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Note

Improved high-performance liquid chromatographic determination of pefloxacin and its metabolite norfloxacin in human plasma and tissue

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Pefloxacin [1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(4-methyl-1-piperazinyl)-quinoline-3 carboxylic acid] is a new broad and potent antibacterial agent active against Gram-negative bacteria, *Staphylococci*, *Mycoplasma pneumoniae* and *Legionella* species [1—4]. Minimal inhibitory concentrations inhibiting 90% of strains are equal to or less than 2 μg/ml, except for *Pseudomonas aeruginosa* (2—4 μg/ml), and the therapeutic levels achieved in man after a 400-mg twice daily oral or intravenous dosage are usually between 3 and 10 μg/ml.

We have described a high-performance liquid chromatographic (HPLC) procedure [5] for the quantitation of pefloxacin in plasma and of pefloxacin and its main active metabolites in human urine, norfloxacin (N-desmethyl-pefloxacin) and oxonorfloxacin [6]. However, the sensitivity of this assay was not sufficient to determine plasma levels of the metabolite. Moreover, it has been recently reported that norfloxacin plasma levels are usually 5% of pefloxacin levels [4].

The present paper reports the development of a new selective HPLC method with fluorimetric detection. The use of the fluorescent properties of pefloxacin and norfloxacin improves the assay sensitivity and allows the quantitation of both compounds in plasma and tissue.

EXPERIMENTAL

Standards and reagents

Pefloxacin mesylate dihydrate, norfloxacin and internal standard [1-allyl-6-fluoro-1,4-dihydro-4-oxo-7-(4-methyl-1-piperazinyl)quinoline-3 carboxylic

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acid] were synthesized in our laboratories. All other reagents were of analytical grade and obtained from usual commercial sources. Stock solutions of pefloxacin mesylate at 1 mg/ml free base concentration were prepared in distilled water; stock solutions of norfloxacin and internal standard at 1 mg/ml were prepared in 0.01 *M* sodium hydroxide. They were stored at 4° C. The daily working standard solutions of pefloxacin and norfloxacin were 10 and 1 μg/ml, respectively; that of the internal standard 10 μg/ml. Plasma standards (volume 0.5 ml) ranging from 0.5 to 6 μg/ml for pefloxacin and from 50 to 600 ng/ml for norfloxacin were prepared by the addition of various amounts of the working standard solution. Tissue standards were prepared by supplementing 1 ml of homogenized extract with 0.125—3 μg of pefloxacin and 12.5—300 ng of norfloxacin.

Chromatography

An HPLC system consisting of a Pye-Unicam Model 4010 pump, a Rheodyne Model 7125 injector in combination with a Varian Fluorichrom fluorescence detector and a Spectra-Physics Model 4270 integrator was used. The mobile phase (pH 4.8), delivered at 2.0 ml/min, was prepared by mixing 150 ml of acetonitrile, 2 g of sodium acetate trihydrate, 2 g of citric acid monohydrate, 1 ml of triethylamine and 850 ml of water.

Separations were carried out on a 10 × 0.5 cm Nucleosil C_{18} 10-μm column (phase from Macherey, Nagel & Co.) equipped with a 2-μm in-line filter (Scientific Systems). The excitation and emission wavelengths of pefloxacin at pH 4.8 are 330 and 440 nm, respectively, so that the fluorimeter was equipped with filters as recommended by the manufacturer (7-54 with 7-60 filters for excitation, 3-72 with 4-76 filters for emission).

Preparation of tissue extract

Tissue was homogenized in 0.5 *M* sodium phosphate buffer pH 7.0 (usually 5 ml per 0.1 to 0.25 g of tissue); 1 ml of the extract was then taken for assay of pefloxacin and norfloxacin.

Assay procedure

Plasma (0.5 ml or 0.25 ml) or tissue extract (1 ml) were spiked with 0.1 ml of internal standard solution; 1 ml of 0.5 *M* sodium phosphate buffer pH 7.0 was added only to plasma. After mixing, a 15-min extraction was performed twice with 10 ml of chloroform—isopentanol (9:1) using a laboratory shaker. The organic layers obtained after centrifugation were combined and evaporated to dryness under a stream of air in a 60° C water bath. The residue was dissolved in 100 μl of 1% ammonia and 25 μl were injected into the HPLC column. Peak heights were used to establish calibration curves.

