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ORIGINAL RESEARCH

Simultaneous determination of enrofloxacin and its primary metabolite, ciprofloxacin, in plasma by HPLC with fluorescence detection

M. A. Garcia,[1]* C. Solans,[1] J. J. Aramayona,[2] S. Rueda,[2] M. A. Bregante[2] and A. de Jong[3]

[1]Department of Analytical Chemistry, Veterinary Faculty, University of Zaragoza, Miguel Servet 177, 50013 Zaragoza, Spain
[2]Department of Pharmacology and Physiology, Veterinary Faculty, University of Zaragoza, Miguel Servet 177, 50013 Zaragoza, Spain
[3]Bayer AG, Business Unit Animal Health, D-51368 Leverkusen, Germany

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ABSTRACT: A simple and sensitive HPLC method has been developed for the simultaneous determination of enrofloxacin (ENR) and ciprofloxacin (CIP) in plasma. Plasma sample preparation was carried out by adding phosphate buffer (pH 7.4, 0.1 M), followed by extraction with trichloromethane. ENR, CIP and the internal standard, sarafloxacin (SAR), were separated on a reversed-phase column, and eluted with aqueous acetonitrile (80:20). The fluorescence of the column effluent was monitorized at λ_{ex} 338 and λ_{em} 425 nm. The retention times were 2.28, 3.30 and 4.40 min for CIP, ENR and SAR, respectively. The detection limit for the two compounds was 10 ng/mL. Standard curves were linearly related to concentration in the range from 1 to 1500 ng/mL. The recovery was 93% for ENR and 75% for CIP. Copyright © 1999 John Wiley & Sons, Ltd.

INTRODUCTION

Enrofloxacin (ENR), 1-cyclopropyl-7-(4-ethyl-1-piperazinyl)-6-fluoro-1,4-dihydro-4-oxo-3-quinoline carboxylic acid (Fig. 1), is a fluoroquinolone antimicrobial which displays a wide antibacterial spectrum (Bauditz, 1990; Semjen *et al.*, 1990). The pharmacokinetics and metabolism of ENR have been extensively studied in animal species (Abadia *et al.*, 1994; Aramayona *et al.*, 1994; Muñoz *et al.*, 1996; Aramayona *et al.*, 1996). In some species, it is de-ethylated to its primary metabolite, ciprofloxacin (CIP), which also displays a potent antimicrobial activity (Brown, 1996).

Several microbiological assays have been developed for the determination of antimicrobial activity after administraction of ENR or CIP (Wise and Donovan, 1987; Walker *et al.*, 1992; Meinen *et al.*, 1995). However, discrepancies between microbiological and chromatographic assays have been observed (Wingender *et al.*, 1984; Joos *et al.*, 1985). In general, findings obtained after a microbiological assay show higher drug levels than those obtained when chromatographic methods are used. These discrepancies have been attributed to the presence of antimicrobial activity by active metabolites. For this reason, liquid chromatography appears to be the most specific and selective method for the simultaneous determination of ENR and CIP and is recommended for pharmacokinetic studies.

The simultaneous determination of ENR and CIP has been described many times, but always with some clear

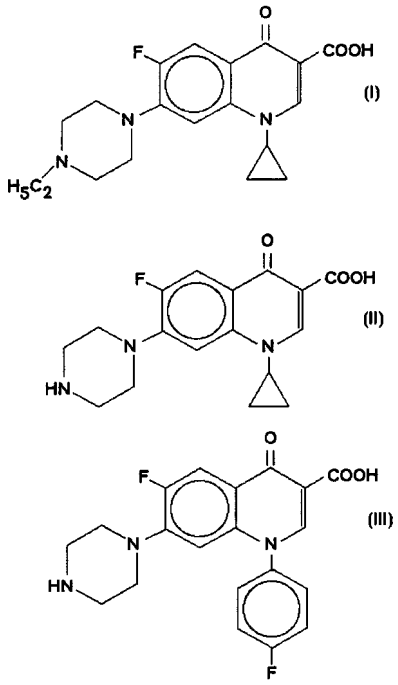

Figure 1. Chemical structure of (**I**) enrofloxacin, (**II**) ciprofloxacin and (**III**) sarafloxacin.

*Correspondence to: M. A. Garcia, Department of Analytical Chemistry, Vetinary Faculty, University of Zaragoza, Miguel Servet 177, 50013 Zaragoza, Spain.

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Abbreviations used: CIP, ciprofloxacin; ENR, enrofloxacin; PBS, phosphate buffered saline; SAR, sarafloxacin.

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shortcomings, such as the use of an external standard (Tyczkowska *et al.*, 1989; Tarbin *et al.*, 1992; Tyczkowska *et al.*, 1994, Fraile *et al.*, 1997), time-consuming sample preparation (Tyczkowska *et al.*, 1989; Tarbin *et al.*, 1992; Tyczkowska *et al.*, 1994) or prolonged elution times (Tyczkowska *et al.*, 1994). Finally, Küng *et al.* 1993 have reported an analytical method for the simultaneous determination of ENR and CIP with an internal standard; this method is expensive because of the ultrafiltration technique used during the extraction procedure.

