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Simple, rapid determination of enrofloxacin and ciprofloxacin in bovine milk and plasma by high-performance liquid chromatography with fluorescence detection

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Abstract

A rapid and simple procedure for determination of enrofloxacin and ciprofloxacin in bovine milk and plasma is described.

Protein precipitation from both milk and plasma samples was achieved by addition of acetonitrile and phosphoric acid. Acetonitrile was removed with methylene chloride, leaving enrofloxacin and ciprofloxacin in the acidic aqueous extract. The aqueous extract was analyzed by high-performance liquid chromatography (HPLC) with fluorescence detection. The limit of quantitation (LOQ) for enrofloxacin and ciprofloxacin in milk was found to be 2 ng/ml. LOQ for enrofloxacin and ciprofloxacin in plasma was found to be 1 ng/ml. Linear calibration curves were obtained with correlation coefficient (r^2) $\geq$0.99. Analysis of quality control (QC) samples gave results within $\pm$10% of the nominal values. Inter-assay precision for the analysis of milk QC samples were in the ranges: 4.63–12.49% (for enrofloxacin) and 4.67–9.86% (for ciprofloxacin). Inter-assay precision for the analysis of plasma QC samples were in the ranges: 6.60–17.31% (for enrofloxacin) and 6.14–13.87% (for ciprofloxacin).

Intra-assay precision for the analysis of milk QC samples were in the following ranges: 3.65–7.21% (for enrofloxacin) and 1.58–14.28% (for ciprofloxacin). Intra-assay precision for the analysis of plasma QC samples were in the following ranges: 2.17–16.95% (for enrofloxacin) and 3.31–16.31% (for ciprofloxacin).

The effectiveness of protein precipitants other than phosphoric acid was investigated. The method described has been applied to a study of the pharmacokinetics of enrofloxacin and ciprofloxacin in lactating dairy cows and beef steers.

Keywords: Enrofloxacin; Ciprofloxacin; Bovine milk; Bovine plasma; HPLC

1. Introduction

Fluoroquinolones (FQs) are antibacterial agents related to nalidixic acid. FQs are used to treat a variety of infections in both human and veterinary medicine [1–4].

Enrofloxacin (Fig. 1) was developed for use exclusively in animals [5,6]. Like other FQs, enrofloxacin exhibits a broad spectrum of antibacterial activity, against both Gram-positive and Gram-negative bacteria, in diseased animals. In the United States, enrofloxacin is approved for use in beef cattle, and calves (excluding veal calves), chicken and turkey not laying eggs for human consumption, and in cats and dogs. Ciprofloxacin is a major, active metabolite

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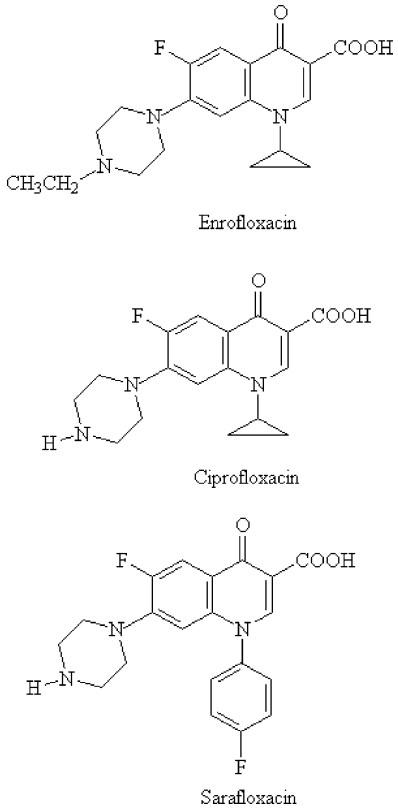

Fig. 1. Chemical structure of enrofloxacin, ciprofloxacin and sarafloxacin.

of enrofloxacin in different species and is formed by the de-ethylation of enrofloxacin. Ciprofloxacin was the first of the two compounds to be developed and it is approved for use only in humans [7,8].

As part of a general study to evaluate whether bovine production class has an effect on the kinetics of different drugs, we wanted to characterize the pharmacokinetics of enrofloxacin and its major metabolite, ciprofloxacin, in lactating dairy cattle and beef steers after intravenously administered enrofloxacin. For this, we needed a sensitive, specific and rapid high-performance liquid chromatographic (HPLC) assay to determine enrofloxacin and ciprofloxacin in milk and plasma.

Several HPLC methods have been reported in the literature for the determination of ciprofloxacin in body fluids, particularly in plasma and serum, as well as in animal tissue.

An extensive review of these methods is included in a recent article on the analysis of fluoroquinolones in biological fluids by HPLC [9].

