Determination of Orbifloxacin in Rabbit Plasma by High-Performance Liquid Chromatography with Fluorescence Detection

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Abstract

A simple and sensitive high-performance liquid chromatography method is developed for the determination of orbifloxacin (ORB) in rabbit plasma. Sample preparations are carried out by adding phosphate buffer (pH 7.4, 0.1M) and extracting with trichloromethane. ORB and the internal standard, norfloxacin (NOR), are separated on a reversed-phase column using an aqueous phosphate buffer–acetonitrile (80:20, v/v) mobile phase. The concentrations of ORB and NOR eluting from the column with retention times of 2.16 and 3.09 min, respectively, are monitored by fluorescence detection at 338 (excitation) and 425 nm (emission). The method is shown to be linear from 4 to 1500 ng/mL (regression coefficient r^2 = 0.999). The quantitation and detection limits are 4 and 9 ng/mL, respectively. Mean recovery is determined as 92% by the analysis of plasma standards containing 150, 750, and 1500 ng/mL. Inter- and intra-assay precisions were 4 and 3%, respectively.

Introduction

In the last decade, we have witnessed the development and manufacture of a large number of fluoroquinolone antimicrobial drugs for treating a variety of bacterial infections in both humans (1,2) and animals (3–6). These agents work through the inhibition of DNA gyrase, interfering with the supercoiling of antibacterial chromosomal material (7). These agents are being used as bacterials primarily against gram-negative bacteria, microplasma, and some gram-positive bacteria. They have little or no activity against group streptococci and anaerobic bacteria (4,8).

1-cyclopropyl-5,6,8-trifluoro-1,4-dihidro-7-(*cis*-3,5-dimethyl-1-piperazinyl)-4-oxoquinoline-3-carboxylic acid, orbifloxacin (ORB), is a new antibacterial fluoroquinolone agent (Figure 1) under development for use in veterinary medicine. It has broad and potent antibacterial activities against gram-negative and gram-positive bacteria, anaerobics, and mycoplasma. Moreover, it's in vivo activity is generally higher than other antibiotics such as oxytetracycline, kanamycine, and ampicillin (9). Until now, ORB has only been used experimentally in veterinary medicine on dogs and cats in circumstances where other agents (such as cephalexin, thiamphenicol, and kanamycin) could not be given because they did not show activity (10).

Recently, some analytical methods have been reported for the determination of ORB in aqueous solution by high-performance liquid chromatography (HPLC) with ultraviolet detection (11–13). To our knowledge, no information has yet been reported on assays of ORB from biological samples. Thus, in this paper, the objective was to develop and validate a simple, specific, rapid, and sensitive method for the determination of ORB in rabbit plasma using HPLC with fluorescence detection. This analytical procedure will permit the routine analysis of plasma samples in future pharmacokinetic and clinical studies.

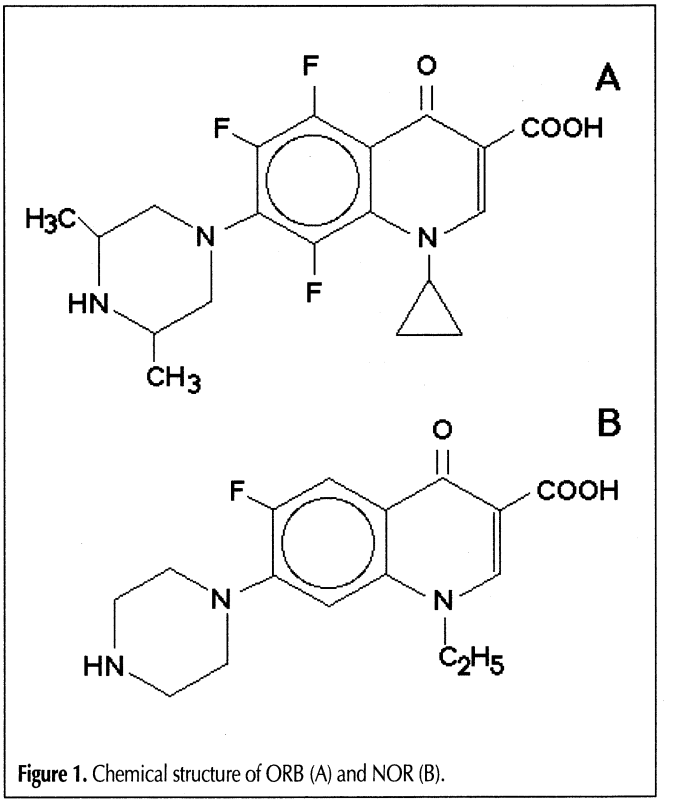

Figure 1. Chemical structure of ORB (A) and NOR (B).

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Experimental

Laboratory precautions

All experiments were carried out in a darkroom using FGS 602N2 sodium vapour lamps and SOX-55 55-W lamps (Philips, Madrid, Spain) in order to prevent ORB photodegradation.

