed with enrofloxacin. The concentrations of ciprofloxacin and enrofloxacin were 88 and 108 ng/ml, respectively, 2 h after dosing.

A representative chromatogram obtained from canine prostatic tissue ultrafiltrate is shown in Fig. 5A. The comparison with the chromatogram from serum obtained at the same time as the tissue was collected revealed an unidentified additional peak just after the ciprofloxacin peak. Although the separation of ciprofloxacin was not baseline-resolved, the fragment of the contour plot of the same chromatogram showed a good analytical window for this analyte (Fig. 5B). In addition, to prove the homogeneity of the ciprofloxacin peak, spectral curves of the analyte standards (15 ng each) and ciprofloxacin from prostatic tissue ultrafiltrate (3.2 ng) were acquired in the auto gain mode of the PDA detector and overlaid for comparison. As seen in Fig. 5C, there was excellent conformity between the two curves. A spectral curve of enrofloxacin from the prostatic tissue ultrafiltrate could not be obtained because of the small amount of enrofloxacin (1.6 ng) and PDA detector limitations.

High recoveries of enrofloxacin and ciprofloxacin with small coefficients of variation were obtained from canine serum at low concentrations (250 and 50

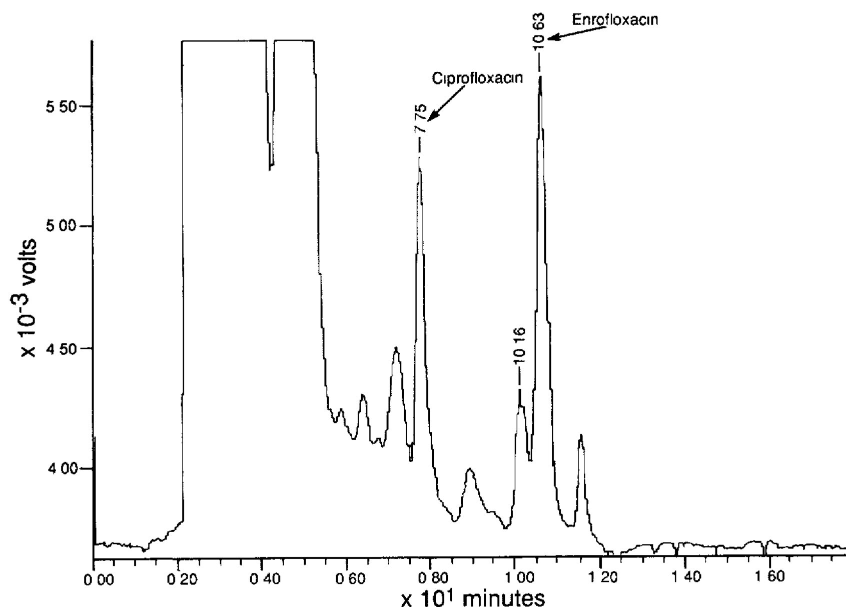

Fig. 4. Typical LC-UV-VIS chromatogram obtained from the photometric detector at 278.6 nm from a canine serum sample of a dog dosed with enrofloxacin. Injection volume was 40 μl.

TABLE I

STATISTICAL SUMMARY OF LC-UV-VIS ANALYSES OF ENROFLOXACIN AND CIPROFLOXACIN IN CANINE SERUM ($n=5$)

Compound	Concentration spiked (ng/ml)	Concentration found (ng/ml)		Coefficient of variation (%)	Recovery (%)
		Range	Mean ± S.D.		
Enrofloxacin	250	234-240	236.8 ± 2.6542	1.12	94.7
	50	37.9-39.9	38.8 ± 0.4213	1.09	77.5
Ciprofloxacin	250	231.1-241.5	235.0 ± 4.3012	1.83	94.0
	50	43.2-49.9	46.8 ± 2.4213	5.17	93.7

