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Note

High-performance liquid chromatographic procedure for the quantitation of norfloxacin in urine, serum and tissues

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Norfloxacin [1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline carboxylic acid; MK-366] is a new quinolone antibacterial agent. Because of its pharmacokinetic properties [1] and broad spectrum of activity, norfloxacin is considered one of the most effective chemotherapeutic agents in urinary tract infections. Experimental studies performed on animals demonstrated that the distribution of the drug in both tissues and organic fluids is rapid, and the concentrations reached are higher than the corresponding serum levels [2]. A recent clinical study [3] demonstrated that norfloxacin eradicates relapsing prostatic infections.

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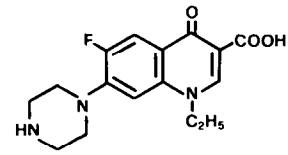
To our knowledge, no previous study has been attempted to correlate the pharmacological effects of norfloxacin with its tissue concentrations. In the present communication, we describe an extraction and quantitation procedure for measuring norfloxacin concentrations in human prostate and kidney after oral administration of the drug. Serum, urine and tissue levels were determined by a high-performance liquid chromatographic (HPLC) method modified from the procedure of Boppana and Swanson [4].

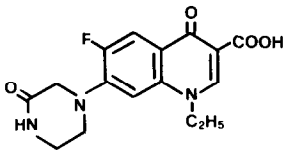
EXPERIMENTAL

Chemicals

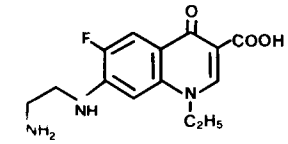
Norfloxacin and three of its metabolites (Fig. 1) with modifications in the 7 position {7-(3-oxo-1-piperazinyl), M-1; 7-[(2-aminoethyl)amino], M-2; and 7-[(2-acetylaminoethyl)amino], M-3} were provided by Merck Sharp & Dohme (West Point, PA, U.S.A.). Acetonitrile (HPLC grade), methylene chloride, and mono- and dibasic phosphates were all from Merck (Darmstadt, F.R.G.)



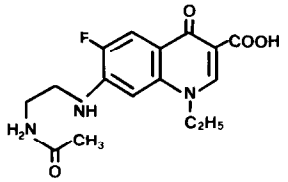
NORFLOXACIN



M-1



M-2



M-3

Fig. 1. Chemical formulae of norfloxacin and of its three major metabolites.

Samples

Specimens were obtained from patients scheduled for either prostatectomy or nephrectomy because of neoplastic and/or chronic infectious pathologies. All patients were below the age of 70 years, had no drug allergies and had serum creatinine concentrations below 2.0 mg/dl. Norfloxacin (400 mg) was given orally at time 0 the evening before surgery and again 11 h later (8.00 a.m.); at the same times urine specimens were collected. The first blood sample was taken before anaesthesia at 12—13 h; a second blood sample and a tissue fragment (either prostate or kidney) was taken during surgery at 14—15 h. All specimens were kept at −20° C until extraction.

Extraction procedure

The tissues were homogenized in 10 vols. of cold 0.4 *M* perchloric acid with an Ultra-Turrax TP 10-N homogenizer (Janke Kunkel, Staufen, F.R.G.) running for two cycles of 30 sec at 20,000 rpm, with a pause of 1 min. The homogenate was centrifuged for 15 min at 1500 *g* (TJ-6 centrifuge, Beckman, Ireland), the pellet discarded and the supernatant adjusted to pH 7.5. A 4-ml fraction was lyophilized and resuspended to 1 ml with 0.05 *M* sodium hydroxide. Serum (1 ml) and urine (200 μl) samples were mixed with 100 μl of 0.05 *M* sodium hydroxide in a 50-ml plastic centrifuge tube (Falcon Plastics, Oxnard, CA, U.S.A.). Methylene chloride (8 ml) and 0.5 *M* sodium phosphate buffer pH 7.5 (0.5 ml) were added to all the samples and the tubes were shaken for 10 min in a Dubnoff mechanical shaker (150 cycles/min). Separation of the two phases was achieved by centrifugation at 1500 *g* for 10 min and 7 ml of the organic phase (lower layer) were transferred to a second tube. Fresh methylene chloride (8 ml) was added to the first tube and the same extraction procedure was repeated twice. The organic phases collected from the three extractions of the same sample were pooled (21 ml). Sodium hydroxide (0.3 *M*) was then added to the serum (250 μl), to the tissue extract (250 μl) and to the urine (500 μl) samples. The tubes were shaken for 10 min and then centrifuged at 1500 *g* for 10 min. The aqueous phase (upper layer) was collected in Eppendorf tubes and frozen (−20° C) until the HPLC assay; 25-μl aliquots were used for chemical analysis.

