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Simultaneous determination of enrofloxacin and its primary metabolite ciprofloxacin in bovine milk and plasma by ion-pairing liquid chromatography

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Abstract

A simple and sensitive high-performance liquid chromatographic method has been developed for the simultaneous determination of enrofloxacin and ciprofloxacin in bovine milk and plasma. Sample preparation consisted of mixing equal volumes of milk or plasma with acetonitrile–0.1 *M* sodium hydroxide (1:1, v/v), followed by ultrafiltration through 3000 Da molecular mass cut-off filters. Separation of these two fluoroquinolones in milk or plasma ultrafiltrate was accomplished by ion-pairing liquid chromatography using a reversed-phase analytical column eluted with acetonitrile–methanol–water. Ultraviolet absorbance of the column effluent was monitored over the 230–350 nm range with a photodiode-array detector (λ_{max} 278 nm). Recoveries of enrofloxacin from bovine milk and plasma were 92–107% and 80–84%, respectively. Recoveries of ciprofloxacin from bovine milk and plasma were 92–105% and 73–75%, respectively. The limit of detection for the two compounds was 5 ng/ml. Enrofloxacin was administered intravenously to a lactating cow at a dose of 2.5 mg/kg. Enrofloxacin was detected in milk within 15 min after injection and the metabolite ciprofloxacin rapidly appeared in plasma and milk. Both enrofloxacin and ciprofloxacin were below the limit of detection (5 ng/ml) by 48 h after drug administration.

1. Introduction

Enrofloxacin is a synthetic antibacterial agent of the fluoroquinolone class (Fig. 1). The fluoroquinolones are bactericidal and act principally by inhibition of bacterial DNA-gyrase [1].

Enrofloxacin has been studied in a variety of domestic animals, including cattle [2–4]. In comparison to many other antimicrobials administered to cattle for the treatment of bacterial infections, enrofloxacin is highly active against most Gram-positive and Gram-negative pathogens and reaches a high tissue/serum concentration ratio. Enrofloxacin is potentially valuable for the treatment of bacterial infections in cattle [4]. However, enrofloxacin and its active

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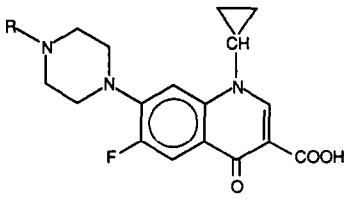

Enrofloxacin, R=CH_3CH_2

Ciprofloxacin, R=H

Fig. 1. Chemical structures of enrofloxacin and ciprofloxacin.

metabolite, ciprofloxacin, are not approved by the U.S. Food and Drug Administration for use in food producing animals and tolerance levels have not been established for these drugs in milk or tissues. The first objective was to develop a sensitive, specific, and rapid assay to detect enrofloxacin in milk and plasma of treated cattle. Because ciprofloxacin is the active metabolite of enrofloxacin, the assay must also be capable of distinguishing between these drugs.

In recent literature, several high-performance liquid chromatographic methods have been developed for the determination of ciprofloxacin in body fluids [5–9]. These methods employ time-consuming sample preparation, which complicates routine analysis. An analytical method for the simultaneous determination of enrofloxacin and ciprofloxacin in canine serum and prostate tissue was developed in our laboratory [10]. This method involved one-step sample preparation for analyzing serum by using an ultrafiltration procedure. The methodology described in the present paper for the simultaneous determination of enrofloxacin and its metabolite ciprofloxacin in bovine milk and plasma involves a one-step sample preparation procedure. Sample preparation was performed by ultrafiltration of diluted milk with an equal volume of acetonitrile–0.1 *M* sodium hydroxide (1:1, v/v) through a 3000 Da molecular mass cut-off filter. Consistent recoveries of enrofloxacin and its metabolite ciprofloxacin and the lack of volume transfers eliminated the need for an internal standard. Therefore, this analytical procedure allows the routine analysis of bovine milk and plasma samples. A second objective of this study was to evaluate this assay in an experiment where enrofloxacin was administered intravenously to a lactating dairy cow, with subsequent collection of milk and plasma samples over a 48-hr period for enrofloxacin and ciprofloxacin quantitation. This paper reports, for the first time, the disposition of enrofloxacin and its metabolite, ciprofloxacin in the milk after systemic administration to a dairy cow.

