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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PEFLOXACIN AND ITS MAIN ACTIVE METABOLITES IN BIOLOGICAL FLUIDS

G. MONTAY*, Y. BLAIN, F. ROQUET and A. LE HIR

Centre de Recherches du Laboratoire Roger Bellon, 90 rue Marcel Bourdarias, 94140 Alfortville (France)

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SUMMARY

We describe a high-performance liquid chromatographic (HPLC) method for the analysis of pefloxacin, a new antibacterial agent, in plasma and urine following administration of a therapeutic dose in humans. HPLC assay of pefloxacin and its two main active metabolites in urine is also described. The applicability of the methods to pharmacokinetic studies of pefloxacin in humans is demonstrated.

INTRODUCTION

Pefloxacin (Fig. 1), 1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(4-methyl-1-piperazinyl)-quinoline-3 carboxylic acid (PF), is a new antibacterial agent, which has shown one of the highest level of in vitro activity against gram-negative bacteria among the compounds of the quinolone group; its broad and potent activity spread to gram-positive bacteria [1, 2].

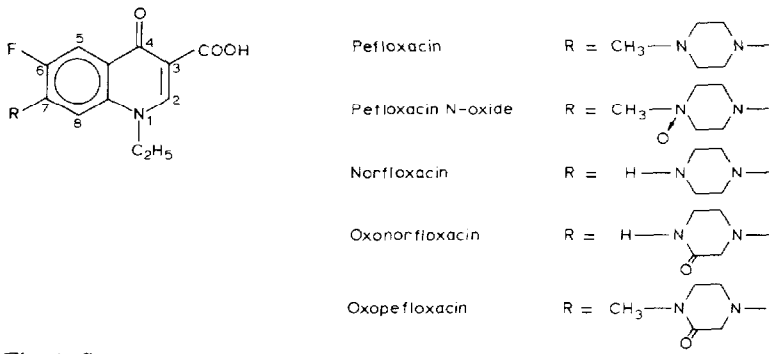

Fig. 1. Structures of pefloxacin and identified metabolites in man.

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Studies in animals and man have shown that PF is transformed into several metabolites [3]. The main metabolites (Fig. 1) identified in man are pefloxacin N-oxide, desmethylpefloxacin or norfloxacin (NF), and oxonorfloxacin (ONF), and also a minor one, oxopefloxacin. PF, NF and ONF have bacteriological activity. PF is the main active compound in human plasma (about 80% of the activity encountered), whereas NF, PF and to a lesser extent ONF account for the activity found in urine.

This paper describes a high-performance liquid chromatographic (HPLC) method for the quantitation of PF in plasma or urine. Quantitation of PF, NF and ONF in urine is also described. The technique is based on separation by reversed-phase ion-pair chromatography using an octadecylsilane stationary phase and a solution of tetrabutylammonium iodide in acetonitrile–aqueous phosphate buffer as mobile phase. An example from a pharmacokinetic study is shown.

EXPERIMENTAL

Chemicals and reagents

PF, NF and ONF, the 6-chloro analogue (I) and the 1-methyl analogue (II) of PF (internal standards) were synthesized in our laboratories. All reagents were of analytical grade.

The mobile phase consisted of a mixture of water and acetonitrile containing 0.4% of $Na_2HPO_4 \cdot 12H_2O$ and 0.2% of tetrabutylammonium iodide, adjusted to pH 9.4 with 1% triethylamine. Two solvents were prepared: solvent A was only distilled water; solvent B was a 2:3 (v/v) acetonitrile–water mixture.

Stock solutions of PF, NF and internal standards were prepared by dissolving 1 mg/ml of each compound in 0.01 *M* sodium hydroxide. A solution, prepared daily, of ONF 0.1 mg/ml in dimethyl sulphoxide (DMSO) was used.

Preparation of samples

Analysis of PF in plasma or urine. Stock solutions of PF and I were diluted in water to obtain working daily solutions which were used to prepare plasma and urine standards.

Plasma standards were between 0.125 and 1.50 μg of PF for 1 ml of plasma with 1.50 μg of I, and between 0.5 and 3 μg of PF for 0.5 ml of plasma with 3 μg of I.

