Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 987 (2003) 221–226
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Determination of enrofloxacin and its metabolite ciprofloxacin in goat milk by high-performance liquid chromatography with diode-array detection
Optimization and validation

A.L. Cinquina[a,*], P. Roberti[a], L. Giannetti[a], F. Longo[a], R. Draisci[b], A. Fagiolo[a], N.R. Brizioli[a]

[a]*Istituto Zooprofilattico Sperimentale del Lazio e Toscana, Via Appia Nuova 1411, 00178 Rome, Italy*
[b]*Veterinary Medicine Laboratory, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy*

Abstract

A high-performance liquid chromatography–diode array detection method (HPLC–DAD) combined with liquid chromatography–mass spectrometry was developed for the determination of enrofloxacin and its metabolite ciprofloxacin in goat milk. The HPLC–DAD method validation was compliant with the ''DG SANCO 1805/2000'' European regulation. The residues were extracted from milk with phosphate buffer, purified on a C_{18} Speedisk cartridge SPE (Baker) and then analysed using HPLC–DAD set at 277 nm. The decision limit (CCα) calculated by spiking samples at 100 μg/kg with both analytes, taking into account the maximum residue limit (MRL) of 100 μg/kg established by the European Union for the sum of enrofloxacin and its metabolite ciprofloxacin in milk, was 105.3 μg/kg for enrofloxacin and 105.5 μg/kg for ciprofloxacin. The detection capability (CCβ) was 110.7 and 110.9 μg/kg for enrofloxacin and ciprofloxacin, respectively. The mean recoveries of the method, calculated by spiking samples at 50, 100 and 150 μg/kg were 84% for enrofloxacin and 88% for ciprofloxacin. The limit of quantification was 20 μg/kg for both analytes. The HPLC–DAD validated method was successfully applied for the first time in goats milk, and proved to be suitable for the sensitive and accurate quantification and confirmation analysis of enrofloxacin and ciprofloxacin for regulatory purposes.

Keywords: Milk; Food analysis; Validation; Enrofloxacin; Ciprofloxacin; Antibiotics; Fluoroquinolones

1. Introduction

The fluoroquinolone antibiotics have a wide range of antibacterial activity and have seen increasing use in veterinary medicine because of their effectiveness in treating bacteria infections [1]. In fact they have been used successfully to treat infection caused by Gram positive, Gram negative bacteria and Micoplasma such as pulmonary infections, urinary infections and digestive infections [1]. These compounds act principally by inhibition of bacterial DNA-gyrase [2] changes in the chemical structure of modern fluoroquinolones increasing the potency and biological spectrum of activity of the drugs [3].

Enrofloxacin, a fluoroquinolone antibiotic, has been approved for use in food animals and is

*Corresponding author. Tel.: +39-6-7909-9402; fax: +39-6-7934-0724.
E-mail address: acinquina@rm.izs.it (A.L. Cinquina).

0021-9673/02/$ – see front matter
PII: S0021-9673(02)01800-9

effective against organisms resistant to antibacterial substances usually used in veterinary medicine, such as β-lactam antibiotics, aminoglycosides, tetracyclines and macrolides [4,5]. The use of antibiotics in lactating breeding animals may leave residues in the milk and tissues. Therefore, animal food may be a potential hazard for the consumers, cause allergic reactions and also lead to the emergence of drug-resistant bacteria. To protect consumers' health the European Union set maximum residue limits (MRLs) for many drugs regarding milk, meat and others foods by means of Regulation no. 2377/90 (EC 1990) [6] and in particular for the sum of enrofloxacin and its metabolite ciprofloxacin in milk the MRL was set as 100 μg/kg for all animal species (EC 1999) [7]. A biologically active metabolite ciprofloxacin [8,9], derived by enrofloxacin deethylation, and which has been restricted to use in human medicine, is excreted with milk after enrofloxacin treatment [10,11]. A HPLC–diode array detection (DAD) method capable of distinguishing between these drugs was developed to detect and quantify enrofloxacin residues and its metabolite ciprofloxacin in milk. This method is more accurate (88% recoveries for ciprofloxacin and 84% for enrofloxacin) than previous methods reported in the literature [12]. To establish the precision and accuracy of the method, internal validation compliant with requirements defined by the Draft SANCO/1805/2000 European regulation was used [13]. For confirmatory assays, however, the same decision recommends that, wherever possible, mass spectrometry should be used in order to increase specificity. We used a turbo-ionspray liquid chromatography–mass spectrometry method to identify the molecules of interest.

