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Analytica Chimica Acta 560 (2006) 227–234
ANALYTICA CHIMICA ACTA
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A liquid chromatographic method, with fluorometric detection, for the determination of enrofloxacin and ciprofloxacin in plasma and endometrial tissue of mares

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Received 31 August 2005; received in revised form 12 December 2005; accepted 16 December 2005

Abstract

The aim of this work was to develop and validate a simple and sensitive analytical method for determining enrofloxacin (EFX) and ciprofloxacin (CFX) in equine plasma and endometrial tissue samples, as a precursor to conducting pharmacokinetic/pharmacodynamic studies on equine endometritis This was achieved in the form of a liquid chromatographic procedure, with fluorometric detection, which also gave good separation of other fluoroquinolones including marbofloxacin (MFX), danofloxacin (DFX) and ofloxacin (OFX). Analytes were separated on a C_{18} reversed phase column using an acidified mobile phase. The exact composition of the mobile phase differed for plasma (16% acetonitrile:methanol [13:1,v/v] 84% water containing 0.4% triethylamine and 0.4% phosphoric acid [35%]) and endometrial tissue (14% acetonitrile, 86% water, without methanol) samples. EFX and CFX were both detected at excitation and emission wavelengths of 294 and 500 nm, respectively. Prior to chromatography, EFX and CFX were purified by solid phase extraction from plasma, and a combination of solvent/solid phase extraction from endometrial tissue.

Mean absolute recoveries for EFX and CFX from plasma were 94.1 and 78.0%, respectively, and from endometrial tissue, 78.0 and 57.8%, respectively, with a percentage residual standard deviation (%R.S.D.) <10% in each case. Mean relative recoveries for EFX and CFX from plasma were 91.3 and 119.4%, respectively, and from endometrial tissue, 80.2 and 108.0%, respectively, with a %R.S.D. <20% in each case.

Standard curves constructed using blank plasma and endometrial tissue samples, spiked with authentic EFX and CFX in the ranges 0.005–10.0 $\mu g\,mL^{-1}$ and 0.05–10.0 $\mu g\,g^{-1}$, respectively, all showed acceptable linearity with correlation coefficients, $r^2 \geq 0.977$. Mean intra- and inter-day precision (expressed as %R.S.D.) was <6 and <13%, respectively, with an associated accuracy (expressed as percentage relative error, %R.E.) of <20% for both analytes in both matrices. Acceptable precision and accuracy was also demonstrated at the pre-assigned LOQs of 0.005 $\mu g\,mL^{-1}$ for both EFX and CFX in plasma, and 0.05 $\mu g\,g^{-1}$ for both drugs in endometrial tissue. EFX and CFX were stable in both plasma and endometrial tissue for at least 60 days at −20 °C.

Keywords: Horses; Endometritis; Fluoroquinolones; Enrofloxacin; Ciprofloxacin; Liquid chromatography; Validation

1. Introduction

Fluoroquinolones are antimicrobials marketed in human and veterinary medicine for treating a variety of bacterial diseases. Enrofloxacin (EFX) is used exclusively in veterinary medicine. It is available in several oral and parenteral formulations and is effective in controlling a wide range of bacteria. EFX undergoes de-ethylation to ciprofloxacin (CFX), (the active compound used in human medicine) (Fig. 1). In both human and veterinary medicine, fluoroquinolones have proved to be highly valuable antimicrobials with appropriate pharmacokinetic properties [1]. EFX and its metabolite, CFX, are effective against microorganisms which are resistant to other antimicrobial agents used for the treatment of endometritis, such as aminoglycosides, tetracyclines, macrolides and β-lactams [2].

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doi:10.1016/j.aca.2005.12.040