Urine standards were between 1 and 10 μg/ml of PF with 5 μg of I, and between 5 and 100 μg/ml of PF with 50 μg of I.

Analysis of PF, NF and ONF in urine. Working solutions of PF, NF and II were prepared by diluting the stock solutions in water. Concentrations of PF and metabolites between 5 and 60 μg/ml in urine with 30 μg/ml of II were obtained from these solutions and the solution of ONF in DMSO.

Plasma and urine analysis of PF

To 0.5 or 1 ml of plasma in a 15-ml glass centrifuge tube were added 300 or 150 μl of the internal standard I solution (10 μg/ml), 0.5 ml of 0.5 *M* phosphate buffer (pH 7.4) and 10 ml of chloroform. The tube was manually shaken for 10 sec, centrifuged and the upper aqueous phase was discarded. The solvent

was transferred into a clean tube and evaporated to dryness in a 60°C water bath under a stream of air. The residue was dissolved in 50 μl of 1% ammonia and 10 μl of this solution were injected into the chromatograph.

To 1 ml of urine in a glass tube were added 50 μl of the internal standard I solution (1 mg/ml or 100 μg/ml), 0.5 ml of phosphate buffer and 10 ml of chloroform. The operation was then carried out as for plasma.

Urine analysis of PF, NF and ONF

To 1 ml of urine in a glass tube were added 300 μl of the internal standard II solution (100 μg/ml) and 0.5 ml of 0.5 *M* phosphate buffer (pH 7.0). The mixture was twice extracted for 20 sec with 10 ml of chloroform–isopentanol (10:1, v/v).

After combining the two extracts in a clean glass tube, the solvent was evaporated to dryness and the residue was dissolved in 100 μl of 1% ammonia; 10 μl of this solution were injected into the chromatograph.

HPLC system

A Varian Model 5020 liquid chromatograph was used, equipped with a Pye-Unicam LC-UV detector operating at 270 nm and a LiChrosorb RP-18 column, particle size 10 μm (Merck, Darmstadt, G.F.R.), 100 × 4.6 mm I.D.

The mobile phase was 52% of solvent A + 48% of solvent B for PF assay; for PF, NF and ONF assay a gradient of B was used starting from 20% of B and rising at a rate of 2.5% per min for 10 min. The flow-rate was 2 ml/min.

RESULTS AND DISCUSSION

PF assay in plasma or urine

Fig. 2 depicts representative chromatograms from blank or spiked plasma or urine and plasma or urine samples from a subject given pefloxacin. Under the described chromatographic conditions, PF and internal standard had retention times of 2 and 3.6 min, respectively, so that an injection could be made every 6 min. No interference was observed except by sulfamethoxazole and trimethoprim.

Calibration curves plotted as the peak height ratio of PF to the internal standard versus the concentration of PF were constructed and linear curves were obtained. The equations for the calibration curves were as follows:

Plasma	
0.125–1.5 μg/ml:	$Y = 1.2980X + 0.0136; r = 0.9997$
1–6 μg/ml (0.5-ml sample);	$Y = 0.571X - 0.072; r = 0.999$
Urine	
1–10 μg/ml:	$Y = 0.201X + 0.099; r = 0.990$
5–100 μg/ml:	$Y = 0.0230X + 0.0416; r = 0.9997$

The concentration ranges studied in plasma (0.1–6 μg/ml) and urine (1–100 μg/ml) covered the expected concentrations of PF in biological fluids after a single administration of the drug in humans. The detection limit was 0.05 μg/

