Development of a Method for the Determination of Ibafloxacin in Plasma by HPLC with Fluorescence Detection and Its Application to a Pharmacokinetic Study

Pedro Marín1, Carlos M. Cárceles1, Elisa Escudero1, Ruperto Bermejo2, and Emilio Fernández-Varón1,*

1Department of Pharmacology, Faculty of Veterinary Medicine, University of Murcia, Campus de Espinardo, 30.071-Murcia, Spain and
2Department of Physical and Analytical Chemistry, University of Jaen. EUP Linares, 23700, Jaen, Spain

Abstract

A simple, rapid, and sensitive high-performance liquid chromatographic method is developed for the determination of ibafloxacin in rabbit plasma. Plasma proteins are precipitated with acetonitrile, and after extraction with methylene chloride followed by desiccation, ibafloxacin is determined by reversed-phase chromatography with fluorescence detection exciting at 330 nm and emission at 368 nm. Peaks corresponding to ibafloxacin and the internal standard (salicylic acid) are obtained at 9.8 and 5.2 min, respectively. The method is validated for a limit of quantitation of 10 ng/mL. The intraday relative standard deviation ranges from 4.78–7.15%, and the interday precision ranges from 1.32–4.03%. The method shows linearity for the two calibration curves used (10–100 ng/mL and 100–2000 ng/mL). The procedure described is applied successfully to a pharmacokinetics study of ibafloxacin in rabbits.

Introduction

In recent years, a high number of new fluorquinolones have been developed for use worldwide, and a wide variety of determination methods for these drugs have been reported (1–5). Ibafloxacin (6,7-dihydro-9-fluoro-5,7-dimethyl-1-oxo-1H, 5H-benzo-[i,j]-quinolizine-2-carboxylic acid) (Figure 1) is a new fluorquinolone antimicrobial agent that has been developed for veterinary use. As other fluorquinolones, it binds to DNA and inhibits the topoisomerase enzyme, DNA gyrase interfering supercoiling of DNA. Ibafloxacin shows a broad bactericidal activity against gram-negative and gram-positive aerobic bacteria as well as mycoplasmas (6). Currently, no specific high-performance liquid chromatography (HPLC) method reports on the determination of ibafloxacin in plasma. One study (6) reported pharmacokinetics of this drug in dogs using an HPLC method with fluorescence detection, and the same authors (7) reported pharmacokinetics of ibafloxacin in cats, using microbiological assay, liquid chromatography tandem mass spectrometry assay, and capillary zone electrophoresis. As in the case of other fluorquinolones after their introduction in market, the pharmacokinetics of ibafloxacin will be studied in other animal species. Therefore, it is of interest to propose a reference method for its determination. The aim of the present study was to propose a simple, isocratic, and rapid method for the quantitation of ibafloxacin in rabbit plasma using fluorescence detection and to test its application in a pharmacokinetics study.

Materials and Methods

Reagents

Ibafloxacin was kindly provided by Intervet Pharma (Salamanca, Spain). Chemicals used were analytical reagent grade. Acetonitrile and water for HPLC was from Scharlau (Barcelona, Spain). Salicylic acid, sodium phosphate dibasic,
potassium dihydrogenophosphate, tetrabutylammonium hydrogen-sulphate, and methylene chloride were purchased by Sigma-Aldrich (Madrid, Spain). Drug free plasma was obtained from health rabbits. For the interference study, danofloxacin (Pfizer, Sandwich, UK), difloxacin (Fort-dodge, Madrid, Spain), moxifloxacin (Bayer AG, Wuppertal, Germany), Ciprofloxacin (Vita Lab, Madrid, Spain), and enrofloxacin (Bayer AG, Leverkusen, Germany) were used.

**Standard solutions**
Ibafloxacin standard solutions (20 mg/L) were prepared by dissolving ibafloxacin in methanol. Quality control (QC) samples were prepared from the stock solution of ibafloxacin by dilution with blank rabbit plasma. Calibration standards were prepared by addition of 50 µL of each standard solution to 950 µL of drug-free plasma.

**Chromatographic system and conditions**
The HPLC system was equipped with a model LC-10Asvp pump, a RF-10Axl Fluorescence Detector, and a model SIL-10Advp auto-injector (Shimadzu, Kyoto, Japan). The previously mentioned system was connected to a computer with a Shimadzu Class-VP chromatography data system program (Shimadzu, Columbia, MD).

