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# Determination of marbofloxacin in plasma samples by high-performance liquid chromatography using fluorescence detection

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**Abstract**

A simple and sensitive HPLC method has been developed for the determination of marbofloxacin (MAR) in plasma. Sample preparations were carried out by adding phosphate buffer (pH 7.4, 0.1 *M*), followed by extraction with trichloromethane. MAR and the internal standard, enrofloxacin (ENR), were separated on a reversed-phase column and eluted with aqueous solution–acetonitrile (80:20). The fluorescence of the column effluent was monitored at $\lambda_{ex}$=338 and $\lambda_{em}$=425 nm. The retention times were 2.20 and 3.30 min for MAR and ENR, respectively. The method was shown to be linear from 15 to 1500 ng/ml ($r^2$=0.999). The detection limit was 15 ng/ml. Mean recovery was determined as 90% by the analysis of plasma standards containing 150, 750, and 1500 ng/ml. Inter- and intra-assay precisions were 3.3% and 2.7%, respectively. 

*Keywords:* Marbofloxacin

## 1. Introduction

Fluoroquinolones have been used for the treatment of a variety of microbial infections in animal and human medicine [1–4]. Marbofloxacin (MAR) (Fig. 1), is a new fluoroquinolone antibacterial drug developed exclusively for use in veterinary medicine [5,6]. It shows a wide spectrum of activity against both gram-negative and gram-positive pathogens and against Mycoplasma spp [7,8].

Microbiological assays have been developed for the determination of antimicrobial activity after administration of fluoroquinolones [9–12]. However, discrepancies between microbiological and chromatographic assays have been observed in the determination of fluoroquinolones. In general, findings obtained after a microbiological assay show higher drug levels than those obtained when chromatographic methods are used [13,14]. 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 determination of fluoroquinolones and is recommended for pharmacokinetic studies [15].

Recently, a number of analytical methods have

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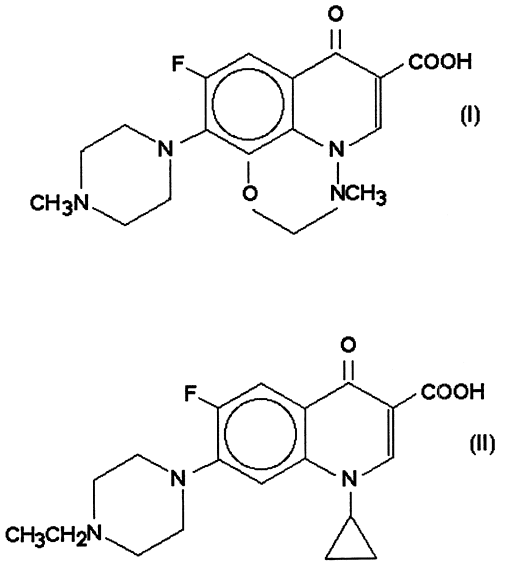


Fig. 1. Chemical structure of (I) marbofloxacin, (II) enrofloxacin.

been reported for the determination of MAR in biological samples by way of high-performance liquid chromatography with ultraviolet detection [5,6]. However, these exhibit some clear shortcomings, such as the use of ultrafiltration techniques during the extraction procedure.

To our knowledge, no information has yet been presented on assays of MAR in plasma samples by HPLC using fluorescence detection. Thus, in this paper our objective has been to develop and validate a simple, specific, rapid, and money-saving method for the determination of MAR in plasma by HPLC using fluorescence detection. In our view this analytical procedure will permit the routine analysis of plasma samples in future pharmacokinetic and clinical studies.

## 2. Experimental

### *2.1. Chemicals and reagents*

MAR and the internal standard, enrofloxacin (ENR), were received as a gift. Acetonitrile and trichloromethane were obtained from Riedel-deHaën AG (Seelze, Germany). The ion-pairing reagent, tetraethylammonium bromide, was obtained from Sigma (St. Louis, MO, USA). Orthophosphoric acid, disodium hydrogenophosphate, and potassium dihydrogenophosphate were purchased from Scharlau (Barcelona, Spain). The water was HPLC grade.

### *2.2. Standard solutions*

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

### *2.3. Extraction procedure*

Aliquots (200 μl) of plasma samples were diluted with 800 μl of 0.1 *M* phosphate buffer (pH 7.4) containing 1500 ng/ml of ENR as the 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 (10–80 μl) was injected into the chromatographic system.

### *2.4. HPLC procedure*

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 was used between the injector and the analytical column to effectively minimise 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 was 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 eluante was continuously monitored by a fluorescence detector ($\lambda_{ex}$=338 nm and $\lambda_{em}$=425 nm) using a Waters 420-AC fluorescence detector. Area integrations, peak height measurements, calculations and the plotting of the chromatograms were all carried out by an Integration pack program (Kontrol Instruments, Spain).

### 2.5. Calibration procedure

The calibration curve was constructed by spiking appropriate volumes of stock solutions of MAR to 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 characterised by its regression coefficient, slope, and intercept, and it 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 ratio of MAR to the internal standard, with these ratios being interpolated in the standard curves obtained for the calibration samples.

### 2.6. Recovery, precision, and accuracy

Recoveries were determined by extracting samples containing 150, 750, and 1500 ng/ml MAR as described in the section on sample preparation ($n$= 10), followed by the addition of further MAR to five of them. All samples were analysed, and the ratios of MAR 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 to its true value.

