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High-performance liquid chromatographic determination of rufloxacin and its main active metabolite in biological fluids

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ABSTRACT

A high-performance liquid chromatographic method is described for the determination of a fluoroquinolone, rufloxacin, and its N-desmethyl metabolite in plasma, urine and bile. Samples are chromatographed on a poly(styrene–divinylbenzene) column, the eluate being monitored with a fluorescence detector. The method was validated and a detection limit of 10 ng/ml for both rufloxacin and its metabolite in all the biological matrices considered was found. The method was successfully applied in pharmacokinetic studies.

INTRODUCTION

Rufloxacin, a fluoroquinolone with a broad-spectrum activity [1,2] against Gram-positive and Gram-negative microorganisms [3], is currently undergoing clinical trials and has been observed to have a long half-life [4].

Two analytical methods useful for pharmacokinetic studies have been published [1,5]. One method [1] involves a single liquid–liquid extraction of plasma and urine spiked with an internal standard (I.S.) not structurally related. The other method [5] requires a triple liquid–liquid extraction for plasma and urine purification. Ofloxacin, another fluoroquinolone, is used as I.S. Both purification procedures are followed by a high-performance liquid chromatographic (HPLC) method with spectrophotometric detection. Neither method is suitable for the determination of the main active metabolite.

In order to investigate the pharmacokinetics of rufloxacin and its active N-desmethyl metabolite, we developed a simple HPLC procedure for the simultaneous measurement of the drug and its metabolite in plasma, urine and bile. The method has a sensitivity suitable for the determination of low levels of drug found at the longest sampling times. Compounds of interest are detected by spectrofluorimetry to improve the selectivity and sensitivity.

EXPERIMENTAL

Reagents and chemicals

Rufloxacin hydrochloride (ISF 09334/MF 934; I) and its N-desmethyl metabolite (II) were obtained from Mediolanum Farmaceutici (Milan, Italy). Pipemidic acid was obtained from Dainippon (Osaka, Japan) (Fig. 1). Acetonitrile, tetrahydrofuran (THF), dichloromethane (all of analytical-reagent grade) and 0.2-μm Anotop 10 filters were supplied by E. Merck (Darmstadt, Germany). Triethylamine (TEA) (HPLC grade) was obtained from Pierce (Rockford, IL, USA). Anhydrous disodium hydrogenorthophosphate, monohydrated sodium dihydrogenorthophos-

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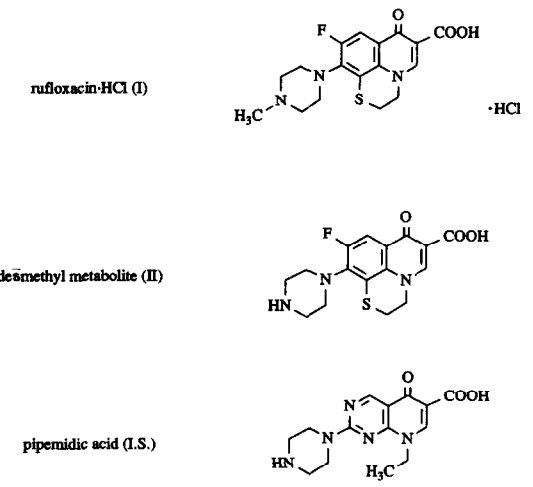

Fig. 1. Structures of rufloxacin hydrochloride (I), its N-desmethyl metabolite (II) and the internal standard pipemidic acid (I.S.).

phate, 85% phosphoric acid and 70% perchloric acid were purchased from Carlo Erba (Milan, Italy).

Internal standard. Pipemidic acid was used as I.S. for the analysis of plasma and extracted urine samples. A stock standard solution was prepared in 0.17% phosphoric acid (1.4 mg/ml). From this stock solution, two working standard solutions (350 μg/ml for addition to plasma samples and 35 μg/ml for addition to urine samples) were prepared daily by dilution in water.