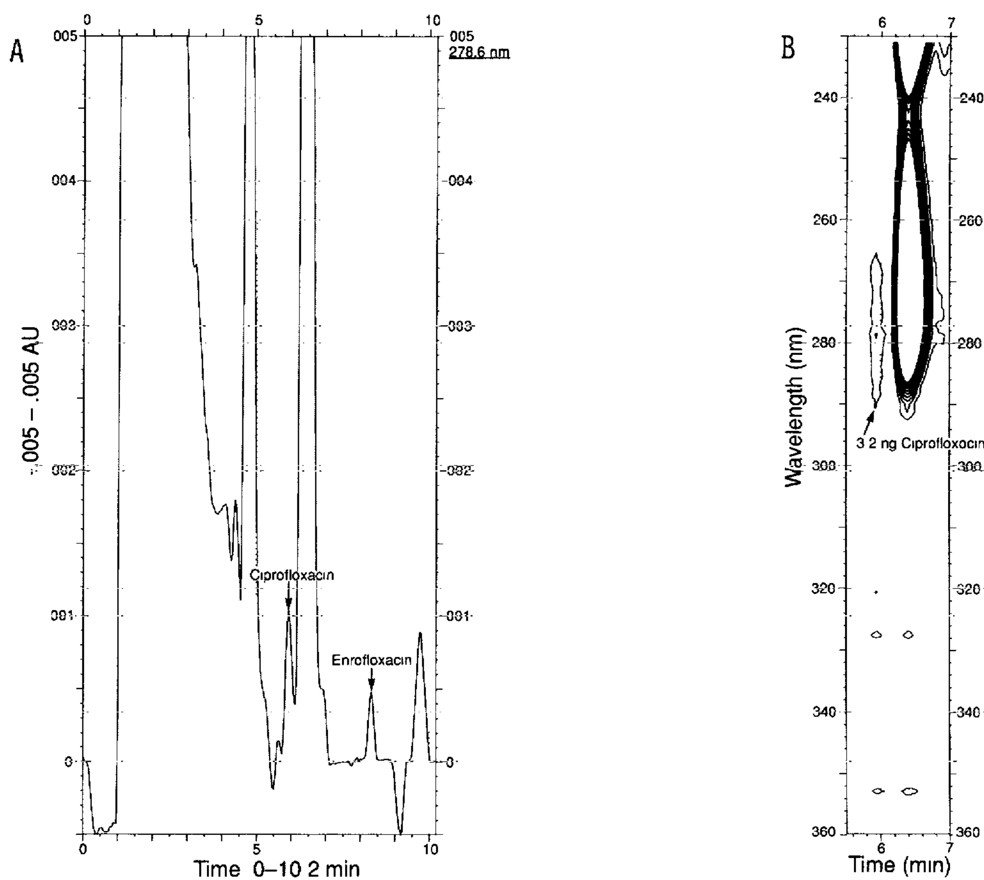

Fig. 5.

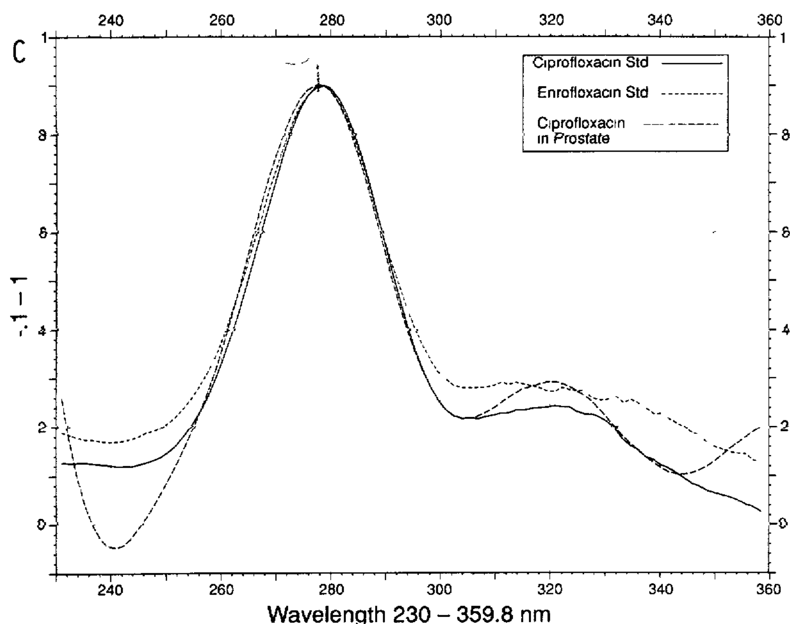

Fig. 5. (A) Chromatogram of a prostatic tissue ultrafiltrate with UV-VIS photodiode array detection of the column effluent at 278.6 nm. Injection volume was 80 μl, corresponding to 40 mg of prostatic tissue. (B) Contour plot obtained from the fragment of chromatogram shown in (A). (C) UV-VIS spectra obtained for 15 ng of enrofloxacin standard, and 15 and 3.2 ng of ciprofloxacin standards from canine prostatic tissue ultrafiltrate displayed in the auto gain mode of the photodiode array detector.

ng/ml) (Table I). Using the photometric detector, the limit of detection, defined as a peak at least three times the height of baseline noise, was 4 ng/ml for enrofloxacin and 2 ng/ml for ciprofloxacin.

In conclusion, an original method for the simultaneous determination of enrofloxacin and its metabolite ciprofloxacin in canine serum and prostatic tissue has been developed and provides a simple, sensitive, and reliable analytical procedure for use in animal studies. Due to its ability to detect low concentrations, the assay is appropriate for the study of enrofloxacin disposition in serum and tissues.

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