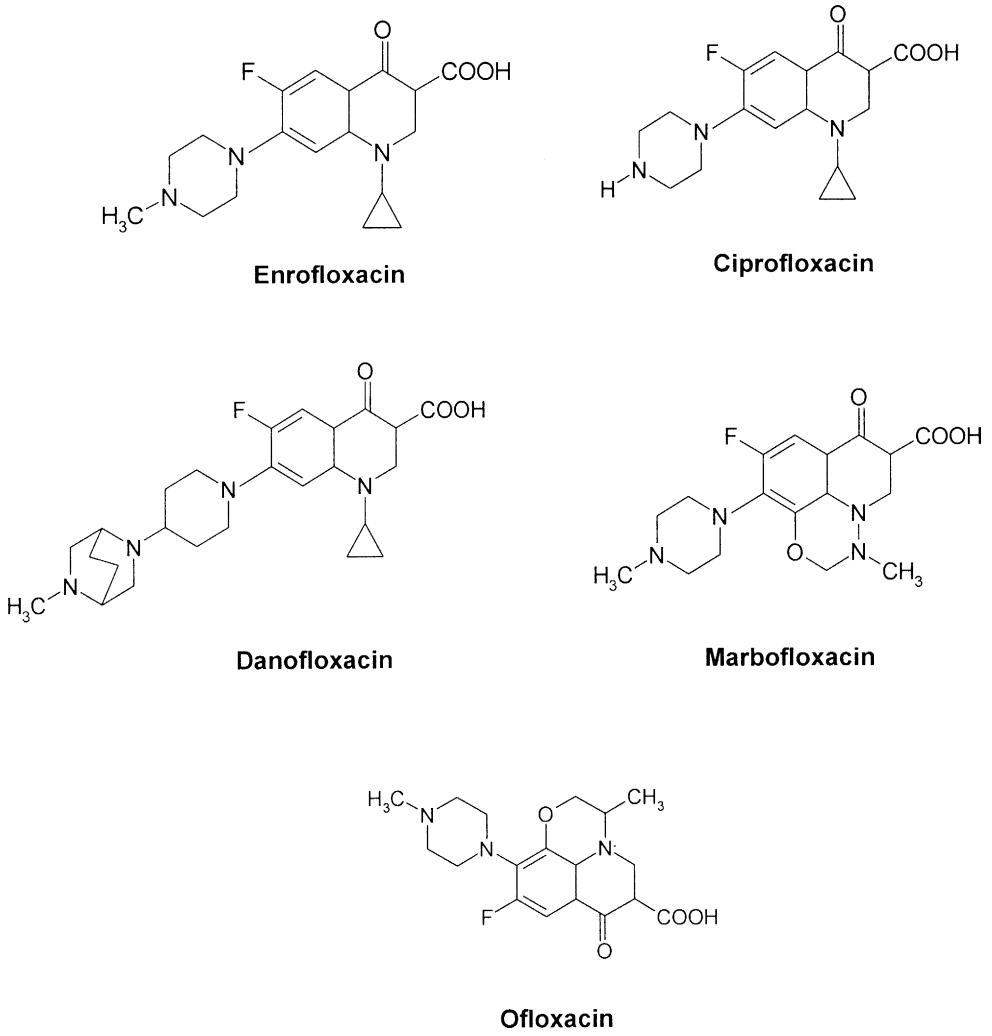

Fig. 1. Chemical structures of the fluoroquinolones tested.

There are numerous potential claims for fluoroquinolone treatment in equine medicine and these include infections caused by most Gram-negative aerobes. *Enterobacteriaceae* strains are commonly isolated from musculoskeletal infections, peritonitis, pleuropneumonia, cholelithiasis/cholangitis and endometritis [3]. Bacterial endometritis is a common cause of fertility problems in mares. Chronic infectious endometritis and venereal diseases are caused mainly by *Streptococcus zooepidemicus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomona aeruginosa* and *Taylorella equigenitalis* [4,5]. In addition, fluoroquinolones have lower, but often therapeutically useful, activity against Gram-positive aerobes, with CFX being the most effective [6,3].

EFX was the first fluoroquinolone marketed specifically for veterinary use in North America. It is a lipophilic and amphoteric antibacterial which is extensively distributed from the bloodstream into different tissues including those colonised by pathogenic bacterial strains [7,8]. Despite its obvious potential, EFX is not currently recommended for use in horses. One reason for this is the concern raised by some authors over the possible association between intravenous and/or oral EFX administration and arthropathy in young horses. The pharmacokinetic behaviour of EFX in horses has not been fully characterized although several recent studies have been undertaken in adult animals [9–15] and foals [16] with the aim of optimising the use of EFX in the equine.

The chemical extraction of EFX from biological samples is a major challenge due to the presence of two ionizable functional groups, in the molecular structure. These acidic carboxyl and basic piperazynil chemical groups are involved in pH-dependent interactions between fluoroquinolones and biological sample matrices [2,17]. Liquid chromatography provides a sensitive technique for detecting EFX [2], and several quantitative methods have been validated for its determination in different biological matrices [18,19]. There has been one previous report on the liquid chromatographic determination of EFX and its metabolite CFX in equine endometrial tissue using relatively non-specific UV detection [15]. A more specific and formally validated method is desirable for undertaking future drug biotransformation studies in this tissue.