2. Experimental

2.1. Chemicals and reagents

The chemical and chromatographic reagents used were HPLC or analytical grade acetonitrile, methanol, orthophosphoric acid, trichloroacetic acid, formic acid and sodium hydroxide obtained from J.T. Baker (Deventer, The Netherlands), potassium dihydrogenphosphate, trifluoroacetic acid and triethylamine obtained from BDH Laboratory Supplies (Poole, UK).

Enrofloxacin was kindly supplied by Bayer (Leverkusen, Germany) and ciprofloxacin was obtained from Serological (Kankakee, IL, USA). Water was purified in a Milli-Q system (Millipore, Bedford, MA, USA). For the extraction procedure, a phosphate buffer of pH 7.4 (0.05 *M*) was prepared by diluting 250 ml of KH_2PO_4 (0.2 *M*) and 197.5 ml of NaOH (0.2 *M*) to 1 l with water.

2.2. HPLC–DAD equipment and conditions

A HP-1100 high-performance liquid chromatograph was used with a diode-array detector from Agilent Technologies (Palo Alto, CA, USA) and equipped with an autosampler and a 20-μl sample loop. Analytical separation of drugs was achieved on a C_{14} Zorbax Bonus-RP column (150 mm×4.6 mm, 5 μm) from Hewlett-Packard (PA, USA) with a C_{14} Zorbax guard column (12.6 mm×4.6 mm, 5 μm). The mobile phase was a mixture of 0.05 *M* orthophosphoric acid (pH 3.4)–acetonitrile (87:13, v/v) at a flow-rate of 1 ml/min; the detector was set at 277 nm.

Mass spectral analyses were performed on an API 2000 triple quadrupole tandem mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a turbo-ionspray interface. The LC–MS interface was set at 450 °C; the orifice potential voltage was set at 80 V using a sprayer voltage of 5300 V. A Perkin-Elmer (Boston, MA, USA) Series 200 pump liquid chromatograph equipped with a Valco (Houston, TX, USA) injection valve fitted with a 5-μl sample loop was used. Separation of enrofloxacin and ciprofloxacin was carried out on a column packed with C_{14} Zorbax Bonus-RP (150 mm×2.1 mm, 5 μm) from Hewlett-Packard at room temperature, operating under isocratic conditions with a mobile phase of 1% formic acid–methanol–acetonitrile (87:5:8, v/v), at a flow-rate 0.3 ml/min.

2.3. Standard solutions

Stock standard solutions of enrofloxacin and ciprofloxacin were prepared by dissolving 10 mg of each compound in 10 ml of acetonitrile to obtain a final concentration of 1 mg/ml. These solutions were

stored at −20 °C and remained stable for at least 4 weeks. Working standard solutions were prepared daily in the range 0.05–1.00 μg/ml by diluting the stock standard solutions in the mobile phase.

The sample concentration was calculated by comparing peak area with external calibration curve. The calibration curves were obtained in the range of 0.05–1.00 μg/ml.

2.4. Sample preparation and extraction

An aliquot (5 g) of milk was accurately weighed, 2.5 ml of trichloroacetic acid (20% in methanol) were added to the sample, the mixture shaken for 15 s and centrifuged at 1500 *g* for 10 min. Phosphate buffer (12.5 ml, pH 7.4) was added and the mixture centrifuged for 15 min at 1500 *g*. The supernatant was purified by solid-phase extraction (SPE) using a C_{18} Speedisk cartridge (Baker, 200 mg, 6 ml) which had previously been conditioned with 6 ml methanol, 6 ml water and 6 ml phosphate buffer, pH 7.4. After the extract had passed through the cartridge, it was rinsed with 2 ml of water and dried under vacuum for 3 min. The fluoroquinolones were eluted from the column with 2 ml of 1% trifluoroacetic acid in acetonitrile.

The eluate was evaporated to dryness at 40 °C under a stream of nitrogen. The residue was resuspended in 100 μl of acetonitrile and diluted to 900 μl with water. Twenty μl of this mixture were injected into the HPLC system.

2.5. Specificity

The specificity of the method was assessed by testing a number of representative blank milk samples ($n=20$), from different origins, to verify the absence of potential interfering compounds.

2.6. Decision limit (CCα) and detection capability (CCβ)

Two different options for the calculation of the decision limit (CCα) and detection capability (CCβ) were chosen; the two procedures described are denoted as variants A and B.

Variant A

CCα-A. Twenty blank goat's milk samples were spiked with the analytes at 100 μg/kg;

CCβ-A. Twenty blank goat's milk samples were spiked with the analytes at the decision limit (CCα).