Compared to ciprofloxacin, enrofloxacin is a relatively recent drug and there have been only a few reports on methods for the simultaneous determination of enrofloxacin and ciprofloxacin in biological fluids and animal tissue [7,10–15].

While there have been several methods reported for these drugs in plasma and serum, only five have been reported for FQs in milk [13,16–19].

In general, HPLC methods for the determination of fluoroquinolones in biological material involve tedious extraction and clean-up steps prior to chromatography. As such, these methods do not allow for high throughput analysis that would be required for a pharmacokinetic study. Our objective, therefore, was to develop a method for the simultaneous determination of enrofloxacin and ciprofloxacin in bovine milk and plasma that involves minimal sample pre-treatment and rapid analysis times.

2. Experimental

2.1. Chemicals

Enrofloxacin was obtained from Bayer Corporation (Shawnee Mission, KS, USA). Ciprofloxacin was obtained from US Pharmacopeia (Rockville, MD, USA). Sarafloxacin hydrochloride (internal standard) was obtained from Abbott Laboratories, North Chicago, IL 60064.

o-Phosphoric acid (H_3PO_4), 85%, was obtained from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid and acetic acid (glacial) were obtained from Sigma (St. Louis, MO, USA). Acetonitrile (HPLC grade), dichloromethane and methanol were purchased from VWR Scientific Products (Brigdeport, NJ, USA) Ethanol (dehydrated and not denatured) was obtained from Spectrum Quality Products, Inc. (New Brunswick, NJ, USA).

2.2. Standard solutions

Standard stock solutions of ciprofloxacin, enrofloxacin, and sarafloxacin (100 μg/ml as free base) were prepared in 10% aqueous acetic acid, and stored

at −20 °C. Working standard aqueous solutions of the drugs were prepared daily by serial dilution of the stock solutions with water. Working standard solutions of the drugs in milk or plasma were prepared by adding appropriate microliter aliquots of the aqueous solutions to 1 ml of milk or plasma.

Quality control (QC) samples were also prepared by adding microliter aliquots of the standard aqueous solutions to milk or plasma.

2.3. Extraction procedure

An aliquot (1 ml) of plasma (or milk) containing enrofloxacin and ciprofloxacin was placed in a 15 ml, glass centrifuge tube containing 200 μl of standard solution of sarafloxacin, the internal standard.

To the mixture was added 100 μl of concentrated *o*-phosphoric acid for plasma sample (or 50 μl for milk sample.) After shaking the mixture briefly (about 5 s) on a vortex-mixer, 2 ml of acetonitrile was added.

The mixture was vortexed for about 10 s at high speed and then centrifuged for 5 min at 4000 rpm (2840 × *g*) and 4 °C.

The supernatant was decanted into another 15 ml glass centrifuge tube, and 3 ml of methylene chloride was added. The mixture was vortexed for about 10 s at high speed and then centrifuged for 5 min at 4000 rpm and 4 °C.

The upper, aqueous layer was transferred into an autosampler vial, using a Pasteur pipet. The aqueous plasma or milk extracts were then analyzed for ciprofloxacin and enrofloxacin using the HPLC conditions described below.

2.4. Chromatographic conditions

A Hewlett Packard Series 1100 chromatographic system was employed, together with a Hewlett Packard 1046A fluorescence detector (λ_{exc} 280 nm and λ_{em} 460 nm). Data acquisition was done using a Perkin-Elmer Turbochrom Chromatography System through a PE Nelson Series 900 Interface.

Chromatographic separations were performed on a PLRP-S polymer column (150 mm × 4.6 mm, particle size 100 Å, 5 μ), with guard column of the same packing (Phenomenex, Inc., Torrance, CA). Column temperature was maintained at 35 °C.

The mobile phase consisted of 2.0% aqueous formic acid–methanol–acetonitrile (75:13:12, v/v/v).

2.5. Method validation

During application of this method to samples from a pharmacokinetic study, it was noticed that after analysis of about 100–120 extracts, a change in column performance was indicated by a significant decrease in the retention times of ciprofloxacin, enrofloxacin and sarafloxacin (internal standard). Column performance was restored by flushing the column with pure acetonitrile for about 2 h (flow rate 1 ml/min). Also, when not in use (for example, over the weekend), the column should be stored in acetonitrile.

Quantitation of enrofloxacin and ciprofloxacin was achieved from calibration curves in the range of 1–100 ng/ml in plasma and in the range of 2–100 ng/ml in milk. (During subsequent application of the method to study the pharmacokinetics of enrofloxacin and ciprofloxacin in bovine plasma, calibration curves in the range of 2–1000 ng/ml were also used for quantitation.) The calibration curve was evaluated by its regression coefficient, slope and intercept and was used to automatically calculate the analyte concentrations in the quality control and unknown samples by the Turbochrom® data collection system. Calculations of calibration curve and analyte concentrations were based on the peak area ratios of enrofloxacin or ciprofloxacin to the internal standard, sarafloxacin.