Chemicals and reagents

ORB and the internal standard, norfloxacin (NOR), were received as a gift. Acetonitrile and trichloromethane were obtained from Riedel-deHaín (Seelze, Germany). The ion-pairing reagent tetraethylammonium bromide was obtained from Sigma (Madrid, Spain). Orthophosphoric, disodium hydrogenophosphate, and potassium dihydrogenophosphate were purchased from Scharlau (Barcelona, Spain). The water was HPLC grade.

Standard solutions

Stock solutions of ORB and NOR were prepared in water (0.1 mg/mL). Drug-free rabbit plasma samples were spiked with these solutions to determine the recovery, precision, accuracy, and quantitation and detection limits. All standards were protected from light with aluminum foil and kept at 4°C until they were used.

Sample preparations

Aliquots (200 µL) of plasma samples from rabbits were diluted with 800 µL of 0.1M phosphate buffer (pH 7.4) containing 1500 ng/mL of NOR as an internal standard. After adding 6 mL of trichloromethane, 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 a stream of nitrogen. The residue was dissolved in 200 µL of phosphate buffered saline (PBS), and an aliquot was injected into the HPLC system.

Table I. Extraction Recovery for ORB from Plasma Samples

Concentration (ng/mL)	Recovery* (%)
150	93 ± 4.0
750	94 ± 3.0
1500	90 ± 4.6
Mean ± standard deviation	92 ± 2.0

* Each value represents the mean of 10 independent determinations.

HPLC system

A Waters (Barcelona, Spain) 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 preceded by a Novapack C_{18} HPLC precolumn from Guard-Pak (Waters). Both columns were used at room temperature (approximately 25°C).

The mobile phase consisted of a mixture of acetonitrile and phosphate buffer solution (20:80). The buffer solution was prepared by dissolving potassium dihydrogenphosphate (0.020M), phosphoric acid (0.006M), and tetraethylammonium bromide (0.012M) in water. The pH of the mobile phase was adjusted to 3.0 by the addition of 2N NaOH and filtered through a 0.45-µm Lida filter (Waters). The HPLC system was operated isocratically at a flow rate of 1.0 mL/min. The eluate was continuously monitored by a Waters 420-AC fluorescence detector (excitiation λ_{ex}, 338 nm; emission 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, Milan, Italy).

Calibration procedure

Calibration curves were constructed by adding appropriate volumes of stock solutions of ORB into glass centrifuge tubes containing plasma in appropriate amounts in order to give a final concentration in the range 4–1500 ng/mL (n = 10). These calibration samples were then taken through the sample preparation procedure described previously.

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. Final sample concentrations were calculated by determining the peak height ratios of ORB to internal standard, and these ratios were then 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 ORB as described in the section on sample preparation (n = 10) and adding more ORB to 5 of them. All samples were analyzed, and the ratio of ORB to internal standard for the 2 sets of samples was then compared.

The precision (inter- and intraday) of the method was calculated at 3 concentrations (150, 750, and 1500 ng/mL). The variability in the peak height ratios at each concentration was determined as the precision of the assay. The accuracy was determined by comparing the measured concentration with the added concentration (n = 6).

Table II. Accuracy and Precision of the HPLC Method for the Determination of ORB

Analyte	Concentration added (ng/mL)	Concentration found* (ng/mL)	Accuracy (%)	Precision† (coefficient of variation, %)
Orbifloxacin	150	157 ± 37	105	4.7 (5.2)
	750	715 ± 73	95	4.7 (5.0)
	1500	1515 ± 25	101	1.0 (2.0)

* Each value represents the mean of 6 independent determinations.
† The intrassay variations were the mean coefficient of variation of the peak height ratios calculated on the day of analysis (n = 6), whereas the interassay variations were calculated using the mean peak height ratios obtained on each day of analysis. Interassay variations appear in parentheses.

Interferences

In order to study the selectivity of the method,

the interferences of ORB with other fluoroquinolones were carried out. For this reason, other fluoroquinolones such as marbofloxacin, ciprofloxacin, danofloxacin, enrofloxacin, sarafloxacin, and difloxacin were spiked (1500 ng/mL) in plasma samples containing ORB. These samples were prepared as previously described and injected into the HPLC system.

Results and Discussion

Analytical results

ORB exhibits ionic properties throughout the whole range of pH because of the presence of 2 ionizable groups in its molecule. Therefore, ion-paired reversed-phase chromatography was used to enhance the separation from other observable plasma components.