Variant B

CCα-B and CCβ-B. Three matrix calibration curves were prepared by spiking blank milk samples with a mixture of the enrofloxacin and ciprofloxacin to obtain concentrations of 25, 50, 100, 150 and 175 μg/kg.

2.7. Precision and accuracy

Inter- and intra-day repeatability in goat milk was assessed by performing six replicated analyses of spiked milk samples with enrofloxacin and ciprofloxacin at three concentrations (50, 100, 150 μg/kg), on 3 different days.

2.8. Limit of quantification (LOQ) and limit of detection (LOD)

The limit of detection (LOD) is the smallest concentration from which it is possible to deduce the presence of the analyte with reasonable statistical certainty.

The limit of quantification (LOQ) is the smallest measured content of the identified analyte in a sample that may be quantified with a specified degree of accuracy and within-laboratory reproducibility. The LOQ was calculated by spiking milk samples with enrofloxacin and ciprofloxacin at concentration of 20 μg/kg, on 3 different days.

2.9. Stability

The enrofloxacin and ciprofloxacin stability was determined in solution and in matrix. Stability of stock standard solutions had been verified at room temperature (in darkness and in the light), at +4 °C and −20 °C for 20 weeks at concentrations around the MRL.

Stability of spiked milk samples with 1 μg/ml of ciprofloxacin and enrofloxacin had been verified at −20 °C for 20 weeks.

3. Results and discussion

We validated the HPLC–DAD method in compliance with the Draft SANCO/1805/2000 European regulation. The validation procedure includes the determination of specificity, decision limit (CCα), detection capability (CCβ), precision, accuracy and stability.

Fig. 1A and B show the chromatograms of a blank milk sample and a spiked milk sample with a mixture of enrofloxacin and ciprofloxacin, respectively.

In Fig. 1A we observe that the blank sample chromatogram contained no peaks at the retention times of the two analytes.

The same purification procedure was applied to the 20 blank milk samples from different origins in order to verify the specificity of the method. No interference around the retention time of two analytes was observed in any of the samples analyzed.

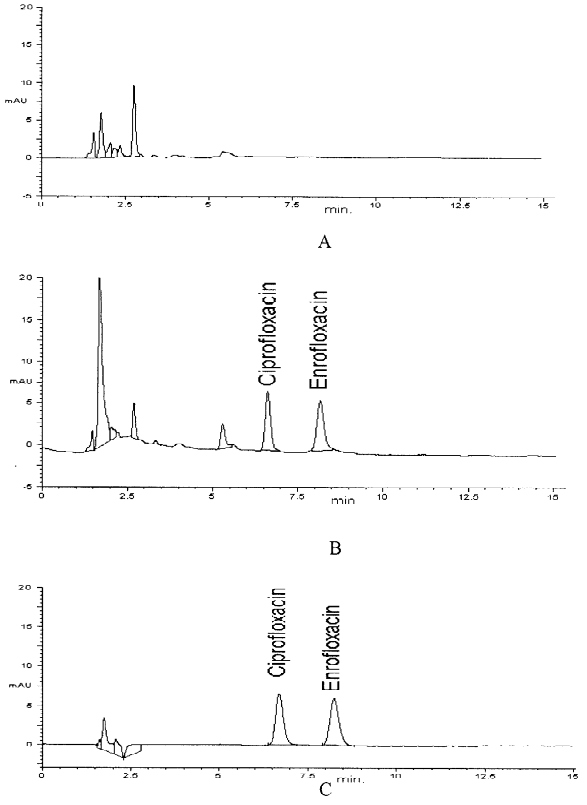

Fig. 1. LC–DAD analysis: (A) blank milk sample, (B) spiked milk sample with a mixture of enrofloxacin and ciprofloxacin and (C) enrofloxacin and ciprofloxacin standards.

The CCα is defined as ''the concentration above which it can be determined, above which it can be decided with a statistical certainty of $1-\alpha$ that the identified analyte content is truly above MRL'' and in the case of MRL substances it can be established with two different modes as described in the Experimental section. CCβ is ''the concentration at which the method is able to detect MRL concentrations with a statistical certainty of $1-\beta$. The β error should be less than or equal to 5%'' [13].

CCα and CCβ values obtained via modes A and B are reported in Table 1. The results revealed no substantial differences between the two procedures adopted.

