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Letter to the Editor

Determination of ofloxacin in plasma and urine by liquid chromatography

Sir,

Ofloxacin is a new fluoroquinolone carboxylic acid antimicrobial [1–3]. High-performance liquid chromatography (HPLC) with UV or fluorescence detection allows its quantification in biological fluids [4–6]. Most of the published methods are not sufficiently sensitive to measure levels below 20 μg/l. Moreover, we have observed interferences in samples from patients in intensive care. We have therefore developed a procedure for the quantification of ofloxacin in plasma and urine which is rapid, specific and has a detection limit of 0.5 μg/l in plasma, allowing therapeutic monitoring and pharmacokinetic studies. Notarianni and Jones [7] have also proposed a sensitive assay for pharmacokinetic investigations.

EXPERIMENTAL

Reagents

Ofloxacin was obtained from Diamant Lab. (Puteaux, France). 1-Allyl-6-fluoro-7-(4-methyl-1-piperazinyl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (internal standard) was a gift from Roger Bellon Lab. (Paris, France).

Apparatus

The HPLC system consisted of a Chromatem 380 pump (Touzart et Matignon), a 710 B Waters Wisp automatic injector (Millipore), a Novapack C_{18} column (4 μm, 150 mm × 4.6 mm I.D.), an RF 530 Shimadzu fluorescence detector (band width 18–22 nm, time constant 1.5 s) and a Hewlett-Packard 3390-A integrator.

Standards

Standard curves were made by spiking pooled human plasma to obtain final concentrations in the ranges 9.8–312 μg/l and 312–7500 μg/l. Urine was spiked to obtain final concentrations in the range 12.5–100 mg/l.

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Extraction procedure and chromatographic conditions

For plasma, 200 μl of 0.1 *M* phosphate buffer (pH 6.8) containing 10 mg/l internal standard were added to 200 μl of the standard or patient plasma. After the addition of 4 ml of dichloromethane, the tube was stoppered and gently shaken at 100 cycles/min for 10 min at room temperature. The aqueous layer was removed and the organic layer was transferred to a fresh tube and dried under nitrogen at 40°C. The residue was dissolved in 200 μl of eluent, and 20 μl were injected into the chromatograph. For pharmacokinetic studies the quantification of residual concentrations employed 500 μl of standard or patient plasma and 500 μl of a 0.1 *M* phosphate buffer (pH 6.8) containing the internal standard (0.2 mg/l). The extraction procedure was the same.

For urine, 5 ml of internal standard (5 mg/l in the mobile phase) were added to 100 μl of the patient urine and 100 μl of the standard. A 20-μl aliquot was injected directly into the chromatograph.

The mobile phase was acetonitrile–0.01 *M* potassium dihydrogenphosphate–triethylamine (140:860:2, v/v). The pH was adjusted to 2.8 with formic acid. The flow-rate was 1 ml/min. The excitation wavelength was set to 307 nm, and the emission signal was monitored at 510 nm.

RESULTS AND DISCUSSION

Our purpose was to develop a selective HPLC assay for use in pharmacokinetic studies and to monitor patients in intensive care. To improve the selectivity of the method, it was necessary to optimize the detection, chromatography and extraction.

In the first step, it was observed that the fluorescence spectrum of ofloxacin showed higher maximal excitation and emission wavelengths than usually reported for fluoroquinolones: λ_{ex} 290 nm, λ_{em} 432 nm [4,6]. Moreover, the fluorescence spectrum of ofloxacin depends on the nature of the mobile phase. When the excitation and emission wavelengths were set to 307 and 510 nm, respectively, a fifteen-fold increase in sensitivity was observed. This is in agreement with the Notarianni and Jones method [7], but offers improved specificity.

In the second step, we studied the resolution in methanol–water and acetonitrile–water mixtures. Two mobile phases can be proposed: methanol–0.01 *M* potassium dihydrogenphosphate (pH 2.8) (25:75, v/v) and acetonitrile–0.01 *M* potassium dihydrogenphosphate (pH 2.8) (14:86, v/v). The less viscous acetonitrile–water mixture was chosen. The influence of the counter-ion (heptane-1-sulphonic acid) on the separation was also studied. For both compounds, capacity factors increased with increasing counter-ion concentration (2.5-fold for a $2 \cdot 10^{-3}$ *M* heptanesulphonic acid solution), but the selectivity remained constant. It appeared that ion-pair chromatography was not necessary. Unlike Notarianni and Jones [7], we added triethylamine to the mobile phase; this significantly reduced the asymmetry of the peak.

Under the chromatographic conditions described above, the retention times for ofloxacin and the internal standard were 3.5 and 5 min, respectively (Fig. 1). Some compounds often associated with ofloxacin in therapeutics were tested for

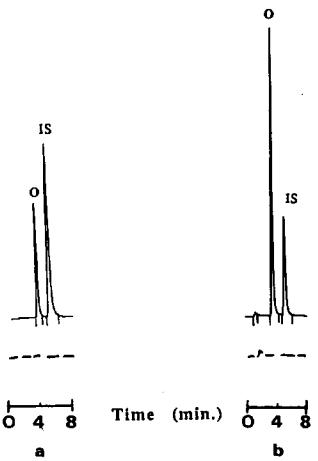

Fig. 1. (a) Chromatograms of plasma samples from a patient receiving ofloxacin. (——) Sample containing ofloxacin, 9.5 μg/l; (---) blank. (b) Chromatograms of urine samples from a patient receiving ofloxacin. (——) Sample containing ofloxacin, 100 mg/l; (---) blank. Peaks: O = ofloxacin; IS = internal standard.

TABLE I

WITHIN-DAY AND BETWEEN-DAY REPRODUCIBILITY OF THE ASSAY

Expected	Observed			
	Within-day ($n=5$)		Between-day ($n=5$)	
	Mean	C.V. (%)	Mean	C.V. (%)
Urine (mg/l)				
12.5	12.7	1.1	12.7	4.6
50	49.7	1.2	49.0	4.1
100	99.7	0.6	100.0	0.5
Plasma, low levels (μg/l)				
9.8	10.1	6.4	9.8	1.7
39	38.7	1.5	38.0	2.9
156	156	0.6	157.0	1.9
Plasma, high levels (mg/l)				
0.31	0.34	5.9	0.30	10
1.25	1.24	4.4	1.20	7.5
5.00	5.00	1.2	5.09	3.5

interference: gentamicin, amikacin, netilmicin, tobramycin, vancomycin, cefotaxim and ceftriaxone did not interfere when injected directly into the column.

The extraction procedure required a small volume of sample (100–500 μl) and was also simplified compared with the Notarianni and Jones method [7]. Ofloxacin was extracted at its isoelectric point (pH 6.8) and the recovery averaged

$81 \pm 8\%$ ($n=15$). The linearity and reproducibility were satisfactory. Coefficients of variation (C.V.) both within-day and between-day were below 4% for urine and below 10% for the plasma assay (Table I).

The limits of detection (signal-to-noise ratio 2:1) for the urine and the "high" and "low" plasma level assays were 500, 9 and 0.5 μg/l, respectively. This sensitivity has proved useful in the acquisition of pharmacokinetic data 50 h after intravenous doses of ofloxacin. Moreover, the method allows the measurement of ofloxacin in plasma of patients treated for a gram-negative septicemia. This had previously been impossible because of interferences and shows the influence of the conditions of detection on the specificity and sensitivity of the assay.

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