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# Determination of Norfloxacin, a New Nalidixic Acid Analog, in Human Serum and Urine by High-Performance Liquid Chromatography

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A high-performance liquid chromatographic method for the analysis of norfloxacin (MK-366) [1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid], a new nalidixic acid analog, in human serum and urine is described. A statistical evaluation of the assay data showed acceptable accuracy and precision for 0.1 to 10.0 μg of MK-366 per ml of serum and for 1.0 to 500 μg of MK-366 per ml of urine. MK-366 was extracted from serum and urine at pH 7.5 with methylene chloride and back-extracted with sodium hydroxide solution. Chromatography was performed on an anion-exchange column with acetonitrile-phosphate buffer as the mobile phase; UV absorbance was monitored at 273 nm. The method was used to measure MK-366 in clinical specimens.

Norfloxacin (MK-366) [1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid] is a new broad-spectrum antibacterial agent that is structurally related to nalidixic acid (Fig. 1). MK-366 exhibits greater antibacterial activity against both gram-positive and gram-negative bacteria than other nalidixic acid analogs and exhibits greater activity against *Pseudomonas aeruginosa* than gentamicin (1, 2). The in vitro activity of MK-366 against *Neisseria gonorrhoeae* is comparable to that of rosoxacin (3). Activity against several microorganisms has been demonstrated in mice. About 30% of each dose of MK-366 is excreted in the urine as unmetabolized drug, with lesser quantities of glucuronide conjugate and six active metabolites with modifications in the piperazine ring being excreted (Merck Sharp & Dohme Research Laboratories, personal communication).

The potential usefulness of MK-366 in the treatment of urinary tract infections justifies in-depth studies into its pharmacokinetics, metabolism, toxicity, and mode of action. A rapid, specific, and sensitive assay method is essential for such studies. Microbiological assay, the only previous method available for MK-366 measurement, does not distinguish between MK-366 and its active metabolites. This report describes a rapid and specific assay for the measurement of MK-366 in human serum and urine by anion-exchange high-performance liquid chromatography (HPLC).

## MATERIALS AND METHODS

**Chemicals.** MK-366 and six metabolites with modifications in the 7 position {7-(3-oxo-1-piperazinyl), *M-1*; 7-[(2-aminoethyl)amino], *M-2*; 7-[(2-acetylaminoethyl)amino], *M-3*; 7-(4-acetyl-1-piperazinyl), *M-4*; 7-amino, *M-5*; and 7-(4-formyl-1-piperazinyl), *M-6*} were obtained from Merck Sharp & Dohme Research Laboratories, West Point, Pa. Standard solutions of MK-366 were prepared in 0.05 N sodium hydroxide and were stable at 4°C. HPLC-grade water, acetonitrile, and methylene chloride were purchased from Fisher Scientific Co., Pittsburgh, Pa.

**Extraction of samples.** Serum (1 ml) or urine (200 μl) was thoroughly mixed with 100 μl of 0.05 N NaOH (containing standards preparing the standard curve) in a 40-ml glass extraction tube. Methylene chloride (11 ml) and 0.5 M sodium phosphate buffer (0.5 ml, pH 7.5) were then added, and the tubes were gently shaken for 10 min on a mechanical shaker. After phase separation by centrifugation at 1,500 × *g* for 5 min, 10 ml of the methylene chloride layer was transferred to a second 40-ml extraction tube. Fresh methylene chloride (11 ml) was added to the first extraction tube, and a second extraction was performed; 10 ml of the second extract was pooled with the first organic phase. Pooled extracts were equilibrated with 0.3 N NaOH (250 μl for serum samples and 500 μl for urine samples) on a mechanical shaker. After centrifugation, a portion of the final aqueous phase was transferred to autosampler vials, and 20 μl was injected into the liquid chromatograph.