The aim of this study was to firstly establish a reliable liquid chromatographic method, based on specific fluorescence

detection, for the separation of different fluoroquinolones, including EFX, CFX, marbofloxacin (MFX), ofloxacin (OFX) and danofloxacin (DFX) (see Fig. 1), and then to develop and validate specific methods for the extraction and quantification of EFX, and its active metabolite CFX, in equine plasma and endometrial tissue as a precursor for undertaking pharmacodynamic/pharmacokinetic studies to optimise the conditions of use for fluoroquinolones in the treatment of endometritis in mares. Method validation adhered to principles set out in the Commission of the European Communities document, 2002/657/EC.

2. Experimental

2.1. Reagents and materials

Pure reference standards of EFX (Vallée, Campo Grande, Brazil), CFX hydrochloride (Labyes, Buenos Aires, Argentina), MFX (Sigma, St. Louis, MO, USA), DFX (Pfizer, Groton, CT, USA) and OFX (Sigma) were used for the development of the analytical methodology.

Methanol and acetonitrile (ACN) (HPLC grade) used during the extraction and chemical analysis were purchased from Sintorgan® (Buenos Aires, Argentina). Anhydrous dibasic sodium phosphate (Na_2HPO_4); monobasic potassium phosphate (KH_2PO_4), sodium hydroxide (NaOH), 85% phosphoric acid (H_3PO_4) were obtained from J.T. Baker (Phillipsburg, NJ, USA) and triethylamine from Sigma. Water was double distilled and deionized using a commercial water purification system (Simplicity®, Millipore, Brazil). All solutions prepared for HPLC were passed through a 0.45 μm nylon filter before use. C_{18} cartridges (Strata™ × 33 μm Polymeric Sorbent, 30 mg/1 mL) purchased from Phenomenex® (Torrance, CA, USA) and disposable C_{18} Supelclean™ solid phase extraction tubes (Supelco Inc. Bellefonte, PA, USA) were used for extraction purposes.

Normal, drug-free equine plasma was collected in heparinized tubes (Sobrius Fadapharma®, Buenos Aires, Argentina) from animals at a military farm ("Haras General Lavalle", Tandil, Argentine). Endometrial tissue was obtained by biopsy from the uterine horn and body of horses killed at Rio Cuarto's slaughterhouse, Córdoba, Argentina.

2.2. Equipment and chromatographic conditions

Chromatography was performed on a Shimadzu (Shimadzu Corporation, Kyoto, Japan) LC system comprising an LC-10AS liquid chromatograph with an RF-10A spectrofluorometric detector, a CTO-10A VP column oven set at 30 °C, a manual sample injector with a 100 μL loop, and a Communications Bus Module-101. Data were collected and analysed using the Shimadzu Class LC10 software (SPD-10A) package. A Prodigy 5 μm particle size, 250 mm × 4.6 mm C_{18} reversed-phase column (Phenomenex® Torrance, CA, USA) was used for separation. Plasma extracts were eluted, at a flow rate of 1.2 mL min^{-1}, using a mobile phase containing 16% ACN:methanol (13:1, v/v) and 84% water with 0.4% triethylamine and 0.4% phosphoric acid (85%) adjusted to pH 2.5 with phosphoric acid [20–22]. For the elution of endometrial tissue extracts, the composition of the mobile phase was slightly modified with respect to the ACN content (14% instead of 16%) and the omission of methanol (see Section 3). All analytes were detected by fluorescence at excitation and emission wavelengths of 294 and 500 nm, respectively.

2.3. Standard solutions

Stock solutions of the EFX and CFX (1000 μg mL^{-1}) were prepared by dissolving 10 mg of pure drug in 10 mL of methanol. Working solutions (100 μg mL^{-1}) of each drug were then prepared by diluting 1 mL of stock solution with 9 mL phosphate buffer, pH 7.0 in a 10 mL volumetric flask. Working standard mixtures of EFX and CFX were prepared at 0.25, 0.5, 1, 2, 10 and 50 μg mL^{-1} by pipetting the appropriate volumes of the individual working solutions into a 10 mL volumetric flask and diluting with phosphate buffer, pH 7.0. *Note*: Phosphate buffer, pH 7.0 was prepared by dissolving 3.54 g of KH_2PO_4 and 5.82 g of Na_2HPO_4 in 1000 mL de-ionised/distilled water and then adjusting the pH with 1 M NaOH.

Stock solutions of internal standards (IS) were prepared by dissolving 10 mg MFX in 10 mL (1000 μg mL^{-1}) of phosphate buffer, pH 7.0 or 1 mg DFX, purity 75.6%, in 100 mL (100 μg mL^{-1}) of the same buffer. The stock solutions of MFX and DFX were diluted 1:10 and 1:100, respectively, in phosphate buffer to give working IS solutions which were used to fortify test samples. Ofloxacin (OFX), included in the development of the separation method, was prepared by dissolving 10 mg (OFX; 1000 μg mL^{-1}) in 100 mL of phosphate buffer, pH 7.0. All standards and working solutions were stored in the dark at 0–4 °C.