Standard solutions and calibration standards. A stock standard solution of I was prepared in water at a concentration of 1 mg/ml. A stock standard solution of II was prepared in 0.17% phosphoric acid (1 mg/ml). These solutions were then diluted further to yield appropriate working standard solutions. For the simultaneous determination of I and II, calibration standards were prepared by adding small volumes of the corresponding working standard solutions to drug-free plasma, urine and bile. The concentration ranges of plasma and bile calibration standards were 0.05–10 μg/ml for both I and II. Urine standards were prepared within the concentration ranges 2.5–155 μg/ml for I and 2.5–25 μg/ml for II; for the determination of lower levels of I and II, calibration standards within the ranges 0.05–50 μg/ml for I and 0.05–5 μg/ml for II were prepared.

Apparatus

The HPLC system consisted of the following components: Waters M45 pump and WISP 712 autosampler from Waters Assoc. (Milford, MA, USA), Merck–Hitachi F1000 fluorescence detector (band width 15 nm, time constant 1 s) from E. Merck and SP 4290 computing integrator from Spectra-Physics (Darmstadt, Germany).

Chromatographic conditions. The column was a Hamilton 150 mm × 4.1. mm I.D. poly(styrene–divinylbenzene) PRP-1, 10 μm particle size (Carlo Erba), equipped with a 15 mm × 3.2 mm I.D. Polymer RP, 7 μm particle size precolumn (Brownlee Labs., Santa Clara, CA, USA). The excitation and emission wavelengths of the fluorimetric detector were set at 350 and 510 nm, respectively. The mobile phase was 0.17% H_3PO_4–acetonitrile (88:12), adjusted to pH 5.6 with TEA, followed by addition of THF (5 ml/l). The flow-rate was 1.0 ml/min, with a column pressure of about 2000 kPa. The mobile phase was not recycled.

Sample preparation

Frozen samples were allowed to thaw at room temperature and were then homogenized on a vortex mixer.

Plasma. A 500-μl volume of each sample was pipetted into a 1.5-ml microtest-tube and 25 μl of the I.S. working solution and 50 μl of 70% perchloric acid were added. After vortex-mixing (20 s), sonicating (10 min) and centrifuging (5 min, 14 926 *g*), the supernatant was transferred to an autosampler vial (glass-capped, with PTFE septa). An aliquot of 10 μl was injected for analysis.

Urine: dilution procedure. A 1-ml volume of each sample was diluted to 50 ml with water. This was transferred to an autosampler vial and 10 μl were injected for analysis.

Urine: extraction procedure for low-level samples (< 2.5 μg/ml). A 500-μl volume of each sample, 0.5 ml of water, 1 ml of the I.S. working solution and 1.5 g of Na_2HPO_4–NaH_2PO_4 (equimolar, giving a pH of about 6) were mixed in a

centrifuge tube and extracted by horizontal low-speed agitation (40 min) with a 8 ml of dichloromethane. After centrifugation (5 min, 6590 *g*), a 5-ml volume of the organic phase (upper layer) was evaporated under a stream of nitrogen. Samples were reconstituted in 1 ml of the HPLC mobile phase. They were then transferred to an autosampler vial and 10 μl were injected.

Bile. A 500-μl volume of each sample was pipetted into a 1.5-ml microtest-tube and the tubes were centrifuged (15 min, 14 926 *g*). The supernatant was filtered through a 0.2-μm filter, transferred to an autosampler vial and an aliquot of 5 μl was injected.

Calibration

Calibration graphs, determined with three separate replicates of each of five different concentrations of I and II, were prepared for all the matrices considered.

The best linear relationship between concentration and response was determined by a least-squares linear regression ($y=a+bx$, where y = peak area for bile and diluted urine or peak-area ratio for plasma and extracted urine and x = concentration) and linearity was tested by analysis of variance.

On each analysis day, three to five plasma, urine or bile standards, covering the expected concentration range, were carried through the procedure routinely together with the unknown samples.