**Chromatography.** A gradient liquid chromatograph (model 272; Waters Associates, Milford, Mass.) equipped with an autosampler (Waters Intelligent Sample Processor model 710B) and a data module (Waters Data Module model 730) were used for drug analysis. A Vydac 10-μm anion-exchange column (25 cm by 4.6 mm; Separations Group, Hesperia, Calif.) with a LiChrosorb 10-μm anion-exchange guard column (Rainin Instrument Co., Woburn, Mass.) was used. A variable-wavelength detector (model 8400; Spectra Physics, Santa Clara, Calif.) was used to monitor the eluate at 273 nm. The mobile phase

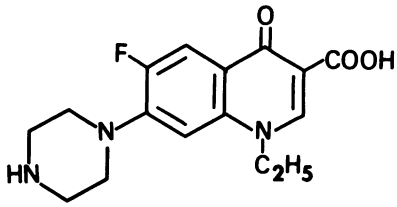


FIG. 1. Structure of MK-366.

consisted of a mixture of acetonitrile and 0.05 M (pH 7.0) phosphate buffer (20:80, vol/vol) at a flow rate of 1.2 ml/min. The mobile phase was prepared daily, filtered through a 0.8-μm membrane filter, and then continuously degassed with helium throughout the day.

To assess interassay variation, we added known amounts of MK-366 to serum and urine and stored portions at −20°C. These quality control samples were assayed daily along with clinical specimens.

**Clinical samples.** Blood and urine were collected from a normal male volunteer after oral administration of 400 mg of MK-366. Serum and urine samples were stored at −20°C.

## RESULTS AND DISCUSSION

HPLC methods have been developed for nalidixic acid (5) and the closely related drugs rosoxacin (4) and pipemidic acid (6). We observed that MK-366 could not be eluted from Spherisorb (silica), LiChrosorb $NH_2$, Vydac cation exchange, and Spherisorb ODS columns (Applied Science Laboratories, State College, Pa.) under a variety of mobile phase conditions. Elution from both Vydac anion-exchange and LiChrosorb RP-8 columns could be accomplished; however, anion-exchange chromatography was clearly superior in separating MK-366 from its major metabolites as well as from endogenous substances in serum and urine (Fig. 2 and 3). *M-3*, *M-4*, and *M-6* eluted as a cluster of peaks just before MK-366, whereas *M-5* eluted about 1 min after the parent drug. In clinical specimens, total peak area for these metabolites was less than 3% of the area for the MK-366 peak. When *M-1*, the major urinary tract metabolite, was injected directly into the column, its retention time (5.3 min) was close to that of MK-366 (6.1 min). However, after extraction into 0.3 N NaOH, *M-1* was rapidly converted to another compound (retention time, 3.5 min) which coeluted with *M-2*, suggesting that *M-2* may be an artifact of isolation procedures rather than an

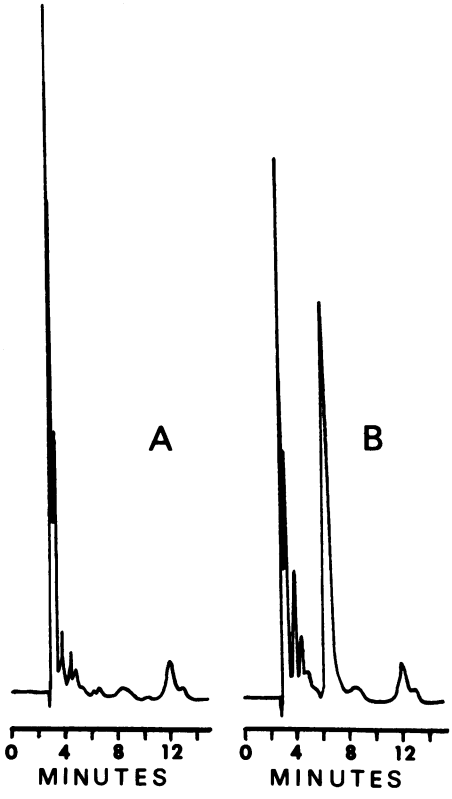


FIG. 2. Chromatograms for serum obtained before (A) and 2 h after (B) oral administration of 400 mg of MK-366. The concentration of MK-366 was 1.5 μg/ml.