2.4. Sample preparation

Plasma was obtained from heparinised whole blood by centrifugation at 500 × *g* for 10 min. Endometrial tissue biopsies were washed with distilled water and then wrapped in aluminium foil. Pools of both plasma and endometrial tissue were kept at −20 °C, until analysis.

2.5. Clean up procedure

2.5.1. Plasma

For the routine analysis of EFX and CFX, portions (1 mL) of plasma were placed into 5 mL glass tubes, and spiked with the required concentrations of the test analytes. Ten microlitre MFX (100 μg mL^{-1}) as IS were also added. The analytes and IS were purified using disposable C_{18} cartridges (see Section 2.1) which were pre-conditioned with 0.5 mL of methanol, followed by 0.5 mL of water. Samples were applied to the cartridges, which were then washed three times with 1 mL de-ionised/distilled water and, after allowing as much liquid as possible to drain off, finally eluted with 2 mL of methanol. Eluents were collected in 5 mL glass tubes and evaporated to dryness under vacuum (Speed-Vac®, Savant, Los Angeles, CA, USA) at 40 °C. Dried extracts were reconstituted in mobile phase (2.4 mL) and mixed by vortexing for approximately 30 s. Aliquots of 250 μL of the

reconstituted extracts were injected directly into the chromatographic system for analysis.

2.5.2. Endometrial tissue

Endometrial tissue samples (150 mg) were carefully minced and spiked with the test analytes as in Section 2.5.1. Then, 37.5 μL DFX (1 μg mL^{-1}) was added as IS, followed by 350 μL phosphate buffer (pH 7). Samples were extracted in 2 × 1 mL portions of ACN by stirring for 10 min, at room temperature, in a multi-tube vortexer. The two ACN extracts were combined and the mixture centrifuged at 2500 × *g*, for 10 min at 5 °C. The resulting supernatant was transferred into glass tube, and evaporated to dryness as in Section 2.5.1. Dried extracts were reconstituted in 200 μL methanol and 800 μL phosphate buffer, pH 7.0. Reconstituted extracts were then subjected to a solid phase extraction process similar to that described above for plasma, except that sample washing was performed with 10 volumes of deionised/distilled water.

2.6. Method validation

2.6.1. Recovery

Absolute and relative recoveries of EFX and CFX from plasma and endometrial tissue were assessed at three different concentration levels (0.1, 0.5 and 5 μg mL^{-1} or μg g^{-1}, respectively) by triplicate analysis. Extraction efficiency was determined by comparison of the peak areas obtained from spiked plasma/endometrial tissue blanks with those given by equivalent authentic standard solutions.

2.6.2. Linearity

Linearity was tested by constructing authentic standard curves and spiked matrix (plasma and endometrial tissue) curves for EFX and CFX. Analyte/IS peak area ratios were determined, in triplicate, for each drug over a calibration range of 0.005–10 μg mL^{-1} for plasma and 0.05–10 μg g^{-1} for endometrial tissue. Data were analysed by the least squares regression method, using the Runs Test and ANOVA to confirm linearity.

2.6.3. Precision and accuracy

Precision and accuracy of the method were determined by analysing replicate ($n = 3$) extracts of blank matrix (plasma or endometrial tissue) spiked with EFX and CFX at two concentrations (0.5 and 5 μg mL^{-1} for plasma; 0.1 and 0.5 μg g^{-1} for endometrial tissue). Ten microlitre MFX (100 μg mL^{-1}) and 37.5 μL DFX (1 μg mL^{-1}) were added as IS to plasma and endometrial tissue samples, respectively. Precision was expressed as the percentage residual standard deviation.

For intra-day precision (repeatability) triplicate extracts of plasma or endometrial tissue spiked at two concentrations of EFX and CFX were assayed within a single run. Inter-day precision (within-laboratory reproducibility) was determined by analysing single extracts of plasma and endometrial tissue, spiked at the two test concentrations on three consecutive working days. Accuracy was defined as the closeness between the experimentally measured and true concentration values [23], and was estimated as the percentage relative error where:

$$\%\text{R.E.} = \frac{\text{estimated analyte concentration} - \text{nominal analyte concentration} \times 100}{\text{nominal analyte concentration}}$$

2.6.4. Stability

Long-term stability of EFX and CFX in plasma and endometrial tissue samples stored at −20 °C was tested at two concentration levels (0.05 and 0.1 μg mL^{-1}; 0.5 and 2 μg g^{-1}, respectively, for each matrix samples and assayed in triplicate on day zero and then at 7 day intervals for up to 2 months.

