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Note

High-performance liquid chromatographic determination of ofloxacin in plasma and urine

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Ofloxacin, (±)-9-fluoro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-2,3-dihydro-7*H*-pyrido[1,2,3-*de*]-1,4-benzoxazine-6-carboxylic acid, is a fluoroquinolone that exhibits good in vitro and in vivo activity against Gram-negative and Gram-positive bacteria. The method previously used for the measurement of ofloxacin was microbiological assay, which has been applied in pharmacokinetic studies [1–3]. More recently, a high-performance liquid chromatographic (HPLC) assay of ofloxacin using UV detection was published [4]. This paper described the development of a more sensitive method using HPLC with fluorescence detection.

EXPERIMENTAL

Reagents and materials

Ofloxacin (RU43280) was obtained from Roussel-Uclaf (Paris, France). Formic acid, hydrochloric acid, acetonitrile and dichloromethane (analytical-reagent grade) were purchased from Merck (Interchim, Montluçon, France). Potassium monophosphate was obtained from Fluka (Interchim). A 0.05 *M* borate buffer (pH 9), which contained 0.05 *M* potassium chloride and 0.021 *M* sodium hydroxide, was obtained from Merck (Cofralab, Bordeaux, France). Desmethylpefloxacine, used as internal standard, was purchased from Rhône-Poulenc (Paris, France).

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Instrumentation and chromatographic conditions

The HPLC equipment consisted of an M590 two-piston pump (Waters Assoc., St. Quentin en Yvelines, France), a Wisp 710B automatic sampler (Waters Assoc.), a μBondapak C_{18} (10 μm) HPLC column (300 mm × 3.9 mm I.D.) (Waters Assoc.) and an LS-4 spectrofluorometric detector (Perkin-Elmer, Bois d'Arcy, France). The excitation wavelength was set at 290 nm and the emission wavelength at 500 nm (nominal bandpass, 10 nm) and the sensitivity for ofloxacin determination was 0.25 μA full scale in plasma and 0.125 μA full scale in urine. Chromatograms were recorded on an Omniscribe chart recorder (Houston Instruments, St. Quentin en Yvelines, France) and processed by HP3357 LAS software (Hewlett-Packard, Orsay, France). The mobile phase contained 1.36 g of potassium monophosphate and 40 ml of aqueous tetrabutylammonium phosphate (0.005 *M*) in 1 l of water–acetonitrile (90:10, v/v). The pH was adjusted to 2.9 with formic acid. The mobile phase was filtered before use. The flow-rate was 0.7 ml/min (column temperature 20°C).

Extraction procedure

Plasma (0.5 ml), 50 μl of internal standard solution (200 μg/ml desmethylpefloxacine in 0.01 *M* hydrochloric acid) and 1.5 ml of borate buffer (pH 9) were placed in a 20-ml tube, which was capped and vortexed for 10 s. Dichloromethane (8 ml) was added and the tube was shaken for 5 min. After centrifugation at 1500 *g* for 5 min, the aqueous phase was discarded and 7 ml of the organic phase were transferred into another tube. Hydrochloric acid (0.1 *M*, 0.2 ml) was added and the tube was shaken for 5 min. After centrifugation (1500 *g*) the aqueous solution (50 μl) was transferred into a vial for automatic injection (5 μl).

Urines (1 ml) was diluted with water (1:40, v/v), the internal standard (100 μg/ml) was added and 5 μl were injected directly into the chromatograph.

Calibration

Using an ofloxacin stock solution in 0.01 *M* hydrochloric acid (500 μg/ml) and appropriate dilutions, ofloxacin standards (0.078, 0.156, 0.312, 0.625, 1.25, 2.5 and 5 μg/ml) were prepared in pooled human plasma, spiked with the internal standard, desmethylpefloxacine (200 μg/ml, 50 μl), and stored at −20°C in 0.5-ml aliquots. Calibration graphs were obtained by plotting ofloxacine/desmethylpefloxacine peak-area ratio against ofloxacin concentration.

Precision and accuracy

The precision and the accuracy of the method were determined by analysing replicate spiked plasma samples at selected concentrations (0.02, 0.04, 1.25, 2.5 and 5 μg/ml). The coefficient of variation (C.V.) for each concentration was calculated as C.V. = 100 S.D./mean. The day-to-day precision and accuracy were determined by analysing several groups of control samples. Each group consisted of five sets of replicates, which were prepared at concentrations unknown to the experimenter, using a stock solution different from that used to obtain the calibration graphs. Three control samples were analysed daily during the period necessary to assay ofloxacin in all the samples collected during one bioavailability

study. For each set of replicates the coefficient of variation and the percentage error were calculated.