The HPLC separation was performed using a reverse-phase Discovery C18 column, 150 × 4.6 mm, 5-µm particle size (Supelco, Bellefonte). Autosampler vials and column temperature was set at 5°C. The mobile phase consisted of acetonitrile (40%) and tetrabutylammonium hydrogen sulphate solution (5 g/L) (60%) (pH = 3.5) using an isocratic form with a flow rate of 1.0 mL/min. The fluorescence detection was performed at an excitation wavelength of 330 nm and an emission wavelength of 368 nm.

**Sample preparation**
After addition of 10 µL of the internal standard solution, 1 mL acetonitrile was added to 500 µL of plasma. Plasma proteins were precipitated by shaking in an ultrasonic bath for 5 min, followed by centrifugation for 10 min at 1600 × g. Supernatant was extracted, and 3 mL of methylene chloride were added. The mixture was mixed and centrifuged for 5 min at 1600 × g. The aqueous phase was removed, and the organic phase was subjected to desiccation. The residue was dissolved with 50 µL of 0.06M disodium hydrogen phosphate buffer (pH = 7.5) and transferred to HPLC autosampler vials.

**Linearity**
Quantitation of ibafloxacin was performed from two calibration curves for the low concentration calibration range (10–100 ng/mL) and for the high concentration calibration range (100–2000 ng/mL). The calibration curve was defined by its regression coefficient, slope, and intercept. The concentration of ibafloxacin was determined using five calibration curves from both a low and high range of concentrations on five different days. Analysis was based on the peak area ratios of ibafloxacin to the internal standard. Analysis was performed using the SPSS statistic program (Chicago, IL).

**Recovery**
The absolute recovery from plasma was measured at three concentrations (50, 250, 1000 µg/L, see Table I) by comparing drug peak area of the spiked analyte samples (n = 5) to unextracted analyte of stock solution, which was injected directly into HPLC system. Recovery (%) was computed as: (mean peak area spiked plasma / mean peak area spiked extract) × 100.

**Precision and accuracy**
The intraday precision and accuracy of assays performed in replicate (n = 5) were tested using 3 concentrations of ibafloxacin (50, 500, and 1000 ng/mL). The between-day precision and accuracy of the assay were evaluated from five replicate assays on five different days. Precision was evaluated by the relative standard deviation (RSD) of the measured concentration respective to the true value.

**Limit of detection and quantitation**
The limit of detection (LOD) of ibafloxacin was established as the concentration that provides a signal to noise ratio of 3. The limit of quantitation (LOQ) was accepted as the lowest concentration on the calibration curve that can be determined with acceptable precision (coefficient of variation % < 20) (8).

**Animals**
Six New Zealand white rabbits of both sexes, weighing between 3.2–4.5 kg, were collected from the Laboratory Animal Farm of the University of Murcia. The rabbits were determined to be clinically healthy before the study, based on physical and blood analytical examination.

**Experimental design**
Ibafloxacin was administered by intravenous route at single doses of 15 mg/kg bodyweight. Blood samples were collected at 0, 5, 10, 15, 30, 45 min and 1, 1.5, 2, 4, 6, 8, 10, 12, 24, 32, and 72 h following drug administration. Blood samples (1 mL) were obtained by inserting a 20-gauge needle into the marginal ear vein, and allowing the blood drip into a 2-mL syringe, after which it was placed in a tube. Samples were centrifuged at 1500 × g for 15 min within 10 min after collection. Plasma was immediately removed and stored at −45°C until assayed.

**Pharmacokinetic analysis**
A non-compartmental model was used to determine the area under the concentration-time curve (AUC) using the linear trapezoidal rule with extrapolation to infinity. Mean residence time was calculated as MRT = AUMC/AUC. The systemic clearance as Cl = Dose/AUC. The apparent volume of distribution (area method) and apparent volume of distribution at steady state were calculated as Vz = Dose / (AUC · λz) and Vss = (Dose · λz)/Cl.

<table>
<thead>
<tr>
<th>Table I. Mean Absolute Recovery of Ibafloxacin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nominal concentration (µg/L)</strong></td>
</tr>
<tr>
<td>50</td>
</tr>
<tr>
<td>250</td>
</tr>
<tr>
<td>1000</td>
</tr>
</tbody>
</table>
AUC1/AUC2, respectively. The pharmacokinetic analysis was performed with the WinNonlin computer program (Version 3.1, Pharmasight, Mountainview, CA).

Results and Discussion

Linearity and LOQ

The calibration curves showed a linear response over the concentration range of 10–100 and 100–2000 µg/L for both calibration curves used in this assay. The mean calibration curve was 

\[
y = 0.000187 \pm 0.00 \times - 7.5476 \pm 2.25 \quad (n = 5 \text{ days}) \quad (r^2 > 0.9970). 
\]

The 100–2000 ng/mL calibration range was 

\[
y = 0.0002246 \pm 0.00 \times + 5.7332 \pm 5.38 \quad (r^2 > 0.99).
\]

The lowest concentration of these calibration curves representing the LOQ was 10 µg/L, with an RSD of 9.83% (n = 5). The LOD was 5 µg/L (signal to noise > 3).