2.6.5. Limit of quantification (LOQ)

The LOQ was defined as the lowest drug concentration on the standard curve (performed for each matrix) that could be quantitated ($n = 5$) with a precision not exceeding 20% and accuracy within 20% of nominal, for plasma and endometrial tissue extracts.

3. Results and discussion

This paper describes the development and validation of a reliable analytical method for the quantitation of EFX and CFX in equine plasma and endometrial tissue to facilitate the pharmacokinetic and pharmacodynamic studies needed to exploit their therapeutic potential in the treatment of equine endometritis.

EFX and CFX are ampholytic compounds [19] and a generic concern in developing liquid chromatographic methods for such substances is their association with severe peak tailing on reversed phase columns. To address this and other potential problems, a planned development programme, based on the general strategy of Liang et al. [24], was initially undertaken. Whilst a detailed description is not relevant in the context of this paper, the result of evaluating major chromatographic conditions including column size, column temperature, mobile phase composition, pH, flow rate and detection wavelengths, was an optimized procedure of assured robustness for validation. In addition, the introduction of a solid phase extraction step minimized the occurrence of interfering chromatographic peaks. The chromatographic conditions established achieved good separation among a total of five fluoroquinolones, as indicated by the retention data summarized in Table 1. Subsequently, procedures

Table 1
Absolute retention times (ART; min) and relative retention times (RRT)[a] for ciprofloxacin (CFX), enrofloxacin (EFX), marbofloxacin (MFX), danofloxacin (DFX) and ofloxacin (OFX) in plasma and endometrial tissue of mares

Analyte	Plasma		Endometrium	
	ART	RRT	ART	RRT
CFX	8.5	1.27	11.3	0.78
EFX	12.5	1.87	16.7	1.15
MFX	6.7	1.00	8.5	0.59
DFX	9.6	1.43	14.5	1.00
OFX	7.1	1.06	10.5	0.72

[a] Relative to the internal standard (MFX for plasma; DFX for endometrium).

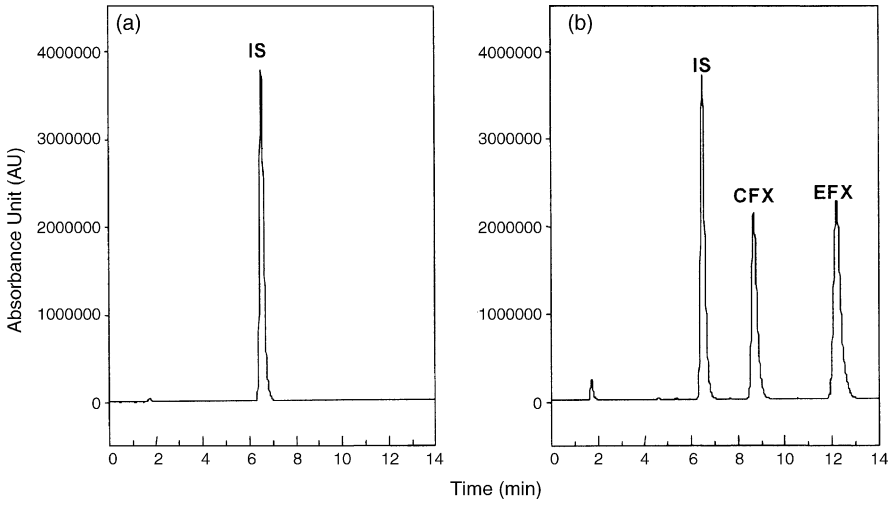

Fig. 2. Typical chromatograms obtained for (a) blank plasma spiked with marbofloxacin as internal standard (IS) and (b) plasma spiked with IS and 1 μg mL^{-1} enrofloxacin (EFX) and its active metabolite ciprofloxacin (CFX).

were specifically optimized and validated for the extraction and analysis of EFX, CFX, along with appropriate internal standards, from equine plasma and endometrial tissue.