QC samples for determining inter- and intra-assay precision and accuracy were prepared at different concentrations, as shown in Tables 3–6.

3. Results and discussion

3.1. Method validation

3.1.1. Calibration curve

Table 1 shows the back-calculated concentrations of enrofloxacin and ciprofloxacin in milk along with other statistical data for the calibration curves. Similar data for enrofloxacin and ciprofloxacin in plasma are shown in Table 2.

3.1.2. Inter- and intra-assay precision and accuracy

The accuracy and precision data of the method are shown in Tables 3–6. These results show that adequate

Table 1
Calibration curve statistics of enrofloxacin and ciprofloxacin in milk

	Nominal concentrations (ng/ml)				
	2	5	10	50	100
Back-calculated concentrations (ng/ml)					
Enrofloxacin					
Mean	1.96	4.43	10.7	55.3	96.6
S.D.	0.18	0.47	1.3	5.6	7.9
CV (%)	9.0	11	12	10	8.2
Accuracy (%)	98	87	107	111	97
Ciprofloxacin					
Mean	1.78	4.19	10.5	53.6	92.9
S.D.	0.23	0.72	0.97	3.6	6.6
CV (%)	12	17	9.2	6.7	7.1
Accuracy (%)	89	84	105	107	93

Note: Calibration curves were prepared in duplicate on each of five different days. On each occasion linear calibration curves were obtained with correlation coefficient (r^2) ≥0.99.

accuracy and precision were obtained for the determination of enrofloxacin and ciprofloxacin milk and plasma by the method.

3.1.3. Limit of quantitation

The lowest possible standard on the calibration curve was accepted as the limit of quantitation (LOQ). LOQ for enrofloxacin and ciprofloxacin in plasma was found to be 1 ng/ml. LOQ for enrofloxacin and ciprofloxacin in milk was found to be 2 ng/ml.

Table 2
Calibration curve statistics of enrofloxacin and ciprofloxacin in plasma

	Nominal concentrations (ng/ml)				
	2	5	10	50	100
Back-calculated concentrations (ng/ml)					
Enrofloxacin					
Mean	2.01	4.82	10.6	50.6	94.7
S.D.	0.17	0.23	0.61	3.6	9.9
CV (%)	8.3	4.8	5.7	7.1	10
Accuracy (%)	101	96	106	101	95
Ciprofloxacin					
Mean	2.00	4.85	10.60	50.6	94.8
S.D.	0.15	0.20	1.3	3.6	9.9
CV (%)	7.7	4.2	12	7.4	10
Accuracy (%)	100	97	106	101	95

Note: Calibration curves were prepared in duplicate on each of five different days. On each occasion linear calibration curves were obtained with correlation coefficient (r^2) ≥0.99.

3.1.4. Specificity

The chromatograms obtained from the analysis of milk extracts containing enrofloxacin and ciprofloxacin are shown in Fig. 2 as well as a chromatogram of an extract of control bovine milk. Similar chromatograms of extracts of control bovine plasma and plasma containing enrofloxacin and ciprofloxacin are shown in Fig. 3. No significant interfering peaks were found at the retention times of enrofloxacin, ciprofloxacin and the internal standard, sarafloxacin. Chromatograms of extracts of pre-dose plasma and plasma obtained 1.5 h after dosing of a steer with enrofloxacin (5 mg/kg body weight) are shown in Fig. 4. Similarly, the method was recently applied to the analysis of ciprofloxacin in milk samples from lactating dairy cows dosed to steady state with ciprofloxacin. Chromatograms of pre-dose milk and milk from a cow having steady state plasma level of ciprofloxacin are shown in Fig. 5. Both Figs. 4 and 5 show there were no interfering peaks when the method was applied in the analysis of samples from dosed animals.

3.1.5. Stability of samples and extracts

Experiments showed that plasma and milk samples containing enrofloxacin and ciprofloxacin that were kept frozen at −80 °C were stable in milk and plasma over three freeze-thaw cycles.

During application of the method to bovine plasma samples obtained during a pharmacokinetic study, no change in the concentration of enrofloxacin and ciprofloxacin could be detected in plasma samples stored at −80 °C over a 3-week period.

No significant change was detected in the concentration of the drugs in the aqueous milk or plasma extracts maintained at room temperature for at least 24 h in autosampler vials before being re-analyzed.

