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Note

High-performance liquid chromatography and preliminary pharmacokinetics of enoxacin and its 4-oxo metabolite in human plasma, urine and saliva

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Enoxacin [1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-1,8-naphthyridine-3-carboxylic acid; CI 919, AT-2266] is a new broad-spectrum antibacterial agent of the quinolone—azaquinolone class. Based on minimal inhibitory concentrations (MIC), enoxacin shows a high activity against *Pseudomonas aeruginosa* [1]. Nakamura et al. [2] described a determination by high-performance liquid chromatography (HPLC) of enoxacin and its metabolites in human plasma and urine; this method, however, requires a relatively large sample volume of 1 ml. Enoxacin is metabolized by hydroxylation and oxidation to form 4-oxo-enoxacin. Another metabolic route is conjugation with formic and acetic acid, ring opening and deamination of the piperazinyl ring. The 4-oxo metabolite accounts for 10–15% of the ingested dose, and the four other metabolites each constitute less than 1% of the dose. This paper describes a reversed-phase liquid chromatographic determination of enoxacin and its 4-oxo metabolite in human plasma, urine and saliva.

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Pharmacokinetic studies of enoxacin were carried out with two healthy volunteers in order to obtain a preliminary kinetic profile.

MATERIALS AND METHODS

Chromatography

A Spectra-Physics 3500B liquid chromatograph was used, equipped with a variable-wavelength spectrophotometer detector (Model SP 770). The detector was connected to a 10-mV recorder (BD7, Kipp & Zonen, The Netherlands): the chart speed was 1 cm/min. A stainless-steel column (15 cm × 4.6 mm I.D.) was packed by a slurry technique with LiChrosorb RP-2, particle size 5 μm (Merck, Darmstadt, F.R.G.). In order to avoid contamination of the analytical column, a guard column (7.5 cm × 2.1 mm I.D.) tap-filled with pellicular reversed-phase material (Chrompack, Middelburg, The Netherlands) was placed between the sampling valve and the analytical column. Detection of enoxacin was effected at 342 nm, detection of its oxo metabolite in plasma and saliva at 265 nm, in urine at 342 nm. The mobile phase was 7 m*M* phosphoric acid–dimethylformamide– ethanol (77:20:3). The chemicals were of analytical grade (Merck). The solvent flow-rate was 1.6 ml/min at a pressure of 23.5 mPa. The injection loop was 100 μl. Chromatographic analysis was carried out at room temperature.

Drugs

Enoxacin and its 4-oxo metabolite were obtained from Warner Lambert (Substantia, Ir. T. van Elzakker).

Subjects and patients

One helathy male (A, 91 kg, 35 years) and one healthy female (B, 63 kg, 30 years) volunteered for this study. A dose of 400 and 600 mg of enoxacin was administered to each volunteer in two different experiments. Blood and saliva were collected at 0.5, 1, 1.5, 2, 3, 4, 6, 9, 12 and 15 h after drug administration. Blood samples were prevented from clotting by 0.5 mg of solid heparin and then centrifuged; the plasma was separated and kept at −20°C until analysis. Spontaneously voided urine was collected for 24 h. All samples were kept frozen at −20°C until analysis.

Sample preparation

Plasma. To 0.1 ml of plasma was added 0.3 ml of 3% trichloroacetic acid and mixed thoroughly on a vortex mixer. The mixture was allowed to stand for 10 min and centrifuged at 2600 *g* for 10 min. A 100-μl volume of the clear supernatant was injected onto the column.

Urine. Urine was diluted 50 times with the mobile phase. The solution was mixed and 100 μl were injected onto the column.

Deglucuronidation of plasma and urine samples. To 0.1 ml of plasma was added 0.1 ml of a β-glucuronidase solution (1000 U per 10 ml of phosphate buffer, pH 6.8; Sigma, St. Louis, MO, U.S.A.). The solution was incubated at 37°C. After 16 h of incubation, 0.2 ml of 5% trichloroacetic acid was added, mixed thoroughly and centrifuged. A 100-μl volume of the clear supernatant was injected onto the column.

To 0.1 ml of urine was added 0.4 ml of 0.1 *M* acetate buffer (pH 5.0) and 10 μl of a β-glucuronidase solution (100 000 U/ml of water; Sigma). The solution was incubated at 37°C for 16 h, then 0.1 ml of the mixture was diluted ten times with the mobile phase and 100 μl were injected onto the column.

Saliva. After thawing, saliva and sputum samples were homogenised either on a vortex mixer or by ultrasonication and centrifugation at 2600 *g* for 15 min. A 0.1-ml volume of the sample was treated as for plasma.

Protein binding

Plasma protein binding was estimated after ultrafiltration by using EMIT® Free Level™ filters (Syva, Palo Alto, CA, U.S.A.; Merck, Amsterdam, The Netherlands). To 100 μl of the ultrafiltrate, 200 μl of water were added, and 100 μl of the mixture were injected onto the column. Filters were pre-tested with known amounts of enoxacin and its metabolite in phosphate buffer (pH 7.4). Both compounds were filtered to 100%.

RESULTS

Chromatography

Enoxacin is well separated from its 4-oxo metabolite (Fig. 1). Absorption maxima of enoxacin in 0.1 *M* sodium hydroxide are at 345 and 265 nm, for the 4-oxo metabolite at 342 and 265 nm. Sensitivity for both compounds is about two times higher at 265 nm. $E^{1\%}_{1\,cm}$ is 1300 for enoxacin and 900 for the 4-oxo metabolite.