As illustrated in Fig. 2b, the chromatographic conditions defined for plasma gave symmetrical and well separated peaks, with minimal peak tailing, for EFX, CFX and the chosen internal standard, MFX, in a total run time of 15 min. Moreover, it was confirmed that no co-eluting peaks were present in plasma blanks (see Fig. 2a). Linearity of the method for EFX and CFX in plasma was confirmed, by least squares regression analysis (see Table 3), at eleven calibration points over a concentration range of 0.005–10 μg mL^{-1}. Mean absolute recovery values, derived from triplicate plasma extracts spiked at three concentrations levels (0.1, 0.5, 5 μg mL^{-1}) of each test compound, were 94.1 (R.S.D. = 8.98%) and 78.0% (R.S.D. = 6.48%) for EFX and CFX, respectively (Table 2). Mean relative recoveries, based on comparison of analyte:internal standard peak area ratios, were 91.3 (R.S.D. = 15.8%) and 119.4% (R.S.D. = 20.0%) for EFX and CFX, respectively. The recovery values obtained in the present study were higher than those previously reported in equine plasma [16] but, for CFX, were similar to those reported for human plasma (Imre et al. [25]) and, for EFX, comparable to those obtained for porcine plasma (Wiuff et al. [26]). In contrast, EFX recovery was lower than that obtained for bovine milk by Marazuela and Moreno-Bondi [27]. Some of these discrepancies may be due to species/matrix effects, but another obvious possibility would be differences in the analytical methodology used. Intra-day precision (repeatability), expressed as the %R.S.D. for triplicate plasma extracts spiked at two concentrations (0.5 and 5.0 μg mL^{-1}) were, respectively, 7.15 and 1.67 for EFX, and 3.40 and 4.60 for CFX (see Table 4). Inter-day precision (intermediate reproducibility), expressed as the % R.S.D., estimated by single injections, on three separate days, of plasma extracts spiked at the same two concentrations was 15.0 and 7.50 for EFX, and 4.90 and 20.0 for CFX, respectively. Mean accuracy data for the precision samples, expressed as percentage relative error (%R.E., see Section 2.6.3) are given in Tables 2 and 4. For the intra-day precision assays accuracy ranged from +4 to −15.2% for EFX and from +4 to −16.6% for CFX. For the inter-day precision study, the ranges were −8.2–11.1% for EFX, and −9.70 to −5.60% for CFX. For plasma analysis both EFX and CFX were pre-assigned LOQ values of 0.005 μg mL^{-1}. R.S.D. values for repeatability ($n = 5$) at that concentration were 6.43 and 5.39%, respectively, for EFX and CFX. Stability studies indicated there was no significant degradation of either component in plasma samples spiked with 0.1 μg mL^{-1} of EFX and CFX and stored frozen for up to 2 months at −20 °C before extraction and analysis (Fig. 4a).

Table 2
Summary of validation parameters for the liquid chromatographic determination of ciprofloxacin (CFX) and enrofloxacin (EFX) in plasma and endometrial tissue from mares

	Plasma		Endometrial tissue	
	CFX	EFX	CFX	EFX
LOQ	0.005[a]	0.005[a]	0.050[b]	0.050[b]
Linearity (r^2)	0.978	0.954	0.967	0.971
Mean intra-day precision (%R.S.D.)	4.00	4.41	4.60	5.90
Accuracy (%R.E.)	20.0	11.0	9.0	0.0
Mean inter-day precision (%R.S.D.)	12.45	11.25	3.75	1.20
Accuracy (%R.E.)	8.4	9.5	3.0	17.0
Mean absolute recovery (%)	78.0	94.0	57.7	77.9
%R.S.D.	6.48	8.98	10.0	1.30
Mean relative recovery (%)	91.3	119.4	80.2	108.0
%R.S.D.	15.8	20.0	3.2	14.2

LOQ, limit of quantitation; r^2, squared correlation coefficient; %R.S.D., percentage residual standard deviation; %R.E., percentage relative error.
[a] Values are in μg mL^{-1}.
[b] Values are in μg g^{-1}.