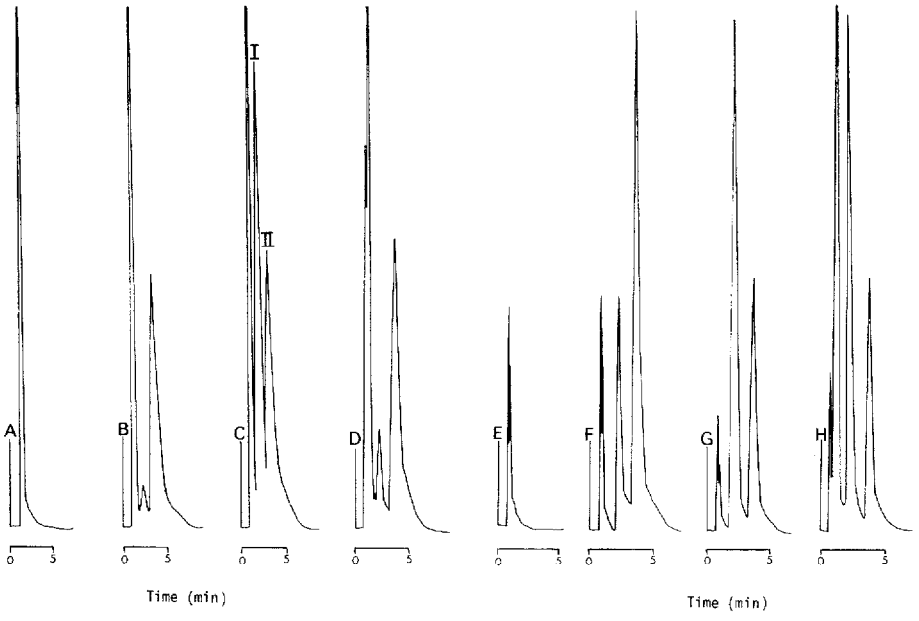

Fig. 2. Chromatograms of plasma and urine samples. (A) Blank plasma extract. (B, C) Extracts from blank plasma spiked with 0.125 μg/ml (B) or 1.5 μg/ml (C) of PF (I) with 1.5 μg of internal standard (II). (D) Plasma extract from a subject given an intravenous dose of 400 mg of pefloxacin. (E) Blank urine extract. (F, G) Extracts from blank urine spiked with 10 μg/ml (F) or 30 μg/ml (G) of PF with 30 μg of internal standard. (H) Urine extract from a subject orally given 400 mg of drug.

ml in plasma and 0.5 μg/ml in urine. The overall recovery of the method was 90% for both PF and I.

Spiked plasma and urine samples were repeatedly analysed to check the reproducibility of the technique. Table I shows the results obtained for intra-day assay and inter-day assays from samples stored at 4°C for eight weeks.

Although the pH of the mobile phase was alkaline, we were able to use every

TABLE I

REPRODUCIBILITY OF PF ASSAY IN HUMAN PLASMA AND URINE

Inter-day assays were performed over an eight-week period.

	Biological fluid (1 ml)	Amount of PF added (μg)	Coefficient of variation (%; n = 10)
Intra-day	Plasma	0.25	6.4
		2	5.2
	Urine	50	2.1
		100	2.1
Inter-day	Plasma	1	9.8
		5	4.8
	Urine	25	5.2
		100	5.2

column for about 400 assays, taking care to clean it with water after each run and periodically to change the head column frit.

PF, NF and ONF assay in urine

Representative chromatograms obtained from blank or spiked urine and from a subject given pefloxacin are shown in Fig. 3. Retention times of ONF, NF, IS and PF were, respectively, 3, 4, 6.7 and 8.8 min. The N-oxide metabolite of PF was contaminated with polar components of the urine which were first eluted from the column (see c, Fig. 3). Using the described conditions, the