Table I shows the extraction recovery of ORB from plasma control samples. A recovery of 92% has been found when the extraction procedure using trichloromethane has been followed. Other methods for sample preparations, such as deproteinization by trichloroacetic acid or acetonitrile, were investigated during the development of the method. The results showed that recovery or accuracy were better when trichloromethane was used than when deproteinization was carried out by other methods. Unfortunately, the results could not be compared because, to our knowledge, assays of ORB in biological samples have not been reported. However, the analytical results of other fluoroquinolones from biological samples have been reported, showing that the recoveries ranged from 60 to 100% (14–16).

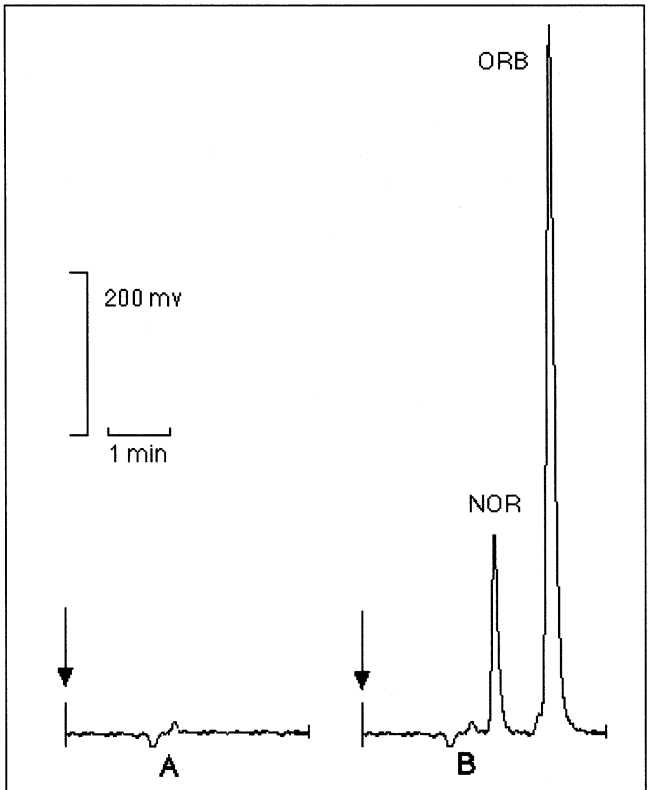

Figure 2. Typical chromatograms for ORB and NOR: blank plasma (A) and blank plasma spiked with 1500 ng/mL of ORB and NOR (B).

Table III. Study of Interferences

Analyte	Retention time (min)
Norfloxacin	2.16
Marbofloxacin	2.20
Ciprofloxacin	2.28
Danofloxacin	2.80
Orbifloxacin	3.09
Enrofloxacin	3.30
Sarafloxacin	4.40
Difloxacin	4.52

In our work, a mixture of acetonitrile and aqueous solutions containing the ion-paired reagent (20:80) was found to be the optimal mobile phase. When the proportion of aqueous solution or tetraetylammonium to acetonitrile was increased, the separation of the ORB and NOR was improved, but the sensitivity was impaired. Other ion-pair reagents, such as heptane sulfonate sodium, were also investigated, but longer elution times were required.

Ideally, an internal standard should display similar physicochemical properties to the analyte. For this reason, norfloxacin, marbofloxacin, enrofloxacin, and sarafloxacin (all having chemical structures very similar to ORB) were investigated as internal standards. The best results were found for norfloxacin, which could be efficiently extracted from plasma samples and whose retention time allowed for a correct separation of ORB.

The column temperature was not controlled but remained at room temperature (approximately 25°C). Retention times were 2.16 and 3.09 min for NOR and ORB, respectively, and were not affected by the small changes observed in the column temperature. No interfering peaks appeared at these retention times (Figure 2). In addition, the resulting run time was suitable for processing numerous samples on a daily basis.

The ORB standard curve was linear over the range 4–1500 ng/mL (regression coefficient $r^2 = 0.999$, $n = 10$). Furthermore, the quantitation and detection limits (calculated by the Miller method) (17) were 4 and 9 ng/mL, respectively. This linearity range will permit the use of this method in future pharmacokinetic studies or for the therapeutic monitoring of this drug. The coefficient of variation (inter- and intraday) was 4 and 3%, respectively. The precision and accuracy results for the proposed method are summarized in Table II. In all instances, the accuracy and precision were satisfactory.

Interferences

Table III shows the retention times of the different fluoroquinolones that were studied in the interference analysis. As can be observed, when these fluoroquinolones and ORB were injected at the same time, no interferences were detected for sarafloxacin, difloxacin, ciprofloxacin, and marbofloxacin.

Conclusion

We have developed a specific, rapid, and economic HPLC method for the determination of ORB in plasma. The assay

involves a simple extraction procedure followed by separation on a reversed-phase column using an internal standard and fluorescence detection. The accuracy, precision, recovery, and detection limit of this analytical method will permit it to be used in pharmacokinetic or clinical studies. In addition, the resulting time was suitable for processing numerous samples on a daily basis.

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