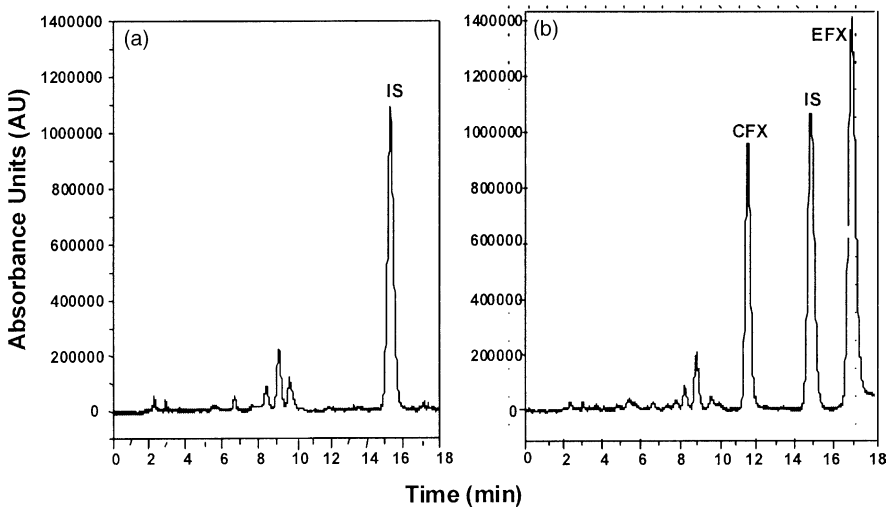

Fig. 3. Typical chromatograms obtained for (a) blank endometrial tissue spiked with danofloxacin as internal standard (IS) and (b) endometrial tissue spiked with IS and 2 μg g^{-1} enrofloxacin (EFX) and its active metabolite ciprofloxacin (CFX).

Table 3
Linear regression equations obtained for enrofloxacin (EFX) and ciprofloxacin (CFX) in spiked matrix calibration curves for plasma and endometrial tissue of mares

Matrix	Drug	Test range	Regression equation	Linearity (r^2)
Plasma	EFX	0.005–10.0[a]	$y = 0.009 + (1.09 \pm 0.008)$ x	0.954
	CFX	0.005–10.0[a]	$y = 0.040 + (0.44 \pm 0.09)$ x	0.978
Endometrium	EFX	0.05–10[b]	$y = -0.010 + (0.71 \pm 0.014)$ x	0.971
	CFX	0.05–10[b]	$y = 0.093 + (0.21 \pm 0.43)$ x	0.967

r^2 = Squared correlation coefficient.
[a] Values are in μg mL^{-1}.
[b] Values are in μg g^{-1}.

When endometrial extracts were analysed under the same conditions as plasma, a number of interfering peaks were detected, particularly around the retention time of the MFX internal standard (data not shown). This problem was successfully resolved (see Fig. 3a and b) by a combination of slightly modifying the original mobile phase composition (see Section 2.2), increasing the total run time to 20 min, and replacing MFX with DFX as internal standard. Linearity of the modified

Table 4
Analytical precision and accuracy for the liquid chromatographic determination of enrofloxacin (EFX) and ciprofloxacin (CFX) in plasma and endometrial tissue of mares

CFX				EFX		
Concentration added	Mean concentration recovered	Precision (%R.S.D.)	Accuracy (%R.E.)	Mean concentration recovered	Precision (%R.S.D.)	Accuracy (%R.E.)
Plasma[a]						
Intra-day ($n = 3$)						
0.50	0.60	3.40	−16.6	0.48	7.15	4.10
5.00	6.00	4.60	−16.6	5.90	1.67	−15.2
Inter-day ($n = 3$)						
0.50	0.53	4.90	−5.60	0.45	15.0	11.1
5.00	5.54	20.0	−9.70	5.45	7.50	−8.20
Endometrial tissue[b]						
Intra-day ($n = 3$)						
0.1	0.09	5.20	10.1	0.10	10.5	0.00
0.5	0.54	4.00	−7.40	0.50	1.30	0.00
Inter-day ($n = 3$)						
0.25	0.24	3.00	4.00	0.29	2.20	−13.8
1.00	0.98	4.50	2.00	1.18	0.20	−15.0

%R.E., percentage relative error; %R.S.D., percentage residual standard deviation.
[a] Concentration values are in μg mL^{-1}.
[b] Concentration values are in μg g^{-1}.

procedure for the analysis of EFX and CFX in endometrial tissue was confirmed (see Table 3) at eight concentration points in the range 0.05–10 $\mu g\,g^{-1}$. Mean absolute recovery values for endometrial tissue samples spiked at 0.1, 0.5, 5 $\mu g\,g^{-1}$ were 77.9% (R.S.D. = 1.30%) and 57.7% (R.S.D. = 10.0%) for EFX and CFX, respectively (see Table 2): mean relative recoveries were 80.2 (R.S.D. = 3.2%) and 108.0% (R.S.D. = 14.2%), respectively. These results are comparable with those reported for other tissues in different animal species [18,19,26]. EFX and CFX concentrations in equine endometrial tissue have also previously been reported following intravenous administration of EFX, but with little supporting method validation data [15]. Repeatability, expressed as the %R.S.D. for triplicate extracts of endometrial tissue spiked at 0.1 or 0.5 $\mu g\,g^{-1}$ for plasma and was, respectively, 10.5 and 1.30 for EFX, and 5.20 and 4.00 for CFX (see Table 4). Intermediate reproducibility, expressed as the %R.S.D., estimated by single injections, on three separate days, of extracts of endometrial tissue spiked at 0.25 or 1.0 $\mu g\,g^{-1}$ was, respectively, 2.20 and 0.20 for EFX, and 3.00 and 4.50 for CFX in endometrial tissue. The accuracy of these estimations expressed as %RE (see above) ranged from zero for EFX and −7.40–10.1 for CFX in the repeatability samples and from −15.00 to −13.8 for EFX and 2.00–4.00 for CFX in the reproducibility study (Table 4). At the pre-assigned LOQ (0.05 $\mu g\,g^{-1}$) for endometrial tissue R.S.D.s for repeatability ($n = 5$) were 9.75 and 7.43% for EFX and CFX, respectively. Both EFX and CFX were stable in endometrial tissue, spiked with 0.1 $\mu g\,g^{-1}$ of each, stored for up to 2 months at −20 °C (Fig. 4b).