RESULTS AND DISCUSSION

Under the described chromatographic conditions, the retention times of pefloxacin, norfloxacin and internal standard were 4.8, 2.8 and 6.6 min, respectively (Figs. 1 and 2) and no overlap with the metabolites pefloxacin N-oxide and oxonorfloxacin [6] was observed: the retention times of these two

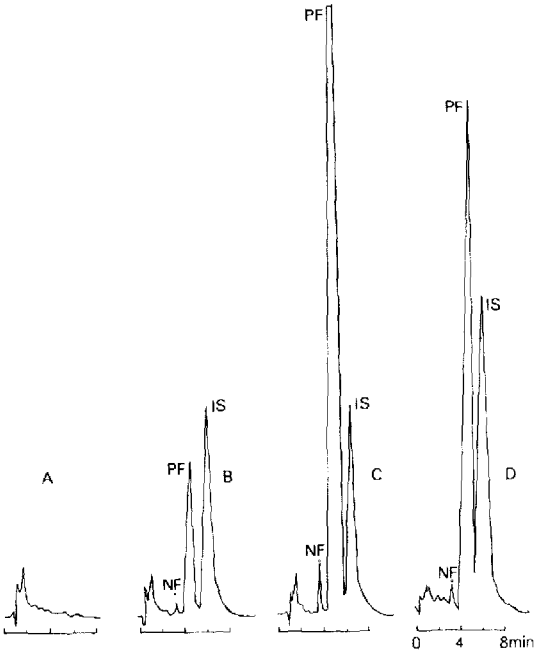

Fig. 1. Typical HPLC profiles of plasma extracts: (A) blank; (B) 0.1 μg/ml norfloxacin and 1 μg/ml pefloxacin standards; (C) 0.6 μg/ml norfloxacin and 6 μg/ml pefloxacin standards; (D) plasma of a subject 6 h after oral administration of 400 mg of pefloxacin mesylate (detector senstivity = 20). Peaks: NF = norfloxacin; PF = pefloxacin; IS = internal standard.

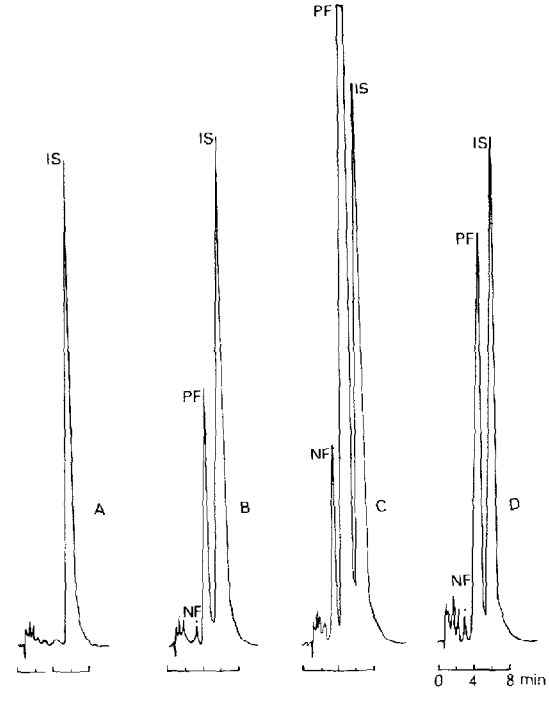

Fig. 2. Typical HPLC profiles of prostatic tissue extracts: (A) blank; (B) 0.025 μg norfloxacin and 0.25 μg pefloxacin standards; (C) 0.30 μg norfloxacin and 3 μg pefloxacin standards; (D) tissue extract of subject 1 (see Table II) (detector sensitivity = 20). Abbreviations as in Fig. 1.

compounds were 8.8 and 11.7 min, respectively. Pefloxacin N-oxide is the main but inactive metabolite of pefloxacin and occurs in plasma [4, 6]; it was poorly extracted (< 3%) by our extraction procedure. To date oxonorfloxacin has only been detected in urine.

The recoveries from plasma spiked with 1 μg/ml pefloxacin and 100 ng/ml norfloxacin were 96 ± 1.9% and 80.0 ± 7.5%, respectively (mean ± S.D., n = 6), whereas those from tissue were 92.9 ± 2.7% and 80.0 ± 4.9%, respectively (n = 6). The method was linear, and typical least-squares regression lines for peak height ratio from plasma standards were $y = 0.715x - 0.027$ ($r = 0.998$) for

TABLE I

PRECISION OF PEFLOXACIN AND NORFLOXACIN ASSAYS IN SPIKED PLASMA SAMPLES OR TISSUE EXTRACTS

Standard	Plasma		Tissue	
	μg/ml	C.V.* (%)	μg/g	C.V. (%)
Pefloxacin	0.5	1.5	2.5	1.9**
	1	1.4	10	1.0
	6	1.7	30	4.8**
Norfloxacin	0.05	8.3	0.25	13.3**
	0.10	5.9	1	7.9
	0.60	5	3	5**

*C.V. = coefficient of variation (n = 10).
**n = 6.