The calibration curves were prepared over the concentration range 0.05 to 1.0 μg/ml, and were repeated on 3 different days. The linearity was good for all analytes in the range of tested concentrations, as shown by the fact that the determination coefficients (r^2) are greater than 0.999 for all curves.

Fig. 1C shows the chromatogram of enrofloxacin and ciprofloxacin standard solution. Recoveries and precision of the analytical method in goat milk data were generated each day for 3 days from analysis of blank samples of milk spiked at three different concentrations (50, 100 and 150 μg/kg) and six replicates at each concentration were analysed each day ($n=18$).

The extraction efficiency of the analytes was determined by comparing the fortified sample peaks with an externally generated calibration curve. The recoveries and precision data are reported in Table 2. Excellent recoveries were observed for both analytes at all fortification levels, the mean recoveries of the method were 84% for enrofloxacin and 88% for

Table 1
Decision limit (CCα) and detection capability (CCβ)

	CCα (μg/kg)		CCβ (μg/kg)		r[a]
	A[b]	B[c]	A[b]	B[c]	
Enrofloxacin	108.4	105.3	115.4	110.7	0.9970
Ciprofloxacin	107.9	105.5	114.2	110.9	0.9966

[a] Representative correlation coefficient of the three matrix calibrations.
[b] Variant A.
[c] Variant B.

Table 2
Intra-day precision and recovery for enrofloxacin and ciprofloxacin in milk samples

Drug	Parameter	Validation sample level: 50, 100, 150 (ng/kg)		
		Day 1	Day 2	Day 3
Enrofloxacin	Average recovery (%)	84.3	86.6	81.8
	SD (ng/ml)	4.7	6.3	5.9
	Precision (RSD, %)	5.5	7.3	7.2
	n	18	18	18
Ciprofloxacin	Average (ng/ml)	89.1	89.7	85.4
	SD (ng/ml)	3.4	5.4	3.5
	Precision (RSD, %)	3.8	6.0	4.1
	n	18	18	18

ciprofloxacin. The percent relative standard deviation for all fortification levels at each day for each analyte was less than 7.3, for each analyte demonstrating excellent method precision. The method performance was in accordance with the requirements defined by the Draft SANCO/1805/2000 European regulation.

The LOD, calculated as the smallest concentration from which it is possible to deduce the presence of the analyte with reasonable statistical certainty, is 15 μg/kg for both analytes.

The LOQ, calculated as the smallest measured content of the identified analyte in a sample that may be quantified with a specified degree of accuracy ($n=18$) and within-laboratory reproducibility, is 20 μg/kg for both analytes. The mean recovery was 80.6% with a SD of 3.7% and an RSD of 4.6% for enrofloxacin and the mean recovery was 90.9% with a SD of 8.8% and an RSD of 9.7% for ciprofloxacin.

The stock standard solutions were stable for 20 weeks in all storage conditions; also the spiked milk samples were stable for 20 weeks.

3.1. LC–MS

In the present work the LC–MS technique was used to achieve the unambiguous identification of enrofloxacin and ciprofloxacin utilising the method of Turnipseed et al. [14] with little modification. Preliminary experiments were carried out by flow injection analysis (FIA) on individual standard solutions of both drugs. Fig. 2A and B show the positive-ion full-scan (mass range m/z 100–500) FIA–MS spectra of enrofloxacin and ciprofloxacin, respectively. The analytes produced an intense protonated molecule $[M+H]^+$ at m/z 360 for enrofloxacin and at m/z 332 for ciprofloxacin. Tandem mass spectrometry was therefore used in order to obtain additional structural information by detecting diagnostic product-ions obtained by collision induced dissociation (CID) of the precursor ion. Fig. 2C and