2. Experimental

2.1. Material and reagents

LC solutions were made from the highest purity solvent grade acetonitrile and methanol (American Burdick and Jackson, Muskegan, MI, USA). LC-grade water was obtained from Hydro Services and Supplies (Research Triangle Park, NC, USA). Phosphoric acid (85%), sodium hydroxide (1 *M* solution) and triethylamine were LC-grade (Fisher Scientific, Raleigh, NC, USA). The ion-pairing reagent, dodecanosulfonate (S12), was obtained from Regis (Morton Grove, IL, USA). The separation system, Centricon-3, employing a molecular mass cut-off filter of 3000 Da, was supplied by American Division of W.R. Grace (Danver, MD, USA). Enrofloxacin and ciprofloxacin standard were kindly supplied by Mobay (Shawnee Mission, KS, USA) and Miles Labs (Elkhart, IN, USA), respectively. Stock solutions of 1 mg/ml enrofloxacin and ciprofloxacin were prepared in acetonitrile–0.1 *M* sodium hydroxide (1:1, v/v). Working concentrations of 100 ng/ml were prepared daily with the same diluent.

2.2. Bovine milk and plasma samples (in vivo study)

Milk and plasma samples were obtained from a lactating dairy cow following an intravenous injection of a 2.27% solution of enrofloxacin (Baytril, Miles, Shawnee Mission, KS, USA) at a dose of 2.5 mg/kg. Blood and milk samples were collected prior to, and at 0.25, 0.5, 1, 2, 4, 6, 8, 24, and 48 h after intravenous injection. The milk sample obtained at each collection time

was a pooled sample from all four quarters of the udder and was stored in a glass vial. Blood samples were collected via venipuncture into heparinized glass tubes and centrifuged at 2000 *g* for 10 min to collect plasma. The plasma and milk samples were refrigerated after collection until analysis, which was performed within 24 h. Blank samples collected before drug administration were spiked with enrofloxacin and ciprofloxacin for assay validation and the in vitro study. Depletion of enrofloxacin and ciprofloxacin was measured by performing least-squares linear regression on the logarithms of the drug concentrations vs. time.

2.3. Sample preparation procedure

Aliquots (500 μl) of bovine milk and plasma samples were diluted with an equal volume of acetonitrile–0.1 *M* sodium hydroxide (1:1, v/v) in the microseparation system equipped with a 3000 Da molecular mass cut-off filter. Samples were vortex-mixed for 10–15 s and centrifuged for approximately 30 min at 4000 g in a 45° fixed-angle rotor. A 50–150 μl aliquot of the colorless ultrafiltrate was injected onto the LC system equipped with a UV-Vis photodiode-array (PDA) detector.