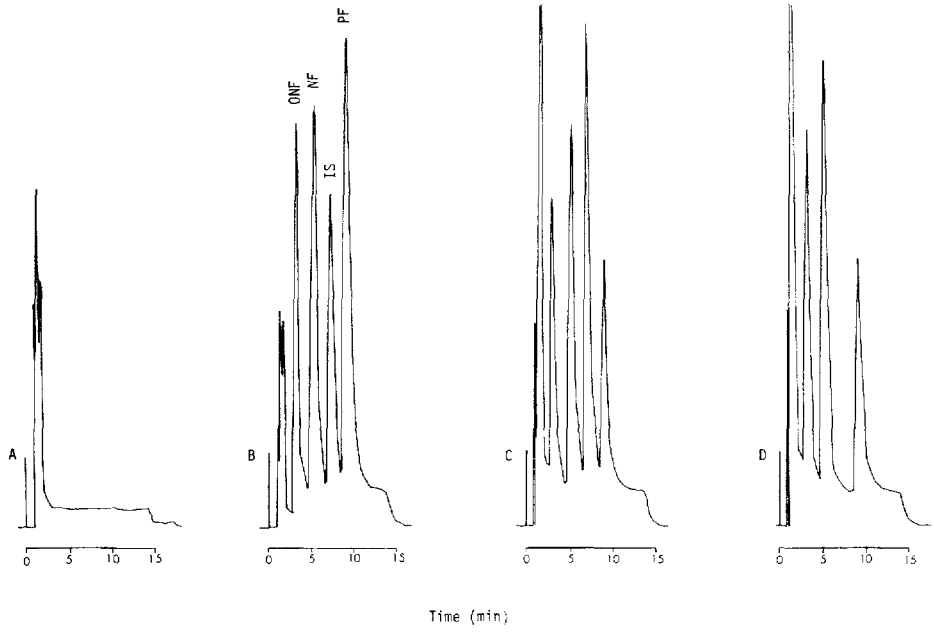

Fig. 3. Chromatograms of urine samples for PF and active metabolites assay. (A) Blank urine extract. (B) Extract from 1 ml of blank urine spiked with 25 μg of ONF, 50 μg of NF and PF and 30 μg of internal standard. (C, D) Urine extracts from a subject orally given PF with (C) and without (D) internal standard.

N-demethylated metabolite was not separated from a minor one, oxopefloxacin; we neglected this metabolite since HPLC dosages from acidified urine extracts showed that it accounted for less than 1% of the dose against 20% for NF.

Standard curves plotted as the peak height ratio of ONF, NF or PF to the internal standard versus concentrations of the metabolites are presented in Fig. 4. The equations for the calibration curves were as follows:

ONF: $Y = 0.050X - 0.015; r = 0.998$
NF: $Y = 0.028X - 0.076; r = 0.997$
PF: $Y = 0.033X - 0.012; r = 0.998$

The detection limit was 1 μg/ml for PF and ONF and 3 μg/ml for NF. Recover-

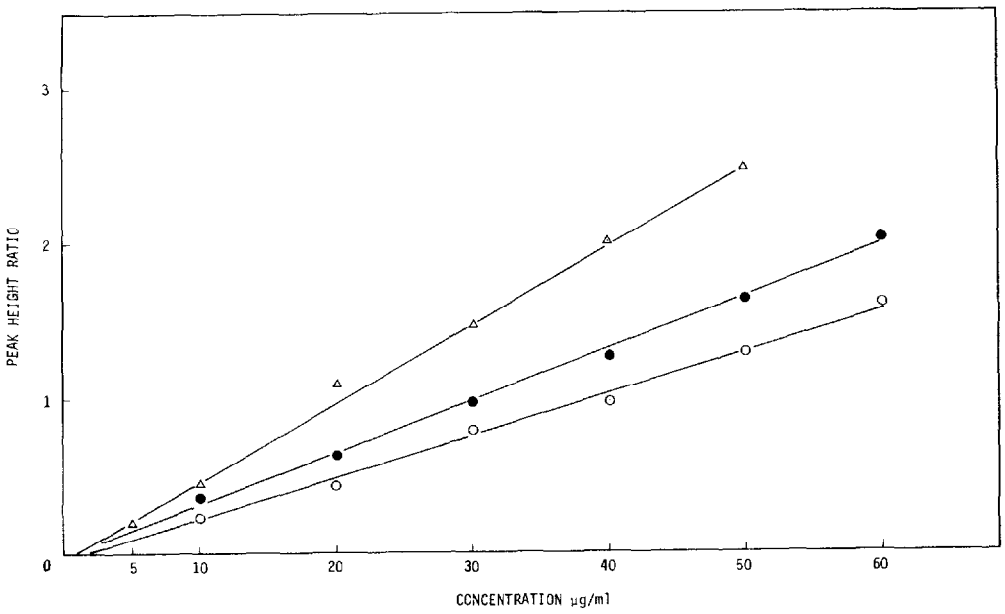

Fig. 4. Standard curves for PF (●——●), NF (○——○) and ONF (△——△) in urine (30 µg of internal standard).