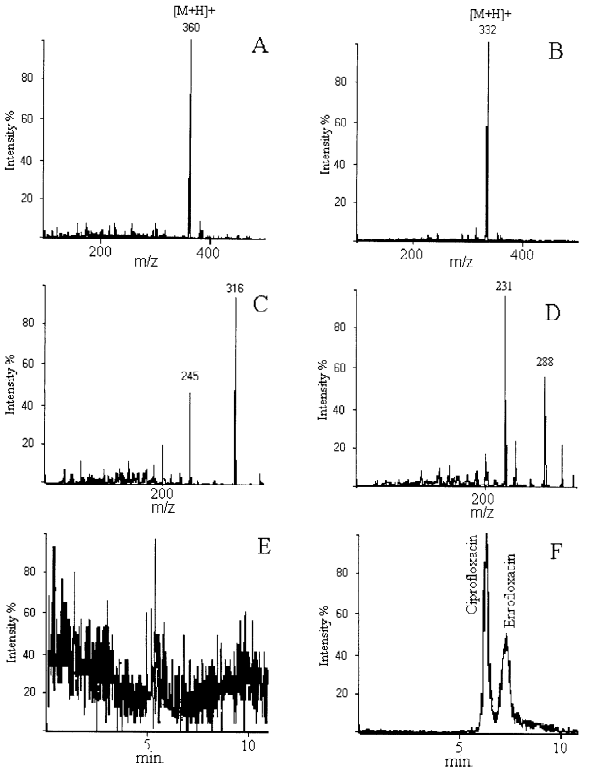

Fig. 2. LC–MS analysis: (A) positive ion mass spectra of enrofloxacin, (B) positive ion mass spectra of ciprofloxacin, (C) positive product ion mass spectrum of enrofloxacin, (D) positive product ion mass spectrum of ciprofloxacin, (E) LC–MS–MS analysis of blank milk samples and (F) spiked milk samples.

D shows the positive product ion mass spectra of the protonated molecule $[M+H]^+$ of enrofloxacin and ciprofloxacin, respectively. The two most abundant product-ions were chosen for LC–MS–MS analyses. Precursor–product ion combination of m/z 360/316, 360/245 for enrofloxacin and m/z 332/288, 332/231 for ciprofloxacin were used, according to the selected reaction monitoring (SRM) technique.

Fig. 2E and F show the LC–MS–MS chromatograms of blank milk samples and spiked milk samples at 100 μg/kg, respectively. A good separation of the two analytes was achieved in 10 min. The high specificity of the LC–MS–MS method was proved by the examination of the LC–MS–MS chromatograms of blank spiked milk sample. No interference was observed in the control sample, whereas the LC–MS–MS chromatogram of spiked milk sample showed only the analyte chromatographic peak.

4. Conclusion

The HPLC–DAD method proved simple, rapid and suitable for routine quantification of enrofloxacin and its metabolite ciprofloxacin in milk samples. Validation results were considered satisfactory in view of the complexity of the biological matrices.

LC–MS was successfully used to achieve an unambiguous identification of enrofloxacin and its metabolite ciprofloxacin as residues in milk, proving specific for the determination of the drug in confirmatory analysis.

References

[1] R. Bauditz, Enrofloxacin, clinical evaluation in several animal species, in: Veterinary Pharmacology, Toxicology and Therapy in Food Producing Animals, University of Veterinary Science–Unipharma, Budapest, 1990, p. 21.
[2] C.J. Gilles, R.A. Magonigle, W.T.R. Grinshaw, A.C. Tanner, J.E. Risk, M.J. Lynch, J.R. Rice, J. Vet. Pharmacol. Ther. 14 (1991) 400.
[3] J.W. Spoo, J.E. Riviere, in: H.R. Adams (Ed.), 7th edn, Veterinary Pharmacology and Therapeutics, Iowa State University Press, Ames, IA, 1995, p. 832.
[4] M. Scheer, Vet. Med. Rev. 2 (1987) 90.
[5] J. Prescott, K.H. Yelding, Can. J. Vet. Res. 54 (1990) 195.
[6] European Commission, Regulation 90/2377/EEC, 18 August, Off. J. Eur. Commun. L224 (1990) 55.
[7] European Commission, Regulation 99/508/EEC, 9 March, Off. J. Eur. Commun. L60 (1999) 305.
[8] K. Tyczowska, K.M. Hedeen, D.P. Aucoin, A.L. Aronson, J. Chromatogr. 493 (1989) 337.
[9] K. Flammer, D.P. Aucoin, D.A. Whitt, J. Vet. Pharmacol. Ther. 14 (1991) 359.
[10] K.L. Tyckowska, R.D. Voyksner, K.L. Anderson, M.G. Papich, J. Chromatogr. B 658 (1991) 341.
[11] L. Kaartinen, M. Salonen, L. Alli, S. Pyorala, J. Vet. Pharmacol. Ther. 18 (1995) 357.
[12] G. Suhren, P. Hammer, W. Heeschen, in: Acta Euroresidue III, Vol. 2, Veldhoven, The Netherlands, 1996, p. 917.
[13] Commission of the European Communities, Draft SANCO/1805/2000 Rev. 1, Laying down performance criteria thereof in live animals and animal products according to Council Directive 96/23/EC.
[14] S.B. Turnipseed, C.C. Walker, J.E. Roybal, A.P. Pfenning, J.A. Hurbult, J. AOAC Int. 81 (1998) 554.