2.4. Ion-pairing liquid chromatography

The LC equipment consisted of Waters Model 600W multi-solvent delivery system with a Waters U6K injector and temperature control accessory set at 50°C. This was coupled to a Model 996 UV-Vis PDA detector (Waters Chromatography Division, Milford, MA, USA). The LC separations were performed using a mobile phase of acetonitrile–methanol–triethylamine–phosphoric acid (85%)–water (9:9:0.45:0.4:81.15, v/v), containing 0.005 *M* dodecanesulfonate. The pH of the mobile phase was approximately 2.5. The mobile phase flow-rate was 1 ml/min, giving a retention time of 10–11 min for ciprofloxacin and 13–15 min for enrofloxacin on an Spherisorb 3-μm phenyl column (250 × 4.6 mm I.D.; Phenomenex, Torrance, CA, USA). The lifetime of this column was approximately 6–9 months without the use of a guard column. The column effluent was analyzed in the wavelength range 230–350 nm with photodiode-array detector. Peak-area measurements were computed on a Millenium 2010 work station (Waters Chromatography Division). After comparing the areas of ciprofloxacin or enrofloxacin in standards and in bovine milk/plasma samples, the quantities of ciprofloxacin or enrofloxacin were calculated as follows: ng/ml = [ciprofloxacin or enrofloxacin std (ng) · 2]/injection volume (ml). Usually, the injection volume was between 0.05 ml and 0.15 ml. The multiplication by 2 in the equation accounts for the dilution of milk plasma (1:1) with the solution used for releasing protein-bound drug.

2.5. Incubation of enrofloxacin with bovine milk and plasma (in vitro study)

Bovine milk and plasma were fortified to concentrations of 10 μg/ml enrofloxacin and then incubated at 37°C for 24 h. At the time of sampling (0.25, 0.5, 1, 2, 4, 6, 8, and 24 h), 500-μl aliquots were vortex-mixed with an equal volume of acetonitrile–0.1 *M* sodium hydroxide (1:1, v/v) and placed in the microseparation systems equipped with a 3000 Da molecular mass cut-off filter, for centrifuging. Ultrafiltrates were injected in the LC–UV-Vis-PDA system.

3. Results and discussion

A previous publication from this laboratory [10] described the determination of enrofloxacin and its metabolite ciprofloxacin in canine serum and prostate tissue. Due to the complex nature of the milk matrix, substantial adaptation of this method has been performed. The major changes were: slightly different sample preparation, different mobile phase composition and different stationary phase. The solution of acetonitrile–0.1 *M* sodium hydroxide was used to release ciprofloxacin and enrofloxacin from milk and plasma proteins. The microseparation systems equipped with 3000 Da molecular mass cut-off filters were used for the extraction/purification procedure

for ciprofloxacin and enrofloxacin from bovine milk and plasma. Previously, we used 10 000 Da molecular mass cut-off filters [10] for the extraction and purification of canine serum and prostatic tissues. The bovine milk and plasma ultrafiltrates obtained after using 3000 Da molecular mass cut-off filters were much cleaner than those obtained using 10 000 Da filters (data not shown).

A typical chromatogram obtained from a milk ultrafiltrate spiked with 100 ng/ml of ciprofloxacin and enrofloxacin is shown in Fig. 2A, while their UV-Vis absorbance contour plots in the range of 230–350 nm are shown in (Fig. 2B). The chromatogram was generated with the photodiode-array detector set at 278 nm (λ_{max} for ciprofloxacin and enrofloxacin). Fig. 3A shows a chromatogram of a blank milk ultrafiltrate and Fig. 3B a UV-Vis absorption contour plot. This chromatogram revealed very clean analytical windows for ciprofloxacin and enrofloxacin in bovine milk. Fig. 4 shows a LC–UV-Vis chromatogram of a bovine plasma sample collected 1 h after intravenous injection of enrofloxacin. There were no interfering peaks near the retention times of ciprofloxacin and enrofloxacin.

Table 1 summarizes the statistical data obtained from bovine milk spiked with 1000, 100 and 20 ng/ml ciprofloxacin and enrofloxacin, and bovine plasma spiked with 200 and 20 ng/ml of these two compounds. Recoveries of ciprofloxacin from bovine milk spiked with 1000, 100 and 20 ng/ml were 92, 92 and 105%, with coefficients of variation of 4.6, 11.1 and 20.5%, respectively. Recoveries of enrofloxacin from bovine milk spiked with 1000, 100, and 20 ng/ml were 92, 99 and 107%, with coefficients of variations of 4.4, 9.2 and 15.1%, respectively. Recoveries of ciprofloxacin from bovine plasma spiked with 200 and 20 ppb were 75 and 73%, with coefficients of variation of 4.0 and 8.7%, respectively. Recoveries of enrofloxacin from bovine plasma spiked with 200 and 20 ng/ml were 80 and 84%, with coefficients of variations of 4.4 and 8.7%, respectively. The UV-Vis detection limit for ciprofloxacin and enrofloxacin in these two matrices was 5 ng/ml, using an injection volume of 150 μl and based on a signal-to-noise ratio of 3 at 278 nm.