RESULTS

Under the described chromatographic conditions, the approximate retention times were 3.5 min for the I.S., 8.9 min for I and 7.1 min for II. Slight differences occurred from column to column.

Selectivity

The pH dependence of the retention of I, II and I.S. was investigated in the range 2–7 (Fig. 2). It can be seen that for I the retention is sensitive to pH and this dependence is sigmoidal, whereas I.S. and II are differently influenced by variations in pH. A pH range of 5.6–6.0 was chosen to achieve a suitable separation between I, II and I.S.

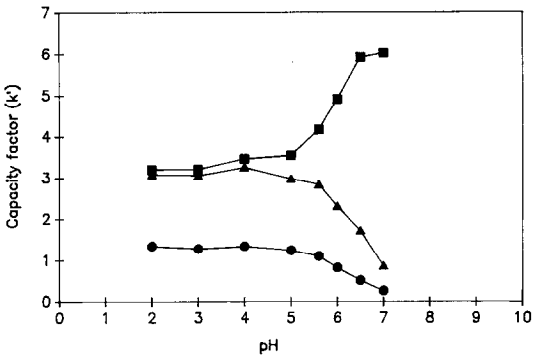

Fig. 2. Plots of the capacity factor of rufloxacin (■), its N-desmethyl metabolite (▲) and the I.S. (●) *versus* the pH of the eluent.

Internal standard

For the determination of I and II in plasma and urine when extraction is required, a structurally related I.S., pipemidic acid, was used. For bile and urine (dilution procedure), the use of an I.S. was not required as I and II are completely recovered.

Linearity and limit of quantification

Under the conditions described, the calibration graphs were linear (at the $p=0.05$ significance level) over the concentration ranges described above; the day-to-day precision of the slopes of the calibration graphs did not exceed 20% for each matrix tested. The limit of quantification (LOQ) evaluated as the concentration that gives a relative standard deviation (R.S.D.) not exceeding 20% was 50 ng/ml for I and II in plasma, urine and bile.

Detection limit

The limit of detection (LOD) in plasma, urine and bile was defined as the lowest concentration of I and II resulting in a signal-to-noise ratio of 3. It was established as 10 ng/ml for both I and II.

Precision and accuracy

The precision and accuracy of the method were calculated for all matrices tested at concentrations of 0.5–100 μg/ml. The precision, calculated as the R.S.D. of replicate assays of the calibration standards containing I and II, was generally around 5% (Table I). The accuracy, expressed as percentage concentration ratio, was in the range 88–116%.

Stability

Rufloxacin, like other quinolones [6], is light-sensitive and each sample manipulation step (thawing, pipetting, etc.) should therefore be done in a darkened room.

Compound I was found to be stable at −20°C in the dark for 21 months (plasma), 4 months (urine) and 5 months (bile). Following analytical treatment, I and I.S. were stable (autosampler plate, room temperature) for at least 18 h.

After three repeated freezings and thawings, I was stable in plasma and urine.

Extraction efficiency

Following the whole sample preparation procedure, the recoveries from plasma were 56.1% for I (concentration 0.4–9.1 μg/ml), 60.8% for II (0.5–5.1 μg/ml) and 80.3% for I.S. (15.7 μg/ml). For diluted urine the recoveries were quantitative: 98.8% for I (concentration 2.5–150 μg/ml) and 98.5% for II (2.5–25 μg/ml), whereas for extracted urine the recoveries were 97.9% for I (concentration 0.5–50 μg/ml), 75.2% for II (0.5 μg/ml) and 74.4% for I.S. (69.9 μg/ml). The recoveries from bile were quantitative (108.7% for I at 2.0 μg/ml and 100.4% for II at 2.8 μg/ml).