4. Extraction of fluoroquinolones from milk and plasma

The extraction procedure described above was adopted after extensive investigation of methods for the extraction of enrofloxacin and ciprofloxacin from milk and plasma. Methods that have been described

Table 3
Intra-day accuracy and precision values for the determination of enrofloxacin and ciprofloxacin in milk

Analyte	Concentration added (ng/ml)	Concentration found (ng/ml) ± S.D.	Accuracy (%)	Precision CV (%)
Enrofloxacin	8	7.43 ± 0.54	93	7.2
	25	23.7 ± 1.2	95	5.0
	30	32.2 ± 1.8	107	5.7
	80	72.5 ± 2.6	91	3.6
Ciprofloxacin	8	8.09 ± 1.2	101	14
	25	24.4 ± 1.5	98	6.1
	30	30.3 ± 0.70	101	2.3
	80	76.8 ± 1.2	96	1.6

$n = 5$.

Table 4
Inter-day accuracy and precision values for the determination of enrofloxacin and ciprofloxacin in milk

Analyte	Concentration added (ng/ml)	Concentration found (ng/ml) ± S.D.	Accuracy (%)	Precision CV (%)
Enrofloxacin	8	7.77 ± 0.97	97	12
	25	22.8 ± 1.2	91	5.2
	30	28.2 ± 1.3	94	4.6
	80	75.8 ± 6.8	95	8.9
Ciprofloxacin	8	7.99 ± 0.79	99	9.9
	25	23.6 ± 1.5	95	6.3
	30	28.7 ± 1.7	96	5.8
	80	76.5 ± 3.6	96	4.7

$n = 15$.

for the extraction of fluoroquinolones include direct solvent extraction, and methods involving protein precipitation with different precipitants. An on-line immunoaffinity extraction method has also been described for fluoroquinolones in milk [17]. During the present work, attempts were made to extract enrofloxacin and ciprofloxacin directly from milk and plasma, without prior protein precipitation. Also, to investigate the effectiveness of different protein precipitants, the extraction procedure described above

Table 5
Intra-day accuracy and precision values for the determination of enrofloxacin and ciprofloxacin in plasma

Analyte	Concentration added (ng/ml)	Concentration found (ng/ml) ± S.D.	Accuracy (%)	Precision CV (%)
Enrofloxacin	5	4.79 ± 0.81	96	17
	20	20.4 ± 1.7	102	8.5
	25	25.l ± l.0	100	4.1
	100	115 ± 2.5	115	2.2
	150	162 ± 4.1	108	2.5
	600	655 ± 29	109	4.4
Ciprofloxacin	5	5.11 ± 0.83	102	16
	20	19.8 ± 1.6	99	7.9
	25	23.1 ± 1.6	92	7.1
	100	109 ± 4.3	109	3.9
	150	163 ± 5.4	108	3.3
	600	646 ± 31	108	4.9

$n = 5$.

Table 6
Inter-day accuracy and precision values for the determination of enrofloxacin and ciprofloxacin in plasma

Analyte	Concentration added (ng/ml)	Concentration found (ng/ml) ± S.D.	Accuracy (%)	Precision CV (%)
Enrofloxacin	5	4.77 ± 0.73	95	15
	20	19.9 ± 1.3	99	6.6
	25	27.5 ± 2.7	109	9.7
	100	109 ± 18	109	17
Ciprofloxacin	5	5.01 ± 0.69	100	14
	20	20.3 ± 1.3	102	6.1
	25	25.9 ± 3.2	104	12
	100	108 ± 14	108	13

$n = 10$.