There are no published reports on the disposition of enrofloxacin and ciprofloxacin in milk of cows after systemic administration of enrofloxacin. This study represents the first published account of the concentrations of enrofloxacin and ciprofloxacin in milk using an HPLC method. Table 2 shows the drug concentration found in milk and plasma after injecting a lactating dairy cow with 2.5 mg/kg of enrofloxacin intravenously. Enrofloxacin rapidly appeared in milk. Thirty minutes post-injection, the enrofloxacin concentration in milk reached a peak of 231 ng/ml. Thereafter the concentration was similar to that in plasma. After thirty minutes, the milk and plasma enrofloxacin concentrations declined at a similar rate. The depletion analysis of enrofloxacin from plasma and milk of this cow, as measured by performing a least-squares regression analysis on the logarithm of the concentrations, showed that enrofloxacin was eliminated from plasma and milk with half-lives of 39 min and 48 min, respectively. Ciprofloxacin appeared rapidly in plasma and milk after the intravenous injection, which suggests a rapid metabolism of enrofloxacin to ciprofloxacin in vivo. Ciprofloxacin appeared in plasma already at 15 min after intravenous injection of enrofloxacin and its concentration decreased with an estimated half-life of 1.5 h. Ciprofloxacin concentrations in milk increased steadily following injection to a maximum of 411 ng/ml at 4 h and then declined with an estimated half-life of 3 h. The dose used for the in vivo study in this cow is a dose that has been approved in other animals for therapeutic use and that has been shown to be clinically effective in experimental injections in calves [4]. Most bacteria are sensitive to concentrations of enrofloxacin above 100 ng/ml. Although enrofloxacin is not approved by the Food and Drug Administration (FDA) for use in food animals, the Food Animal Residue Avoidance Databank (FARAD) [11] has used the available information to recommend discarding milk for 96 h after experimental administration of enrofloxacin to lactating cows [12]. In the cow used in the present study, enrofloxacin and