TABLE II

PEFLOXACIN, NORFLOXACIN AND ANTIMICROBIAL LEVELS IN PLASMA AND PROSTATIC TISSUE SAMPLES FROM PATIENTS GIVEN PEFLOXACIN MESYLATE ORALLY

Patient	Plasma (μg/ml)			Prostate (μg/g)		
	Pefloxacin	Norfloxacin	Antimicrobial	Pefloxacin	Norfloxacin	Antimicrobial
1	9.5	0.41	10.2	14.4	0.64	12.94
2	6.5	0.46	6.3	6.0	2.49	8.10
3	8.0	0.27	8.4	8.5	0.91	8.74
4	9.1	0.37	9.6	8.7	0.46	8.71
5	7.0	0.35	7.5	5.8	0.61	5.71
6	14.1	0.59	14.8	29.1	2.20	32.4
7	7.6	0.27	7.2	10.0	0.87	11.89
8	3.3	0.14	3.8	3.5	0.64	4.51
9	7.9	0.79	9.6	10.5	1.22	12.68
10	7.3	0.47	7.8	7.3	0.91	8.15
11	4.5	0.17	4.8	8.6	1.63	8.74
12	5.9	0.37	6.6	8.5	2.23	9.28
12	10.4	0.66	11.2	10.4	1.07	11.56
14	6.5	0.25	6.6	14.6	1.20	15.01

pefloxacin and $y = 0.321x + 0.015$ ($r = 0.997$) for norfloxacin (y = peak height ratio, x = concentration in μg/ml). The minimum detectable concentration in plasma was 20 ng/ml pefloxacin and 30 ng/ml norfloxacin. A good reproducibility was obtained (Table I). Between-day assays on plasma spiked with 1 μg/ml pefloxacin and 0.1 μg/ml norfloxacin and stored for six weeks at 4°C showed a mean level of 0.97 μg/ml with a coefficient of variation of 6.29% for pefloxacin (n = 19), whereas a mean level of 0.093 μg/ml with a coefficient of variation of 13.82% was found for the metabolite.

Our previously published HPLC method with UV detection [5] has the following disadvantages. The plasma levels of the active N-desmethyl metabolite are below 0.15 μg/ml in healthy subjects receiving a single dose of the drug [4] and are too low to be accurately measured. Moreover, the use of fluorimetric detection is disadvantageous since the alkaline pH of the mobile phase dramatically decreases the fluorescent properties of pefloxacin and norfloxacin. Thus, during the development of this new assay, we investigated an acidic mobile phase on several (C_{18}) reversed-phase HPLC sorbents and found the best efficacy was achieved with the sorbent mentioned in this paper. The acidic mobile phase enables fluorimetric detection with a better sensitivity than that obtained with UV absorption.

The developed method was applied to the analysis of plasma and prostatic tissue samples from fourteen patients orally given pefloxacin mesylate. The subjects received between two and eight administrations of 400 mg of drug (twice daily dosage) and samples were obtained 5—7 h after the last dose. Results were compared with those from the microbiological assay using the cup diffusion method [7] with *Escherichia coli* Kp 1976/712 and good agreement was observed between the values of pefloxacin plus norfloxacin and the antimicrobial levels (Table II).

REFERENCES

1 Y. Goueffon, G. Montay, F. Roquet and M. Pesson, C.R. Acad. Sci. Sér. III, 292 (1981) 37.
2 M. Thibault, B. Koumare, C.J. Soussy and J. Duval, Ann. Microbiol. (Paris), 132A (1981) 267.
3 A. Thabaut and J.L. Durosoir, Pathol. Biol., 30 (1982) 394.
4 J. Gaillot, A. Frydman, Y. Le Roux, J.B. Fourtillan, M.A. LeFebvre, J. Duchier and F. Djebbar, in K.H. Spitzy and K. Karrer (Editors), Proc. 13th Int. Congr. Chemother., Vienna, August 28—September 2, 1983, Part 110, p. 18.
5 G. Montay, Y. Blain, F. Roquet and A. Le Hir, J. Chromatogr., 272 (1983) 359.
6 G. Montay, Y. Goueffon and F. Roquet, Antimicrob. Agents Chemother., 25 (1984) 463.
7 French Pharmacopoea, 8th issue, 1965, p. 1601.