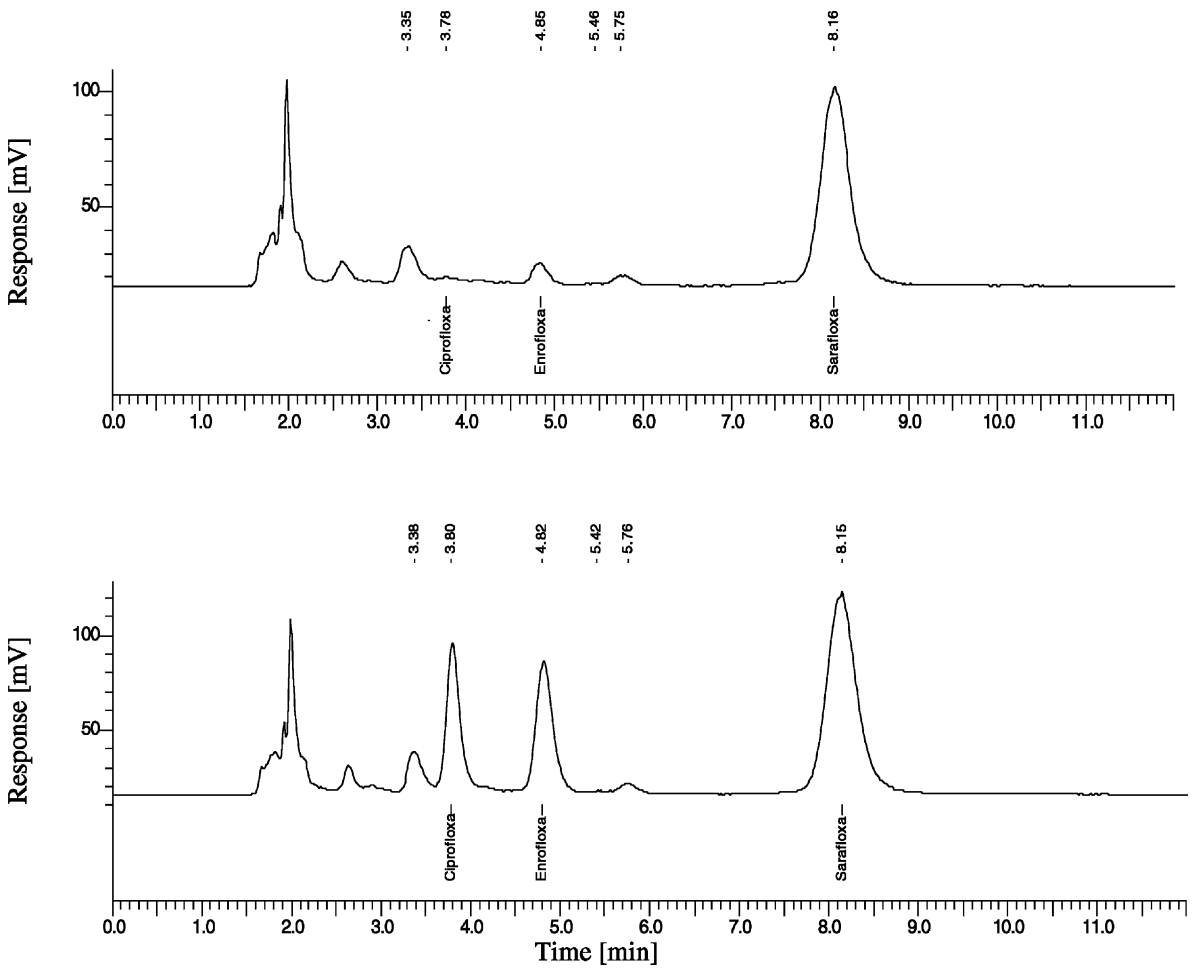

Fig. 2. Chromatograms of blank bovine milk spiked with 200 ng sarafloxacin (upper figure) and milk spiked with ciprofloxacin, enrofloxacin, and sarafloxacin (10, 10 and 200 ng, respectively) (lower figure). Samples were processed as described in Section 2.3.