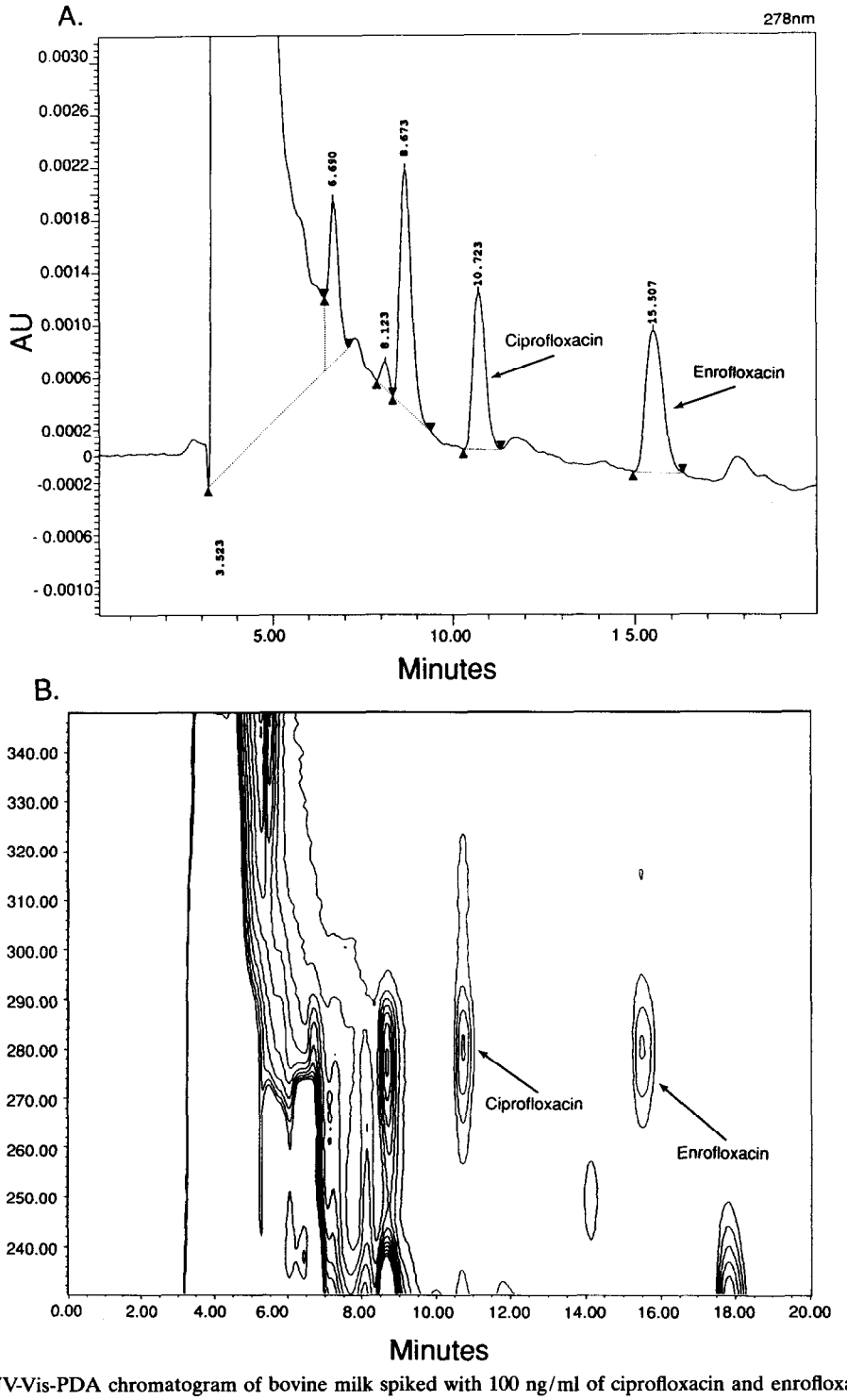

Fig. 2. (A) LC–UV-Vis-PDA chromatogram of bovine milk spiked with 100 ng/ml of ciprofloxacin and enrofloxacin obtained at 278 nm. Injection volume was 100 μl. (B) UV-Vis absorbance contour plot (230–350 nm) for 100 ng/ml ciprofloxacin and enrofloxacin in bovine milk ultrafiltrate.