Our objective has been to develop a sensitive, rapid and economical HPLC method with fluorescence detection and an internal standard for the simultaneous determination of ENR and its primary metabolite CIP in plasma.

EXPERIMENTAL

Chemicals and reagents. ENR, CIP and the internal standard, sarafloxacin (SAR), were supplied by Bayer AG (Leverkusen, Germany). Acetonitrile and trichloromethane were obtained from Riedel-deHaën AG (Seelze, Germany). The ion-pairing reagent, tetraethylammonium bromide, was obtained from Sigma Chemical Co. (St Louis, USA). Orthophosphoric acid, disodium hydrogenophosphate and potasium dihydrogenophosphate were purchased from Scharlau (Barcelona, Spain). The water was HPLC grade.

Standard solutions. Stock solutions of ENR, CIP and SAR were prepared in water (0.1 mg/mL). These solutions were spiked in drug-free plasma rabbit samples to determine recovery, precision, accuracy and detection limit. All standards were protected from the light with aluminium foil and kept at 4°C until used.

Sample preparation. Aliquots (200 μL) of plasma samples were diluted with 800 μL of 0.1M phosphate buffer pH 7.4 containing 1500 ng/mL of SAR as the internal standard. After adding 6 mL of triclhoromethane, the samples were shaken at 200 oscillations/min for 30 min and centrifuged at 13,000 ***g*** for 6 min. After removing the aqueous layer, the organic layer was transferred into a fresh tube and dried at 40°C under nitrogen stream. The residue was dissolved in 200 μL of phosphate buffered saline (PBS) and an aliquot was injected into the chromatographic system.

HPLC system. A Waters 501 HPLC pump and a Waters M717 autosampler were employed. Separations were performed on a Novapack C-18 (150 × 3.9 mm i.d.) reversed-phase column packed with 5 μm particles. A Novapak C-18 precolumn from Guard-Pack inserts was used between the injector and the analytical column to effectively minimize the accumulation of particle matter on the analytical column. Both columns were used at room temperature.

The mobile phase consisted of a mixture of acetonitrile and aqueous solution (20:80). The aqueous solutions were prepared by dissolving potassium dihydrogenophosphate (0.020 M), phosphoric acid (0.006 M), and tetraethylammonium bromide (0.012 M) in water. The pH of the mobile phase was adjusted to 3.0 by the addition of 2 M NaOH. The mobile phase was filtered through a 0.45 μm Lida filter, prior to use. The HPLC system was operated isocratically. The effluent flow-rate was 1.0 mL/min. The eluate was continuously monitored using a Waters 420-AC fluorescence detector (λ_{ex} 338 nm and λ_{em} 425 nm). Area integrations, peak height measurements, calculations and the plotting of the chromatograms were all carried out by an Integration pack program (Kontrol Instruments, Spain).

Calibration procedure. The calibration curves were constructed by spiking appropriate volumes of stock solutions of ENR and CIP in glass tubes containing plasma in appropriate amounts, in order to give final concentrations in the range 1–1500 ng/mL. These calibration samples were then taken through the sample preparation procedure described above.

The calibration curve was characterized by its regression coefficient, slope and intercept, and was used to determine the analyte concentrations in the samples and the detection limits. Finally, the sample concentrations were calculated by determining the peak height ratios of ENR and CIP to the internal standard, with these ratios being interpolated in the standard curves obtained for the calibration samples.

Recovery, precision and accuracy. Recoveries were determined by extracting samples containing 150, 750 and 1500 ng/mL ENR and CIP as described in the section on sample preparation (n = 10), followed by the addition of further ENR and CIP to five of them. All samples were analysed, and the ratios of ENR and CIP to the internal standard for the two sets of samples were then compared.

The precision (inter- and intra-day) of the method was calculated at three concentrations (150, 750 and 1500 ng/mL). The variability in the peak height ratios at each concentration was determined as an indicator of the precision of the assay. The accuracy was determined by comparing the measured concentration with its true value.

RESULTS AND DISCUSSION

Analytical results

ENR and CIP exhibit ionic properties in the whole range of pH values, due to the presence of two ionizable groups in these molecules (ENR pK_1 = 6.1 and pK_2 = 7.8; CIP pK_1 = 6.0 and pK_2 = 8.8). Therefore, ion-paired reversed-phase chromatography was used to improve the separation from other observable plasma components.

The extraction recoveries of ENR and CIP from the control plasma samples for our method are given in Table 1. Mean recoveries obtained were 93% and 75% for ENR and CIP, respectively. The lower recovery of CIP should be attributed to adsorption phenomena and, in particular, to the high aqueous solubility of CIP.