RESULTS AND DISCUSSION

The retention times of ofloxacin and desmethylpefloxacine are 8.7 and 10.2 min, respectively (Fig. 1). Ofloxacin calibration graphs obtained from control human plasma were found to be linear ($R=1.43C-0.0042$, $r=1.00$) for concentrations ranging from 0.078 to 5 μg/ml. The limit of detection for ofloxacin assay was 0.02 μg/ml (C.V. = 9.7%, error = 9.2%). The within-day precision and accuracy of the method are reported in Table I.

Using the described chromatographic conditions, chromatograms obtained from blank plasma did not show any significant signal (Fig. 2). This was confirmed after the analysis of six placebo samples among the 28 control plasma samples analysed, thus confirming the specificity of the assay.

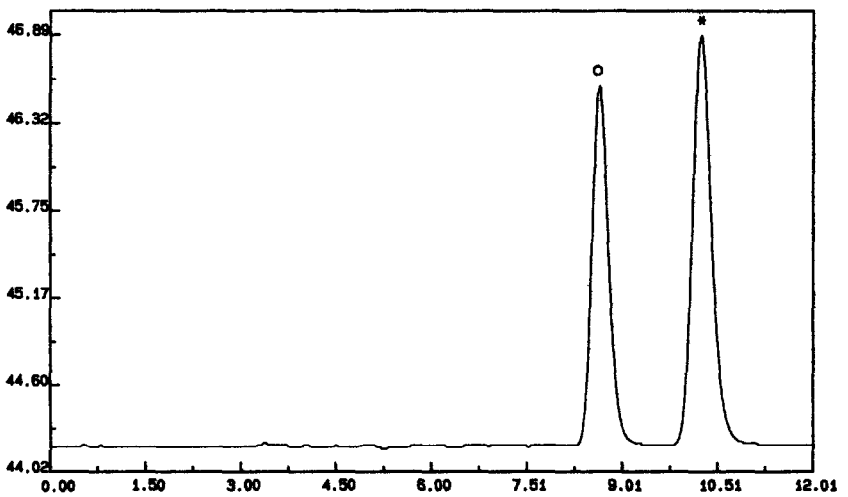

Fig. 1. Typical chromatogram obtained from a plasma sample collected 1.5 h after the fifth oral administration of ofloxacin during a multiple dose (200 mg every 12 h) kinetic study. Ofloxacin concentration, 0.61 μg/ml. Peaks: O = ofloxacin; * = internal standard.

TABLE I

WITHIN-DAY PRECISION AND ACCURACY OF THE HPLC PROCEDURE FOR THE ASSAY OF OFLOXACIN IN PLASMA

Concentration added (μg/ml)	n	Concentration found (mean ± S.D.) (μg/ml)	C.V. (%)	Error (%)
5	10	5.23 ± 0.052	1.0	4.6
2.5	10	2.60 ± 0.096	3.7	4
1.25	5	1.23 ± 0.026	2.1	1.6
0.04	5	0.0416 ± 0.0015	3.5	4
0.02	10	0.0182 ± 0.0018	9.7	9.2

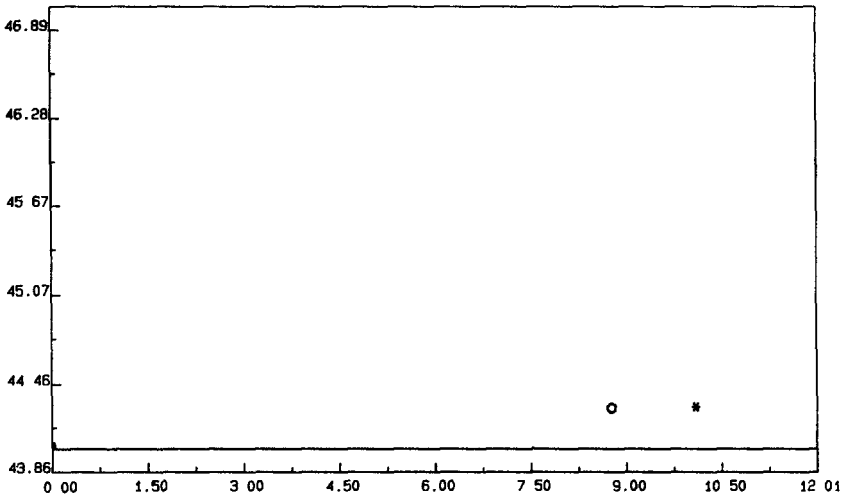

Fig. 2. Typical chromatogram obtained from 0.5 ml of blank plasma, showing the lack of any interference at the retention times of ofloxacin (○) and internal standard (*).