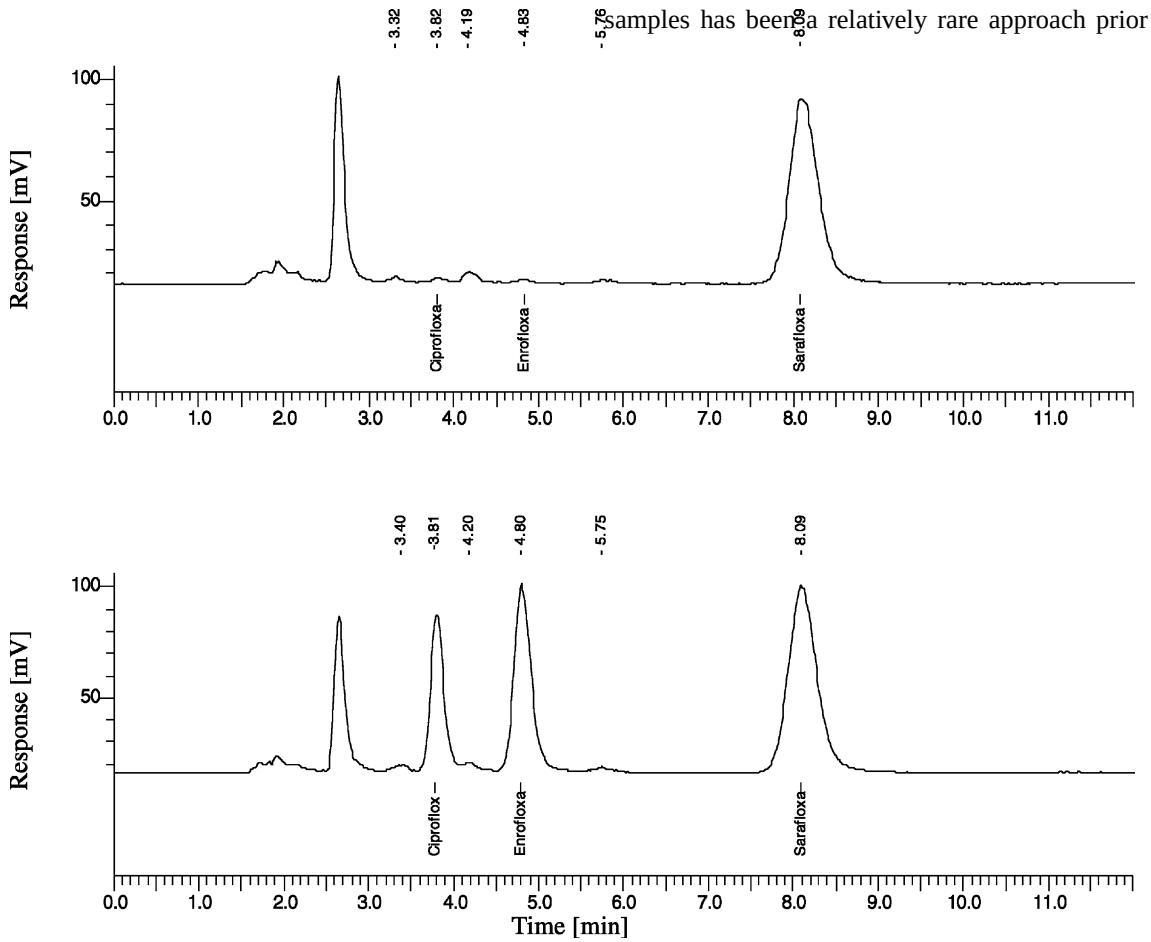

Fig. 3. Chromatograms of blank bovine plasma spiked with 200 ng sarafloxacin (upper figure), and plasma spiked with ciprofloxacin, enrofloxacin, and sarafloxacin (10, 10 and 200 ng, respectively) (lower figure). Samples were processed as described in Section 2.3.

was carried out, using other protein precipitating agents in place of phosphoric acid. The following is a discussion of observations made during this investigation.

4.1. Direct solvent extraction

Ciprofloxacin and enrofloxacin and other fluoroquinolones are amphoteric compounds which exist in ionic forms at both acidic and basic pH. Direction extraction of these compounds from biological sample would, therefore, require an accurate calibration of the pH of the sample to be effective. Direct extraction of fluoroquinolones from biological samples has been a relatively rare approach prior to the HPLC analysis of the compounds. The first reported HPLC method for ciprofloxacin, by Jehl and Brogard involved the direct methylene chloride extraction of ciprofloxacin from serum and urine without prior adjustment of the pH [20,21]. Garcia et al. reported a HPLC method for the simultaneous determination of enrofloxacin and ciprofloxacin in plasma which involved the direct extraction of plasma with chloroform, after adjustment of pH of the sample to 7.4 [22]. While a recovery of 93% was reported for enrofloxacin, a relatively poor recovery of 75% was obtained for ciprofloxacin. Garcia et al. have also reported a recovery of 90% for marbofloxacin from

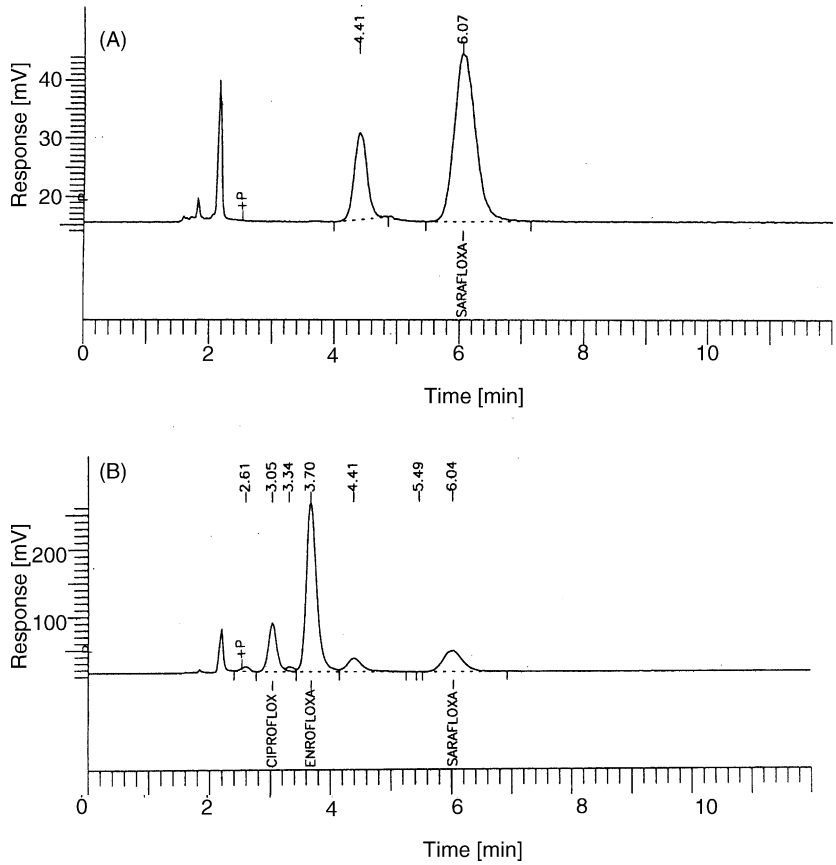

Fig. 4. Chromatograms of extracts of pre-dose plasma (A) and plasma obtained 1.5 h after dosing (B) of a steer with enrofloxacin.

plasma by extraction with chloroform at pH 7.4 [23]. Comparable recoveries have also been reported for the extraction of pefloxacin and its metabolite, norfloxacin, from plasma by extraction with either chloroform or a chloroform–isopentanol mixture [24,25].