Chromatography

The equipment used was a Waters HPLC apparatus with a Waters M 6000A pump (Waters Assoc., Milford, MA, U.S.A.), a U6K injector, a Model 440 absorbance ultraviolet detector (wavelength 280 nm), a Leeds Northrup Speedomax XL-682 linear recorder (paper-speed 0.2 cm/min) and a Hewlett-Packard HP 18850A integrator. The chromatographic separation was achieved in a Vydac 10-μm anion-exchange column (25 cm × 4.5 mm; Separations Group, Hesperia, CA, U.S.A.), connected to an AXGU 10-μm anion-exchange precolumn (Rainin Instruments, Woburn, MA, U.S.A.). The mobile phase used was a mixture of acetonitrile and 0.05 *M* phosphate buffer pH 7 in HPLC grade water (20:80, v/v) at a flow-rate of 1.2 ml/min. The mobile phase was prepared daily. The phosphate buffer was filtered through an HA 0.45-μm filter, while the acetonitrile was filtered through an FA 0.5-μm filter (Millipore, Bedford, MA, U.S.A.). The mixture was continuously stirred under vacuum for 30 min

before use. For our control, known amounts of norfloxacin were added to tissue, serum and urine specimens from untreated patients to measure the efficiency of the extraction procedure and to assess inter-assay variations. Solutions of norfloxacin and of its metabolites, prepared in 0.5 *M* sodium hydroxide and maintained at 4°C, were used as the inter-assay standardization. For the mathematical data processing a Texas Instruments TI-59 programmable calculator and a PC-100A printer were used, with a specifically developed computing program.

RESULTS AND DISCUSSION

Fig. 2A—D shows the HPLC separations of norfloxacin from human prostate, kidney, urine and serum, respectively. Quantitation of the compound was obtained by comparing the peak areas from the samples with the peak areas of known amounts of norfloxacin solutions injected directly into the HPLC system. The retention time of norfloxacin, both in the standards and in the extracted samples, was 7.10 min. No interference was observed during the assay between norfloxacin, its major metabolite (M-1 had a retention time of 4.20 min, unmarked peaks of Fig. 2) and the endogenous substances in the clinical samples.

standard solution in 0.3 *M* sodium hydroxide. Standard curves prepared by adding known amounts of norfloxacin to the samples were linear from 1.0 to 500 μg/ml in urine and from 0.01 to 2 μg/ml in kidney; an analogous linearity was found in both serum and prostate samples.

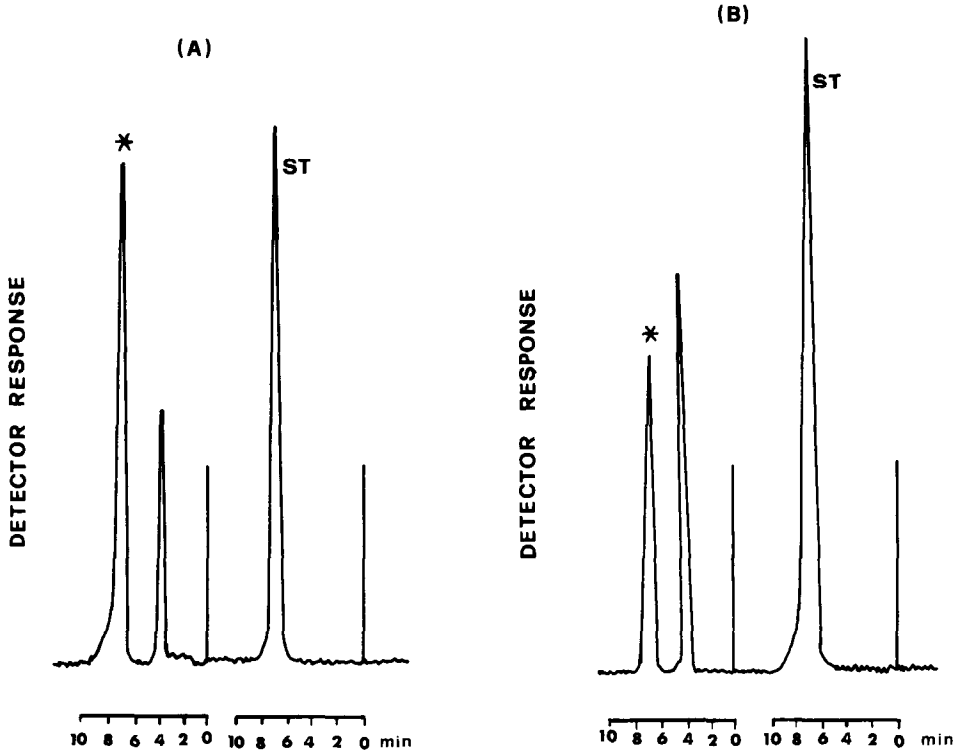


Fig. 2.