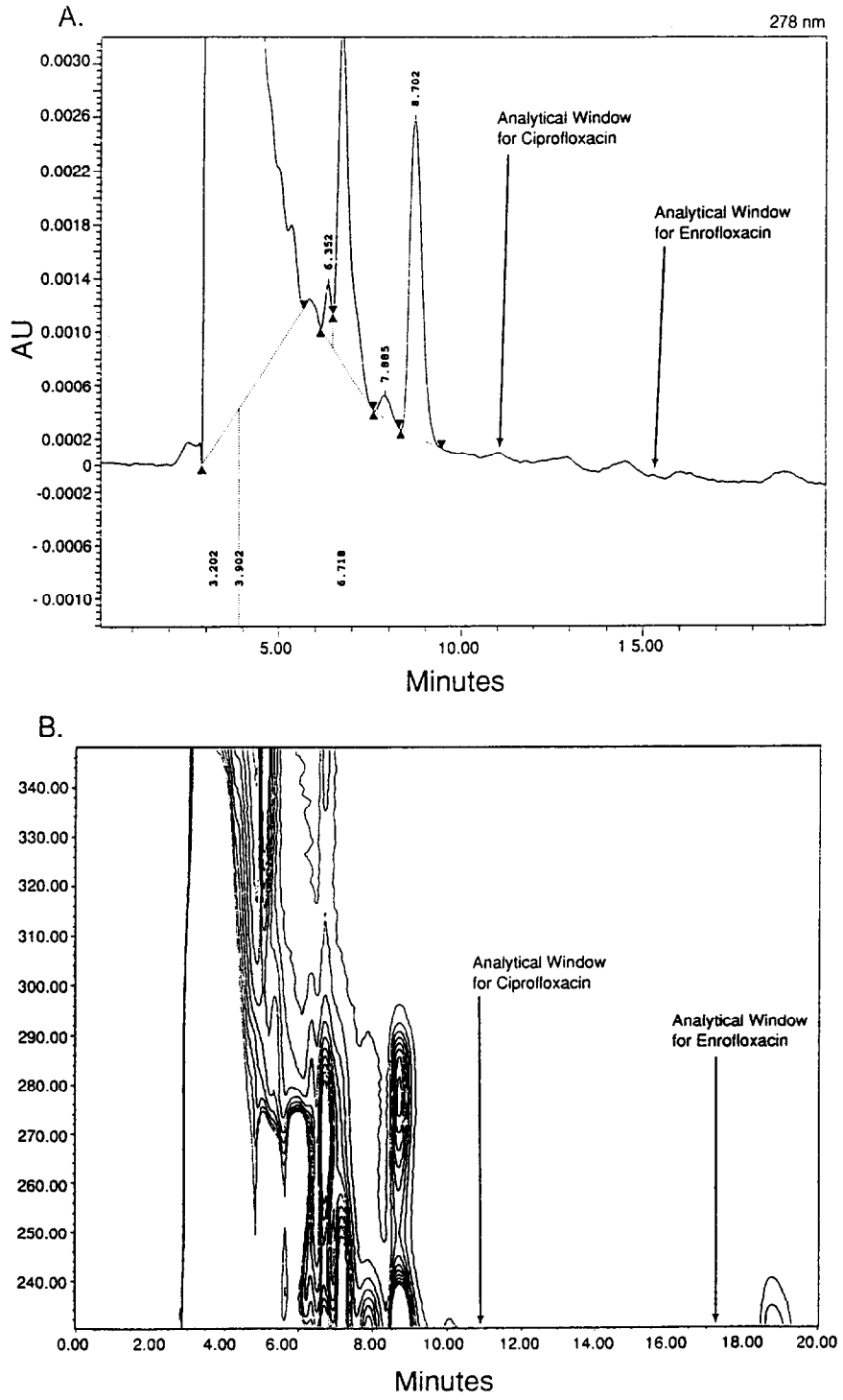

Fig. 3. (A) Blank bovine milk LC–UV-Vis-PDA chromatogram obtained at 278 nm. Injection volume was 100 μl. Note the excellent analytical windows for ciprofloxacin and enrofloxacin. (B) UV-Vis absorbance contour plot for a blank bovine milk ultrafiltrate.