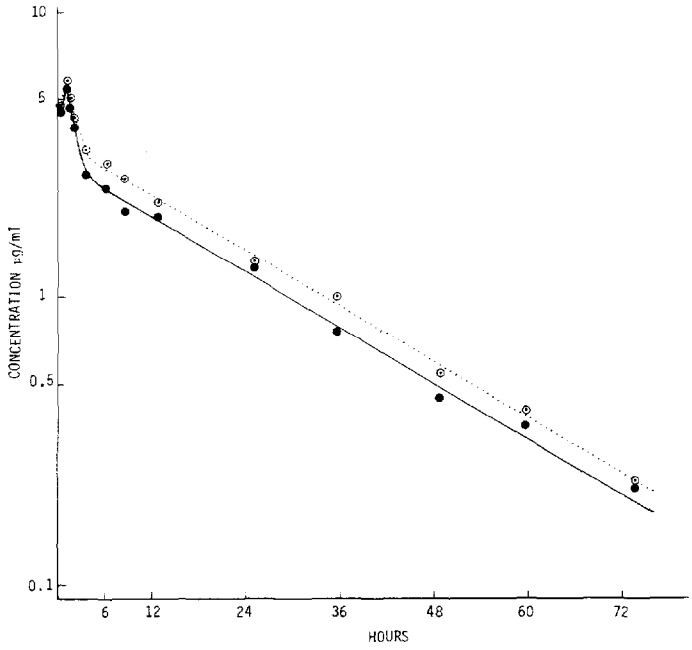

Fig. 5. Plasma level time curve obtained for PF (●——●) and with microbiological assay (○ - - - ○) following a 1-h infusion of 400 mg of pefloxacin to a patient.

ies were 94% for PF and ONF, 65% for NF and 95% for internal standard.

Coefficients of variation, determined on ten samples of 1 ml of urine overloaded with 10 μg of ONF, 40 μg of NF and 20 μg of PF (covering expected ratios of concentrations) were, respectively, 7.1, 5.5 and 4.5%.

KINETIC STUDIES

The method was applied to pharmacokinetic studies of PF in humans. Figs. 5 and 6 show the log plasma levels and cumulative urinary excretion curves versus time after administration of a single intravenous dose of 400 mg of PF to a patient suffering from renal and cardiac insufficiency.

In each figure, values found by microbiological assay are also plotted; they showed that PF was the main active compound in plasma whereas activity found in the urine was due to PF and metabolites.

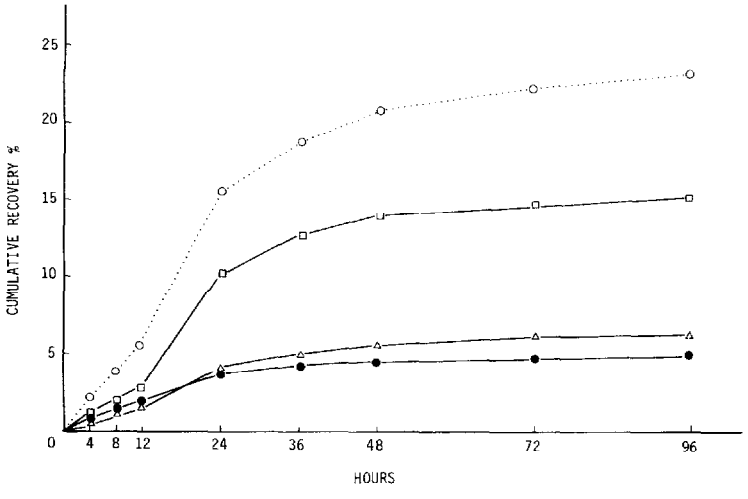

Fig. 6. Cumulative urinary excretion of PF (●——●), NF (□——□) and ONF (△——△), and microbiological assay (○ - - - ○) observed after a single infusion of 400 mg of pefloxacin to a patient.

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REFERENCES

1 Y. Goueffon, G. Montay, F. Roquet and M. Pesson, C.R. Acad. Sci., Ser. III, 292 (1981) 37.
2 M. Thibault, B. Koumare, C.J. Soussy and J. Duval, Ann. Microbiol. (Paris), 132A (1981) 267.
3 G. Montay, Y. Goueffon and F. Roquet, Antimicrob. Agents Chemother., submitted for publication.