### 2.7. Interferences

In order to study the selectivity of our method, the interferences of MAR with other fluoroquinolones were carried out. For this reason, other fluoroquinolones, such as danofloxacin, ciprofloxacin, norfloxacin, sarafloxacin, orbifloxacin, and difloxacin, were spiked (1500 ng/ml) in plasma samples containing MAR. These samples were prepared as described earlier and then injected into the HPLC system.

## 3. Results and discussion

### 3.1. Analytical results

MAR exhibits ionic properties in the whole range of pH, due to the presence of two ionisable groups in its molecule. Therefore, ion-paired reversed-phase chromatography was used to enhance the separation from other observable plasma components.

Table 1 shows the extraction recovery of MAR from the control plasma samples. A recovery of 90% has been found when the extraction procedure using trichloromethane was 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. The recovery of other fluoroquinolones from biological samples has been reported, showing that recoveries ranged from 60 to 100% [16–18]. Moreover, our recovery

Table 1
Extraction recoveries for marbofloxacin from plasma samples

| Concentration (ng/ml) | Recovery (%) (mean±SD, $n$=10) Marbofloxacin |
|---|---|
| 150 | 90.0±3.0 |
| 750 | 87.0±1.3 |
| 1500 | 92.0±6.6 |
| Mean±SD | 89.7±2.5 |

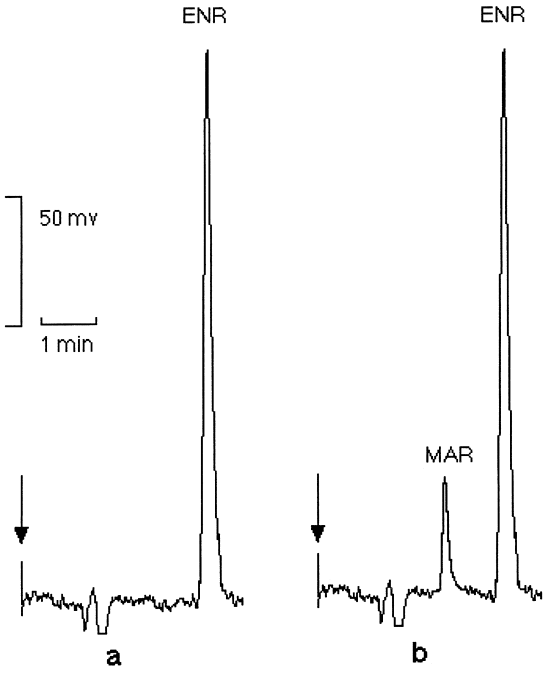


Fig. 2. Typical chromatograms for marbofloxacin and enrofloxacin: (a) blank plasma spiked with 1500 ng/ml of enrofloxacin (injection volume 20 μl); (b) blank plasma spiked with 150 ng/ml of marbofloxacin and 1500 ng/ml of enrofloxacin (injection volume 20 μl).

presents a significant improvement with respect to previous methods for MAR [5,6].

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 tetraethylammonium to acetonitrile was increased, the separation of the MAR and ENR 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, danofloxacin, orbifloxacin, difloxacin, and sarafloxacin (all of them with very similar chemical structures to MAR) were investigated as the internal standards. The best results were found for enrofloxacin, which could be efficiently extracted from plasma samples, and whose retention time allowed for a correct separation of MAR.

The temperature column was not standardized, but remained at around 25°C. Retention times were 2.20 and 3.30 min for MAR and ENR, respectively, and were not affected by the small changes observed in the temperature column. No interfering peaks appeared at these retention times (Fig. 2). Both compounds were eluted as separate symmetric peaks. In addition, the resulting run time was suitable for processing numerous samples on a daily basis.

The MAR standard curve was linear over the range 15–1500 ng/ml ($r^2$=0.999, $n$=10). Furthermore, the detection limit [19] was 15 ng/ml for MAR. Moreover, this linearity range will permit the use of this method in future pharmacokinetic studies and/or for the therapeutic monitoring of this drug. The coefficient of variation (inter- and intra-day) was 3.3 and 2.7%, respectively. The precision and accuracy results for the proposed method are summarised in Table 2. In all instances the accuracy and precision showed satisfactory levels.

### *3.2. Interferences*

Table 3 shows the retention times of the different fluoroquinolones that were studied in the interference analysis. As can be observed, when these fluoroquinolones and MAR were injected at the same time, no interferences were detected for danobofloxacin, orbifloxacin, sarafloxacin, and difloxacin.

Table 2
Accuracy and precision of the HPLC method for the determination of marbofloxacin

| Analyte | Concentration added (ng/ml) | Concentration found (ng/ml)[a] | Accuracy (%) | Precision C.V. (%)[b] |
|---|---|---|---|---|
| Marbofloxacin | 150 | 157±28 | 104.6 | 4.7 (6.0) |
| | 750 | 731±33 | 97.5 | 2.6 (3.0) |
| | 1500 | 1512±135 | 100.8 | 0.8 (1.0) |

[a] Each value represents the mean of six independent determinations.

[b] The intra-assay variations were the mean C.V. of the peak height ratios calculated on the day of analysis ($n$=6), whereas the inter-assay variations were calculated using the mean peak height ratios obtained on each day of analysis. Inter-assay variations appear in parentheses.

Table 3
Study of interferences

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

## 4. Conclusions

We have developed a specific, rapid, and cost-saving HPLC method for the determination of MAR 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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