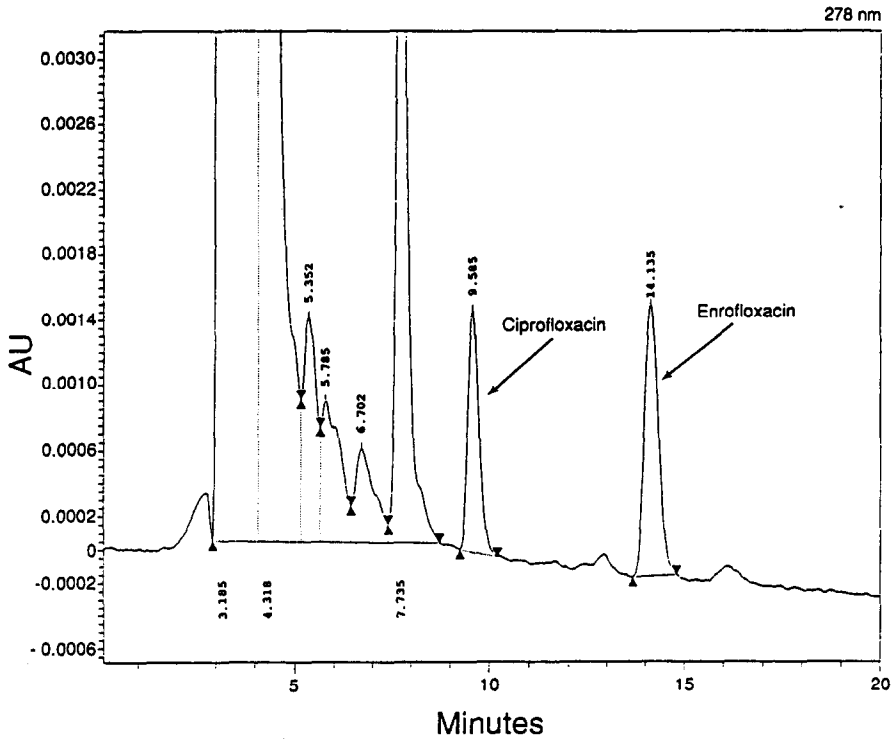

Fig. 4. LC–UV-Vis-PDA chromatogram of bovine plasma sample collected 1 h after intravenous injection of enrofloxacin. This chromatogram was obtained at 278 nm. Injection volume was 50 μl.

ciprofloxacin could not be detected in milk or plasma 48 h after administration. When enrofloxacin was incubated with bovine milk and plasma at 37°C, ciprofloxacin could not be detected.

In conclusion, a simple, rapid and sensitive ion-pairing liquid chromatographic method for the simultaneous determination of ciprofloxacin and enrofloxacin in bovine milk and plasma has been developed. Because of its ability to detect low concentrations, the assay is appropriate to analyze residues of enrofloxacin and ciprofloxacin in bovine milk and plasma.

Table 1
Statistical summary of LC–UV-Vis analysis of enrofloxacin and ciprofloxacin in bovine milk and plasma ($n = 5$)

Matrix	Milk						Plasma			
	Enrofloxacin			Ciprofloxacin			Enrofloxacin		Ciprofloxacin	
Concentration spiked (ng/ml)	1000	100	20	1000	100	20	200	20	200	20
Range (ng/ml)	891–987	86–110	19–25	880–993	82–108	16–26	153–171	14.5–18.4	145–159	12.4–17.5
Mean ± S.D. (ng/ml)	917 ± 40	99 ± 9	21 ± 3	919 ± 43	92 ± 11	21 ± 4	160 ± 7	16.9 ± 1.5	150 ± 6	14.6 ± 1.3
Coefficient of variation (%)	4.4	9.2	15.1	4.6	11.1	20.5	4.4	8.7	4.0	8.7
Recovery (%)	92	99	107	92	92	105	80	84	75	73

Table 2
In vivo biotransformation of enrofloxacin to ciprofloxacin in bovine milk and plasma

Time	Milk		Plasma	
	Enrofloxacin (ng/ml)	Ciprofloxacin (ng/ml)	Enrofloxacin (ng/ml)	Ciprofloxacin (ng/ml)
Pretreatment	Neg[a]	Neg	Neg	Neg
15 min	188	8	512	244
30 min	231	21	366	205
1 h	173	138	197	162
2 h	57	323	45	81
4 h	12	411	14	39
6 h	Neg	354	Neg	14
8 h	Neg	193	Neg	8
24 h	Neg	5	Neg	Neg
48 h	Neg	Neg	ND[b]	ND

[a] Neg = below 5 ng/ml.
[b] ND = Not determined.

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