TABLE I

PRECISION OF HPLC ASSAY

Body fluid	Compound	Concentration (μg/ml)	*n*	R.S.D. (%)
Plasma	I	0.5	5	8.5
		4.2	5	3.6
	II	0.6	5	5.6
Urine	I	0.5[a]	4	4.0
		20.6[a]	5	8.9
		41.1[a]	5	4.1
		9.9[b]	5	4.1
		99.2[b]	5	2.2
	II	0.6[a]	4	4.0
		14.2[b]	4	2.3
Bile	I	1.0	4	4.5
	II	1.4	4	4.8

[a] Extraction procedure.
[b] Dilution procedure.

DISCUSSION

During the set-up of the chromatographic method, the quinolones that were used showed severely tailing peaks when chromatographed on many of the commercially available reversed-phase columns using different mobile phases. This behaviour can be reduced by the use of anti-tailing agents, such as TEA [7,8]. However, poly(styrene–divinylbenzene) supports provided superior chromatograms in phosphate–TEA-based mobile phases. The addition of small amounts of THF to the mobile phase further improved the peak symmetry on this column [9], and a pH of 5.6 optimized separation.

The choice of the detection mode was made by comparison of UV spectrophotometry (at 300 nm) and fluorescence (excitation maximum at 350 nm and emission maximum at 510 nm). Fluorescence detection was 25–200 times more sensitive than UV detection for both I and II in the different matrices.

For fluoroquinolones, various approaches to sample treatment have been proposed. Most workers have developed extraction procedures employing dichloromethane or chloroform [7,10–12]. These procedures offer advantages in terms of concentration of the analyte (*i.e.*, detection limit) and reduced background from endogenous components. We describe here a simple handling procedure for plasma (deproteinization) and bile (centrifugation and filtration). For urine samples a simple 1:50 dilution with water allows the detection of I and II at concentrations as low as 2.5 μg/ml. For lower concentrations (at ≥72 h after a therapeutic dose) an extraction

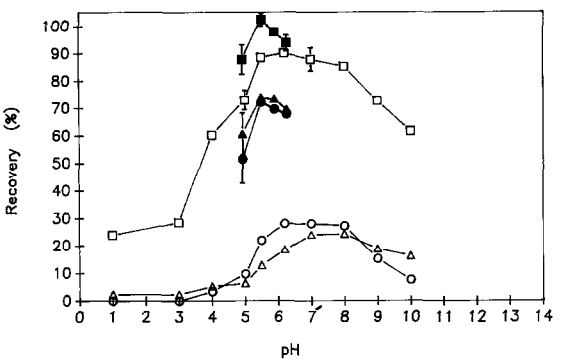

Fig. 3. Extraction recovery from urine *versus* pH. Simple extraction with dichloromethane: (□) I; (△) II; (○) I.S. Extraction after addition of Salts (Na_2HPO_4–NaH_2PO_4) with dichloromethane: (■) I; (▲) II; (●) I.S. (see *Sample preparation*). Standard error bars are shown except when smaller than the data symbol.

procedure with dichloromethane has been developed. To optimize the conditions, the recoveries of I, II and I.S. after a single extraction with dichloromethane were examined as a function of pH (range 1–10) (Fig. 3). The extraction recovery was almost quantitative for I, but not for II and I.S. (<30%). The effect of addition of salts (Na_2HPO_4–NaH_2PO_4 mixture) on the recovery of the three compounds was then investigated in the pH range 5–8 (Fig. 3). Whereas results were irreproducible above pH 6.5, in the pH range 5–6.5 the extraction after saturation of aqueous solution with salts improved the recovery of all the compounds, the best results being obtained at pH ≈ 6.

Application

The method was applied to investigate the pharmacokinetics and the biliary elimination of I. Patients with total external bile drainage through a percutaneous transhepatic catheter were included in this study. Representative chromatograms obtained from subjects given a single oral dose of 400 mg of I are reported in Fig. 4.

Plasma and bile profiles of a representative subject are shown in Fig. 5. Compound II was not detected in plasma or bile. The cumulative urinary excretion curves for I and II are shown in Fig. 6. Urinary concentrations of the N-desmethyl metabolite were below 10% of the concomitantly measured concentration of the parent compound. The cumulative excretion of I into bile during 72 h was less than 2%.