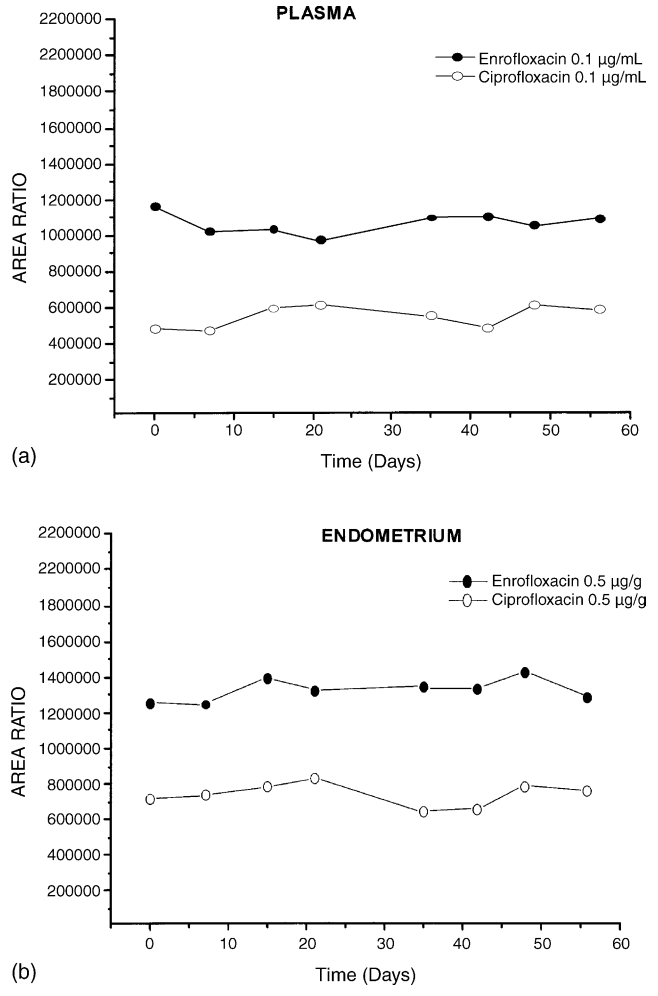

Fig. 4. Absorbance vs. time in vitro stability curves for fortified (a) plasma and (b) endometrial tissue samples stored frozen for up to 2 months at −20 °C.

4. Conclusions

Whilst several analytical liquid chromatography methods have been described for the determination of fluoroquinolones in plasma and/or edible tissues from various species, this is the first report on the formal validation of a specific method for their analysis in equine endometrial tissue.

The method allows the separation of at least five fluoroquinolones (enrofloxacin, ciprofloxacin, marbofloxacin, danofloxacin and ofloxacin), although only the procedures for EFX and CFX were formally validated.

Linearity was maintained for concentrations of up to 10 $\mu g\,mL^{-1}$ and 10 $\mu g\,g^{-1}$ for plasma and endometrium, respectively, and the use of specific fluorescence detection resulted in acceptable precision and accuracy being maintained for concentrations down to the assigned LOQ's of 0.005 $\mu g\,mL^{-1}$ and 0.05 $\mu g\,g^{-1}$, respectively. These levels of sensitivity facilitate precise pharmacokinetic analysis, and definition of the tissue distribution of these molecules in horses. Validation of the analysis in endometrial tissue provides an opportunity for developing pharmacokinetic and pharmacodynamic models which could improve the therapeutic potential of EFX and CFX in the treatment of endometritis in mares.

Acknowledgements

The authors acknowledge the financial support of Fundación Antorchas, Argentine (Project 1397-27) awarded to Prof. S. Sanchez Bruni, and Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), Argentina. The authors also wish to thank staff at the Veterinarian's military farm "Haras Gral. Lavalle" for their collaboration and technical support.

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