In the present study, we were unable to recover either enrofloxacin or ciprofloxacin from plasma by extraction with methylene chloride at pH 7.0. Direct extraction of these compounds from milk was found not suitable due to heavy emulsification and serious interference from fat.

4.2. Extraction after protein precipitation

Most of the methods reported for fluoroquinolones in plasma and tissue involve protein precipitation with an organic solvent in the presence of either an acid or a base. Commonly used organic solvents are acetonitrile, ethanol and methanol, with acetonitrile being the most often used. Phosphoric acid is often used to acidify the sample, while in cases of protein precipitation from basified samples, either sodium hydroxide or ammonium hydroxide is used. With milk samples, Homazabal and Yndestad [16] reported a method involving the use of a combination of acetonitrile and sodium hydroxide for protein precipitation while Roybal et al. [19] used a combination of ethanol and acetic acid, together with sodium sulfate, for the same purpose. Following protein precipitation, the fluoroquinolones are extracted using solid phase extraction with either a strong cation exchange resin column or a reversed phase column. Use of ultra-filtration in the extraction step has also been reported for both plasma and milk samples [7,16].

Because only very limited work has been reported on the extraction of fluoroquinolones from milk, we

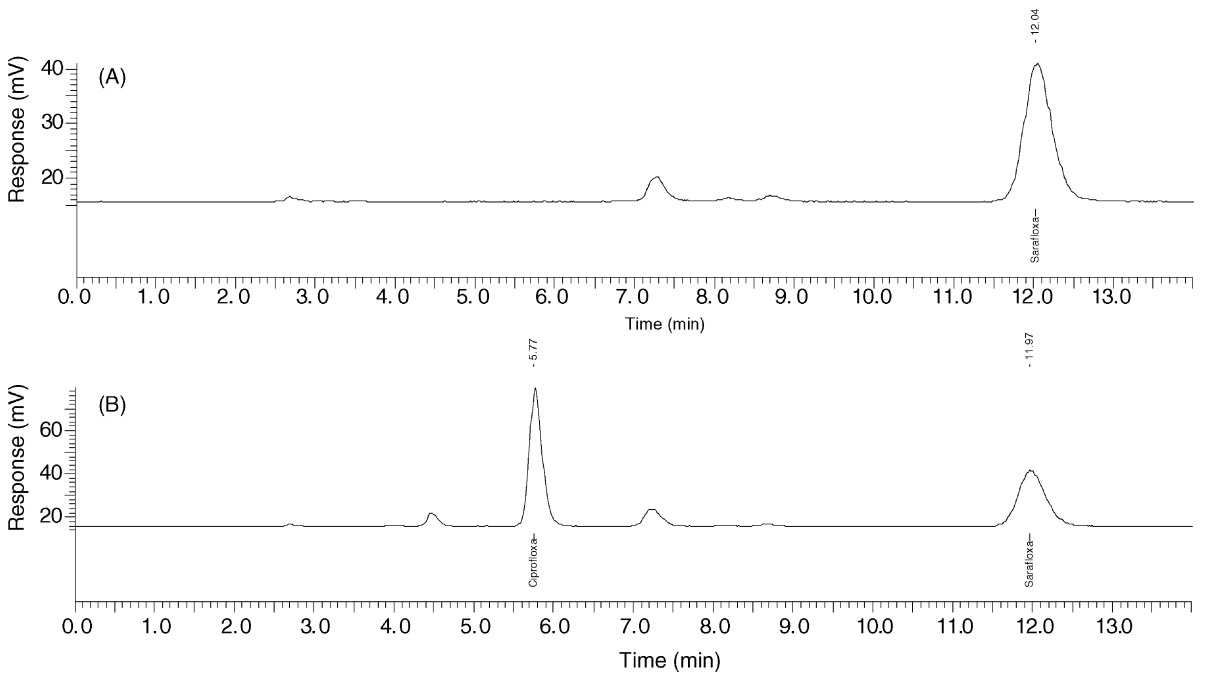

Fig. 5. Chromatograms of pre-dose milk (A) and milk from a cow (B) having steady state plasma level of ciprofloxacin.

decided to examine the extraction of enrofloxacin and ciprofloxacin from milk following protein precipitation under either acid or basic conditions. Since our intention was to avoid the tedious extraction procedures that had been reported, we did not adopt the use of solid phase extraction or ultra-filtration to extract the compounds form the protein-free sample. Instead, taking advantage of the solubility of these compounds in acid or base, we adopted the simple procedure described above whereby following protein precipitation, the organic protein precipitant was removed by extraction with methylene chloride while the fluoroquinolones remained in the aqueous phase, ready for chromatography. A similar approach was reported for the extraction of ciprofloxacin and metabolites from serum, sputum and urine [26].