CONCLUSIONS

We have described a simple HPLC procedure that provides good linearity, recovery, selectivity,

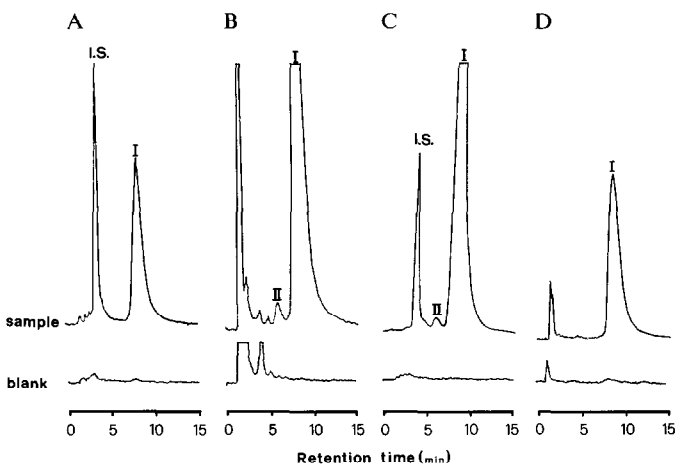

Fig. 4. Chromatograms of samples from subjects following a 400-mg oral dose of rufloxacin. (A) 4-h plasma sample (concentration, 2.62 μg/ml I). (B) 2–4 h urine sample (dilution procedure, see *Sample preparation*; concentrations, 149.40 μg/ml I and 5.23 μg/ml II). (C) 2–4 h urine sample (extraction procedure, see *Sample preparation*; concentrations 34.57 μg/ml I and 0.39 μg/ml II). (D) 1-h bile sample (concentration, 4.09 μg/ml I). For each chromatogram the corresponding predose sample is shown. Peaks: I = rufloxacin; II = N-desmethyl metabolite; I.S. = internal standard.

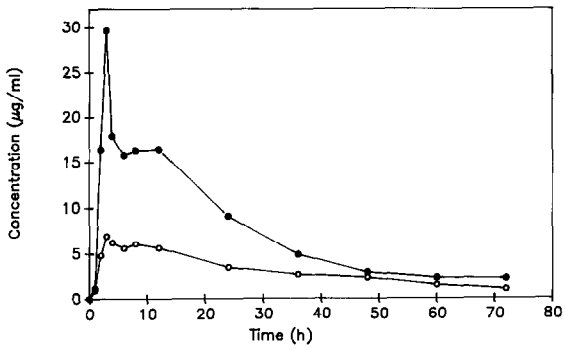

Fig. 5. (○) Plasma and (●) biliary levels of rufloxacin in a subject following oral (400 mg) administration.

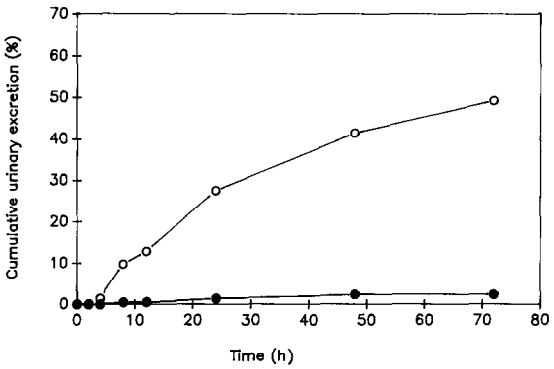

Fig. 6. Cumulative urinary excretion of (○) rufloxacin and (●) its N-desmethyl metabolite in a subject receiving a 400-mg oral dose.

sensitivity and precision for the determination of rufloxacin and its N-desmethyl metabolite in plasma, urine and bile. The limit of detection is 10 ng/ml in all the biological fluids considered. Hence this method is suitable for pharmacokinetic studies in humans.

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