Precision

The intra-day repeatability was determined over one day with repeated analysis (n = 5) at three different concentrations of each drug with an intraday RSD lower than 8% (Table II). The interday reproducibility was determined over a period of 5 days and was approximately 4% (Table III).

Chromatographic separation

The peak corresponding to ibafloxacin and internal standard were obtained at 9.8 and 5.2 min, respectively (Figure 2). The endogeneous compounds did not interfere with ibafloxacin at the retention time. The retention time was checked with two lengths of reversed-phase column (250 and 150 mm), and the best results were shown with the 150 × 4.6-mm column. In order to achieve better resolution of ibafloxacin, the effects of composition of the mobile phase was investigated (9,10). The better separation was obtained when the mobile phase was composed by tetrabutylammonium hydrogen sulphate solution (15mM) and acetonitrile (60:40, v/v) pH = 3.5. Tetrabutylammonium hydrogen sulphate was used as an ion-pairing agent in order to reduce peak tailing, the pH range (2.5–4) was studied for obtaining better results. The retention time of ibafloxacin was prolonged, decreasing the acetonitrile concentration, always using an isocratic method, which was the objective of the study.

Otherwise, different methods had been reported for the extraction of ofluorquinolones from biological samples with direct solvent extraction as well as methods involving protein precipitation with several precipitants (for fluorquinolones in plasma, protein precipitation with an organic solvent is the most often used method) (2). In this case, acetonitrile was used as the protein precipitation agent under basic conditions. Acetonitrile showed a good effectiveness in protein precipitation. The 1:2 plasma–acetonitrile ratio used was sufficient to achieve a good absolute recovery. One study (11) reported that a 1:2 ratio (plasma–acetonitrile) was sufficient to remove 99.4% of the proteins. Absolute recovery values evaluated (Table I) ranged from 88–105%, showing that the procedure was suitable for the determination of ibafloxacin in plasma.

Interference study

Interference of ibafloxacin with other fluorquinolones using the present method was studied. Danofloxacin, difloxacin, moxifloxacin, enrofloxacin, and ciprofloxacin were not detected using the conditions described, perhaps because \( \lambda_{ex} \) and \( \lambda_{em} \) of these fluorquinolones was aproximately 300 nm and 500 nm, respectively, and fluorescence with exciting at 330 nm and emission at 368 nm (ibafloxacin) was nonexistent. Exciting and emission of these five fluorquinolones (danofloxacin, difloxacin, enrofloxacin, ciprofloxacin, and moxifloxacin) were extracted from other studies (12, 24).
Therefore, none of these fluoroquinolones could be used as the internal standard for ibafloxacin analysis. Ibafloxacin has a single asymmetric center at the C5 position of the molecule; therefore, it has two enantiomeric forms (R- and S-).

Ibafloxacin has been marketed as its racemic mixture of R- and S-ibafloxacin (14), and pharmacokinetics studies of this drug, up to date, have been performed measuring the racemic mixture, which is of interest from the pharmacokinetic point of view. A non-stereospecific method has been proposed in order to study the pharmacokinetic of ibafloxacin as its racemic mixture; however, further studies should be developed to separate both isomers.

**Pharmacokinetics study**

The mean (± SD) values for pharmacokinetic parameters after IV administration of ibafloxacin are given in Table IV. The mean ± SD plasma ibafloxacin clearance (Cl) for the IV route was 5.83 ± 0.4 L/h × kg. The steady-state volume of distribution (Vss) was 5.09 ± 0.80 L/kg. The terminal half-life (t1/2) was 2.85 h, and the MRT was 1.03 ± 0.14 h. The systemic clearance of ibafloxacin in our study (5.83 L/kg × h) was higher than that for other fluoroquinolones in rabbits, such as difloxacin (0.59 L/kg × h) (15) and moxifloxacin (0.80 L/kg × h) (16). In summary, the method has been successfully applied to this pharmacokinetics study of ibafloxin in rabbits.

**Conclusion**

The HPLC method described in the present study was simple, rapid, sensitive, and reproducible for the determination of ibafloxacin in rabbit plasma, showing good results when it was applied to a pharmacokinetic study. This method allows a large numbers of biological samples to be analyzed in a relatively short period of time.

**Acknowledgments**

Thanks are due to Intervet Pharma for supplying ibafloxin pure substance.

**References**


Manuscript received January 25, 2006; revision received September 25, 2006.