TABLE II

BETWEEN-DAY PRECISION AND ACCURACY OF THE HPLC PROCEDURE FOR THE ASSAY OF OFLOXACIN IN PLASMA

Concentration added (μg/ml)	*n*	Concentration found (mean ± S.D.) (μg/ml)	C.V. (%)	Error (%)
5	6	5.02 ± 0.256	5.1	0.4
2.5	5	2.38 ± 0.614	2.6	4.8
1.25	6	1.22 ± 0.026	2.1	2.4
0.04	5	0.035 ± 0.005	14.3	12.5

TABLE III

BETWEEN-DAY PRECISION AND ACCURACY OF THE HPLC PROCEDURE FOR THE ASSAY OF OFLOXACIN IN URINE

Concentration added (μg/ml)	*n*	Concentration found (mean ± S.D.) (μg/ml)	C.V. (%)	Error (%)
200	4	196.80 ± 9.97	5.1	1.6
100	3	95.83 ± 6.17	6.4	4.2
25	3	25.07 ± 0.75	3.0	0.3

The between-day coefficient of variation and percentage error (Table II), calculated after analysing the 22 control plasma samples, demonstrate that the long-term precision and accuracy of the assay are suitable for routine analysis. Similar results were obtained with urine samples (Table III).

The recovery was determined by comparing the mean peak area for ofloxacin extracted from plasma with that for ofloxacin injected directly into the chroma-

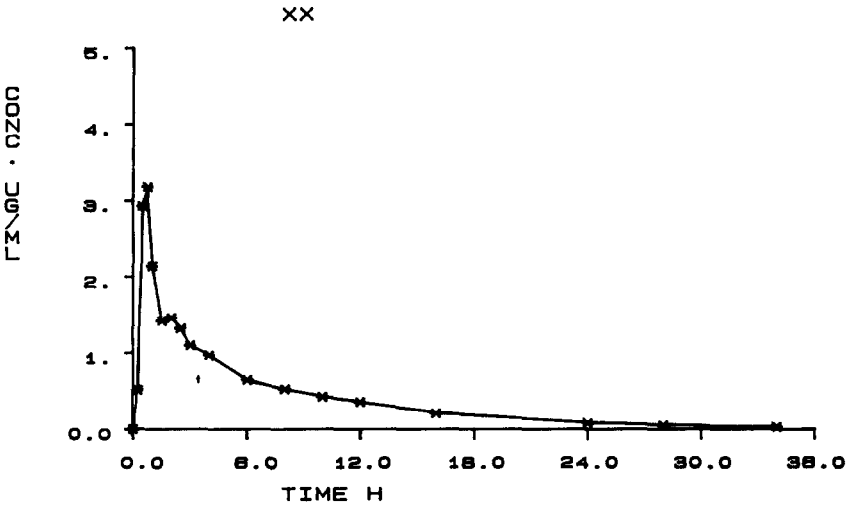

Fig. 3. Example of a plasma ofloxacin concentration-time profile following a 200-mg oral dose to a young healthy volunteer.

tograph. Five determinations were made at a concentration of 0.125 $\mu g\ ml^{-1}$. The mean recovery of ofloxacin was 50.3%, which is comparable to that obtained by Carlucci et al. [4]. As a consequence, the lower limit of detection of our method compared with the previously published method [4] is certainly due to the use of fluorescence detection, which markedly increases the signal-to-noise ratio, as exemplified by the lack of interference between ofloxacin, the internal standard and endogenous compounds, including metabolites (Fig. 2), and by the very weak background noise recorded for the plasma of subjects treated with ofloxacin (Fig. 1).

CONCLUSIONS

A method has been developed for the determination of ofloxacin in human plasma and urine. This method, applied to the analysis of plasma and urine samples collected during several kinetic studies including a bioequivalence study of three oral formulations of ofloxacin [5], was shown to provide consistent analytical results as exemplified by the between-day precision and accuracy, and allowed the quantification of ofloxacin in plasma samples 34 h after a single 200-mg oral dose (Fig. 3).

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