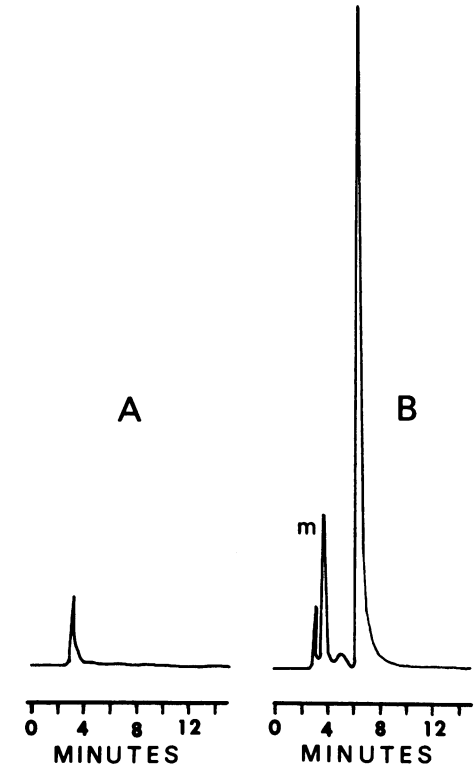


FIG. 3. Chromatograms for urine obtained before (A) and 6 to 8 h after (B) oral administration of 400 mg of MK-366. The concentration of MK-366 was 239 μg/ml. The peak at 3.5 min (m) is a base-catalyzed degradation product of *M-1*, a major metabolite of MK-366.

TABLE 1. Variation in replicate standards extracted from human serum and urine[a]

| Sample type | Standard concn (μg/ml) | Assay concn (mean ± SD) | Coefficient of variation (%)[b] |
|---|---|---|---|
| Serum (1.0 ml) | 0.20 | 0.21 ± 0.01 | 4.8 |
| | 2.0 | 2.04 ± 0.02 | 1.0 |
| | 10.0 | 10.1 ± 0.08 | 0.8 |
| Urine (0.2 ml) | 5 | 4.99 ± 0.21 | 4.2 |
| | 50 | 50.9 ± 2.09 | 4.1 |
| | 500 | 503 ± 4.68 | 0.9 |

[a] Six samples were tested at each concentration.
[b] (Standard deviation/mean) × 100.

actual metabolite of MK-366. This conversion was complete within 90 min of extraction at room temperature. *M-1* would, therefore, not be observed in chromatograms for clinical specimens, as these were not injected onto the HPLC column until 2 h or more after extraction. MK-366 was stable for at least 48 h under the same experimental conditions. Cinoxacin and nalidixic acid eluted earlier than MK-366 and hence did not interfere with the MK-366 assay.

With peak areas as the basis of quantitation, the lower limits of assay sensitivity for MK-366 were 0.1 μg/ml in serum and 1.0 μg/ml in urine. Standard curves for MK-366 were linear from 0.1 to 20 μg/ml in serum and from 1.0 to 500 μg/ml in urine. Extraction recoveries from serum and urine, determined by comparing the detector response to standards injected directly into the column with the response to processed standards of MK-366, were 88 and 95%, respectively. Intraassay variability for the method was assessed by preparing replicate standards from both serum and urine (Table 1). Interassay coefficients of variation were ±6.1% for a 2-μg/ml serum standard (33 assays) and ±5.5% for a 200-μg/ml urine standard (47 assays).

TABLE 2. MK-366 in serum and urine after a 400-mg dose

| Serum | | Urine | | |
|---|---|---|---|---|
| Collection time (h) | Concn (μg/ml) | Collection interval (h) | Concn (μg/ml) | Amt excreted (mg) |
| 0 | 0 | 0–1 | 203 | 12.8 |
| 0.5 | 0 | 1–2 | 710 | 40.5 |
| 1.0 | 1.33 | 2–3 | 611 | 23.2 |
| 1.5 | 1.41 | 3–4 | 387 | 24.4 |
| 2.0 | 1.50 | 4–6 | 155 | 23.3 |
| 3.0 | 1.14 | 6–8 | 239 | 16.0 |
| 4.0 | 1.11 | 8–12 | 157 | 14.0 |
| 6.0 | 0.67 | | | |
| 8.0 | 0.56 | | | |
| 12.0 | 0.38 | | | |

Analytical results for over 1,300 clinical specimens demonstrated that the assay method is sensitive enough to easily measure MK-366 in both serum and urine after standard therapeutic doses of the drug. Typical results for one subject who received 400 mg of MK-366 orally are shown in Table 2.

In conclusion, we have described a highly specific and sensitive assay method for MK-366 in serum and urine which is suitable for pharmacokinetic studies in humans.

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