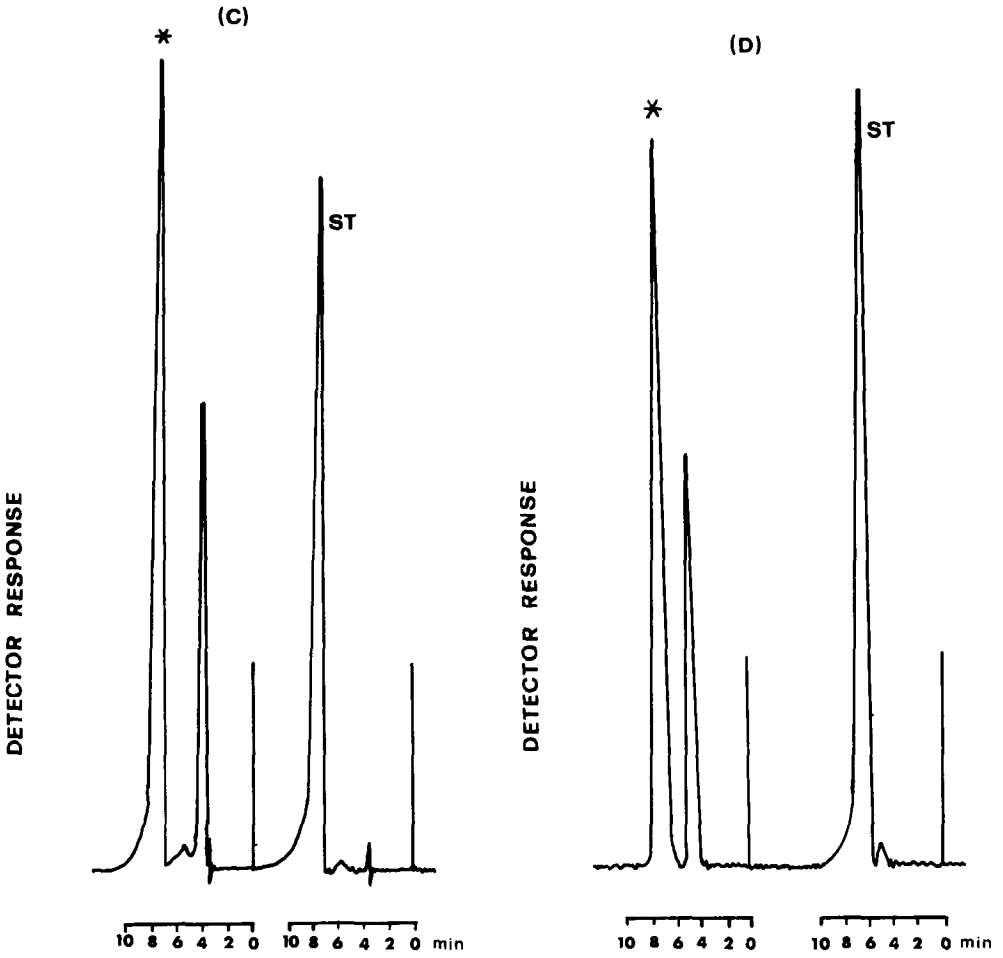


Fig. 2. HPLC chromatograms from human prostate (A), kidney (B), urine (C) and serum (D), after two 400-mg doses of norfloxacin. The norfloxacin peaks are marked with a star. The concentrations of the standard samples of norfloxacin (ST) were 5 μg/ml for A, B and D and 50 μg/ml for C; the calculated norfloxacin concentrations in these samples were 1.14 μg/g of prostate (A), 0.61 μg/g of kidney (B), 115.0 μg/ml of urine (C) and 0.60 μg/ml of serum (D). The unmarked peaks represent the M-1 metabolite of norfloxacin.

Extraction recoveries from prostate, kidney, urine and serum were 90%, 60%, 80% and 60%, respectively. The inter-assay coefficient of variation was 2.2% (eighteen assays) for a 1.25 μg/ml standard.

Table I shows the typical norfloxacin concentrations found in two out of twenty patients examined so far. The data indicate that after only two 400-mg administrations norfloxacin reaches concentrations in the prostate and kidney similar to those attained in the serum. The tissue levels of norfloxacin, compared with the in vitro minimal inhibitory concentrations (MIC, 90%) [5, 6], confirm that the drug attains concentrations in the prostate and kidney that are effective to control infections sustained by the most common pathogens involved in urinary tract infections. The very high concomitant urinary levels are described also by other authors [1, 4] and account for the high rate of success

TABLE I

NORFLOXACIN CONCENTRATIONS IN URINE, SERUM AND EITHER PROSTATE OR KIDNEY IN PATIENTS WHO INGESTED TWO 400-mg DOSES BEFORE SURGERY

Patient	Urine 1 Control (μg/ml)	Urine 2 11 h from 1st dose (μg/ml)	Serum 1 14 h from 1st dose, 30 min from 2nd dose (μg/ml)	Serum 2 15.5 h from 1st dose, 2 h from 2nd dose (μg/ml)	Prostate 15.5 h from 1st dose, 2 h from 2nd dose (μg/g)	Kidney 15.5 h from 1st dose, 2 h from 2nd dose (μg/g)
D.S.R.	0.00	146.25	0.60	1.10	1.14	
I.G.	0.00	115.00	0.40	0.55		0.61

in the treatment of lower urinary tract infections, even due to highly resistant bacterial strains [7].

In our study we have demonstrated a correlation between the clinical observations in complicated and relapsing urinary infections such as prostatitis [3] and the pharmacokinetic properties of norfloxacin, which is able to reach effective antibacterial levels in the examined tissues even after only two 400-mg oral doses.

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