Other methods for sample preparation, such as deproteinization by trichloroacetic acid or acetonitrile, were investigated during the development of the method.

Table 1. Extraction recoveries for enrofloxacin and ciprofloxacin from plasma samples

	Recovery (%) (mean ± SD, $n = 10$)	
Concentration (ng/mL)	Enrofloxacin	Ciprofloxacin
150	93.5 ± 5.0	79.4 ± 5.3
750	95.0 ± 6.3	75.3 ± 2.6
1500	92.5 ± 3.6	71.5 ± 2.8
Mean ± SD	93.7 ± 1.3	75.4 ± 3.9

The results showed that recovery and accuracy were better with the method described in this paper than with other methods. This extraction method also presents a significant improvement with respect to previous methods (Vallée *et al.*, 1986; Teja-Isavadharm *et al.*, 1991; Hormazabal and Yndestad, 1994; Tyczkowska *et al.*, 1994).

In our work, the optimal mobile phase was found to consist of a mixture of acetonitrile and aqueous solutions containing the ion-paired reagent (20:80). When the proportion of aqueous solution or tetraethylammonium bromide to acetonitrile was increased, the separation of the compounds was improved, but the sensitivity was impaired. Other ion-pair reagents, such as heptane sulphonate sodium, were also investigated, but longer elution times were required.

Ideally, an internal standard should display similar physico-chemical properties to the analyte. For this reason norfloxacin, marbofloxacin, orbifloxacin and sarafloxacin (all of them fluoroquinolones with very similar chemical structures to ENR and CIP) were investigated as the internal standard. The best results were found for sarafloxacin, which could be efficiently extracted from plasma samples, and whose retention time allowed for a correct separation of ENR and CIP.

The column temperature was not standarized, but remained at around 25°C. Retention times were 2.28, 3.30 and 4.40 min for CIP, ENR and SAR, respectively, and were not affected by the small temperature changes observed in the column. No interfering peaks appeared at these retention times (Fig. 2). All compounds were eluted as separate symmetric peaks. In addition, the resulting run time was suitable for processing numerous samples on a daily basis.

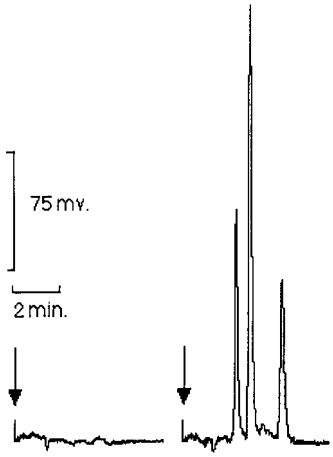

Figure 2. Typical chromatograms for enrofloxacin, ciprofloxacin and sarafloxacin. (a) Blank plasma. (b) Blank plasma spiked with 1500 ng/mL of enrofloxacin, ciprofloxacin and sarafloxacin.

The ENR and CIP standard curves were linear over the range from 1 to 1500 ng/mL ($r^2 = 0.999$ for both drugs, $n = 10$). Furthermore, the detection limit (calculated by the Miller and Miller method, 1993) was 10 ng/mL for both compounds. This linearity range will permit the use of this method for pharmacokinetic studies of these drugs. The coefficient of variation (inter- and intra-day) was 10% for both curves. The precision and accuracy results for the proposed method are summarized in Table 2. In all instances, the accuracy and precision showed satisfactory levels.

In conclusion, this method presents a marked improvement when compared with previous studies (Tyczkowska *et al.*, 1989; Rogstad *et al.*, 1991; Pou *et al.*, 1991; Tyczkowska *et al.*, 1994).

CONCLUSIONS

We have developed a specific, rapid and economical

Table 2. Accuracy and precision of enrofloxacin and ciprofloxacin

Analyte	Concentration added (ng/mL)	Concentration found (ng/mL)[a]	Accuracy (%)	Precision CV (%)[b]
Enrofloxacin	150	138 ± 8	92.0	8.0 (5.2)
	750	787 ± 54	104.9	5.9 (6.0)
	1500	1441 ± 25	96.1	3.4 (2.3)
Ciprofloxacin	150	150 ± 9	100.0	6.0 (8.5)
	750	758 ± 38	101.1	5.1 (1.7)
	1500	1520 ± 20	101.3	1.3 (2.0)

[a] Each value represents the mean of six independent determinations.
[b] The intra-assay variations were the mean coefficients of variance (CV) of the peak height ratios calculated on day of analysis ($n = 6$), whereas the inter-assay variations were calculated using the mean peak height ratios obtained during each day of analysis. Inter-assay variations appear in parentheses.

HPLC method for the simultaneous determination of ENR and its primary metabolite, CIP, in plasma. The assay involves a simple extraction procedure followed by separation on a reversed-phase column using an internal standard, and fluorescence detection. In addition, the resulting run time was suitable for processing numerous samples on a daily basis.

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