A combination of acetonitrile and sodium hydroxide (or ammonium hydroxide) was found to be ineffective in precipitating proteins from either milk or plasma. Homazabal and Yndestad [16] reported the use of a combination of acetonitrile and sodium hydroxide for the pre-treatment of milk during the assay of enrofloxacin in milk. However, after removing acetonitrile (and milk fat) by extraction with a hexane–diethyl ether mixture, the authors had to further treat the aqueous phase with phosphoric acid and methanol and then centrifuge. The relative ineffectiveness of a combination of acetonitrile and base as a protein precipitant would explain why, after treatment with acetonitrile and sodium hydroxide, these authors had to further treat the aqueous sample with phosphoric and methanol followed by centrifuging once more to obtain a supernatant for analysis. Similarly, after protein precipitation from milk, plasma and tissue with a combination of acetonitrile and sodium hydroxide, Homazabal and Yndestad [16] and Tyczkowska et al. [7,13] applied ultra-filtration to the samples, presumably, because the acetonitrile–base combination did not completely precipitate the protein from the samples. The ineffectiveness of the acetonitrile–base combination as protein precipitant from milk was confirmed in the present study. After treating the milk sample with acetonitrile and sodium hydroxide, and then extracting the acetonitrile from the supernatant with methylene chloride, the aqueous layer was treated with 0.1 ml of glacial acetic acid which is more than enough to neutralize the sodium hydroxide. Upon addition of acetic acid, more protein precipitation was

observed. Another disadvantage of protein precipitation under basic conditions during the analysis of the FQs in milk (or plasma) comes from the observation that the detectability of the compounds was found to be reduced upon chromatography of the basified solution. This may have been due to an unwanted change in their fluorescence behavior in the basic medium. Thus, the extra step of treating the supernatant with acid, both to bring protein precipitation to completion and to restore the detectability of the FQs makes pre-treatment with a combination of acetonitrile and a base an unattractive approach during the analysis of FQs in milk or plasma.

As mentioned above, a combination of methanol and ammonium hydroxide was found to be a poor protein precipitant from milk or plasma.

While there have been many reports on the use of a combination of an organic solvent and an acid as protein precipitant during the analysis of fluoroquinolones in plasma and tissue, there has been no report of a similar approach in the analysis of FQs in milk. We, therefore, examined the use of a combination of acetonitrile and different acids as protein precipitant during the analysis of enrofloxacin and ciprofloxacin in milk. Both organic and inorganic acids were examined.

A combination of acetonitrile and trifluoroacetic acid was found to be highly effective in precipitating proteins from the milk samples, giving a clean FQ-containing supernatant, suitable for the chromatographic step of the analysis. Trifluoroacetic acid on its own caused immediate protein precipitation on being added to milk. In contrast, glacial acetic acid on its own does not precipitate proteins from milk. However, a combination of acetic acid and acetonitrile was effective in precipitating proteins from milk. Treatment of milk with formic acid and acetonitrile followed by extraction of the mixture with methylene chloride yielded a cloudy supernatant, suggesting that protein precipitation from milk was incomplete in presence of formic acid.

A combination of acetonitrile with either phosphoric acid or hydrochloric acid was found to be effective in precipitating proteins from milk to give a clean supernatant suitable for the analysis of FQs in milk as described above. Use of hydrochloric acid was, however, not adopted for the routine work because of possible adverse effect on the column material.

Acetonitrile, in combination with any of the above-mentioned acids was effective in precipitating proteins from plasma.

Following from these observations, the procedure described above for the determination of FQs in milk (and plasma) was developed, based on the initial precipitation of proteins with a combination of acetonitrile and phosphoric acid, and the removal of acetonitrile and fat by extraction with methylene chloride. This procedure has the advantages of simplicity and speed relative to earlier methods, thus allowing for high throughput analysis required for a pharmacokinetic study. During actual application of the method to pharmacokinetic study of enrofloxacin and ciprofloxacin in bovine plasma, 41 samples were usually processed within 5 h. These consist of 24 bovine plasma study samples, nine plasma calibration standards, six quality control standards, and two blank (control) plasma.

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