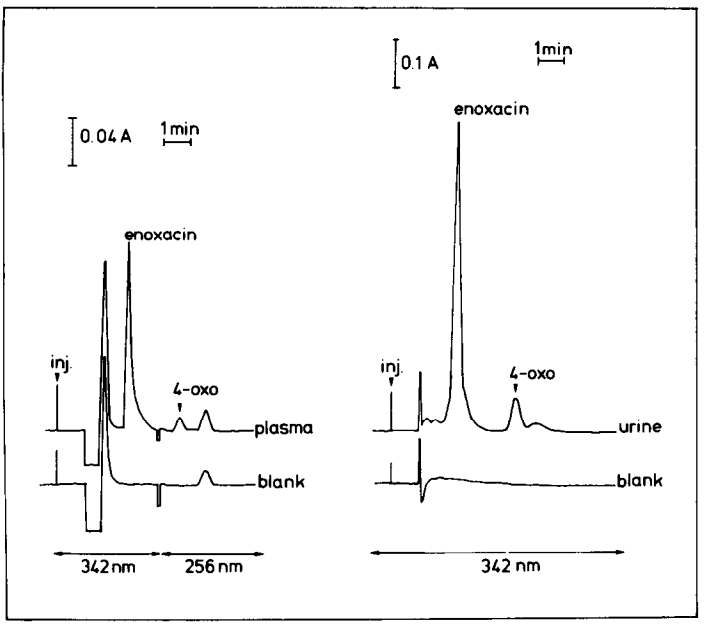

Fig. 1. HPLC profiles of enoxacin and its 4-oxo metabolite (left) in plasma in concentrations of 4.7 μg/ml enoxacin and 0.88 μg/ml 4-oxo-enoxacin, and (right) in urine in concentrations of 282 μg/ml enoxacin and 74 μg/ml 4-oxo-enoxacin.

Owing to interfering peaks at 265 nm at the beginning of the chromatogram of plasma samples, enoxacin was measured at 342 nm, and once the enoxacin peak appeared, the detection wavelength was set at 265 nm to estimate the 4-oxo metabolite. Urine samples were measured at 342 nm. The minimum detectable concentrations of enoxacin and its metabolite in plasma and saliva were 0.05 μg/ml, and in urine 0.5 and 1 μg/ml, respectively.

The recovery of enoxacin added to human plasma in the concentration range 0.8–12 μg/ml was 81.7 ± 2.8%. The recovery of the 4-oxo metabolite was 51.9 ± 3.9% in the concentration range 0.1– 2.5 μg/ml.

Calibration curves of enoxacin and its 4-oxo metabolite were linear: r = 0.9998 and r = 0.9990, respectively. The recovery for both compounds added to the urine and saliva was 100 ± 2%.

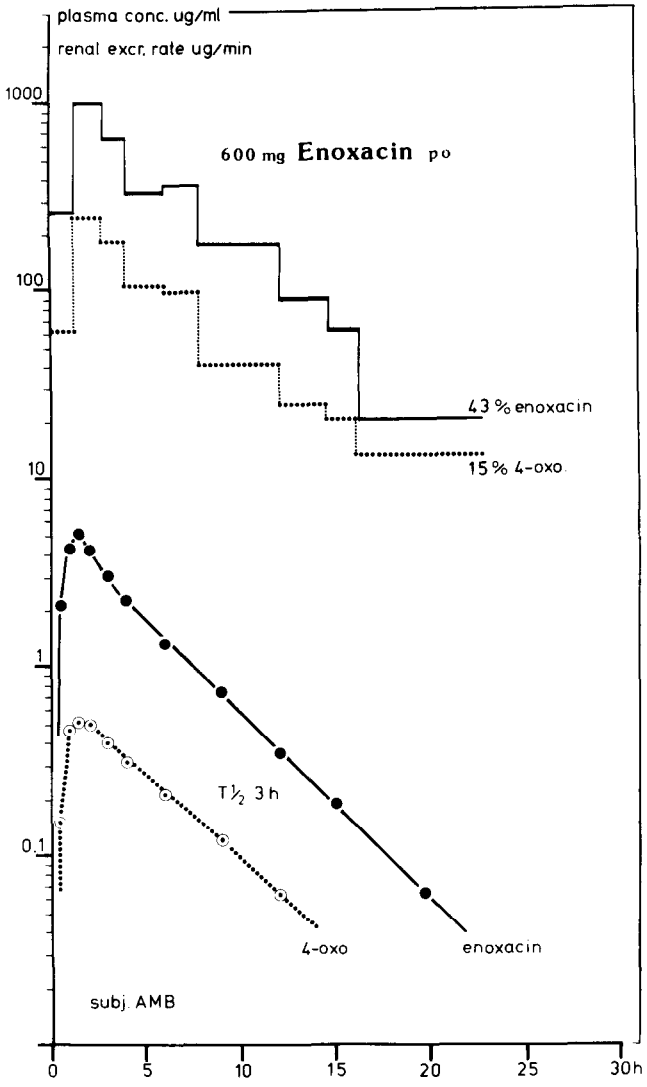

Fig. 2. Plasma concentration—time curves and renal excretion rate—time profiles of enoxacin and its metabolite 4-oxo-enoxacin in a volunteer after an oral dose of 600 mg of enoxacin.

In order to estimate enoxacin and its metabolite in saliva it is necessary for the subject to abstain from drinking tea and coffee, to prevent interfering peaks appearing in the chromatogram.

Pharmacokinetics

Fig. 2 shows the plasma concentration–time curves and renal excretion rate- time profiles of enoxacin and its 4-oxo metabolite in a volunteer after an oral dose of 600 mg of enoxacin. The half-lives of enoxacin and its metabolite are identical (3 h in this subject) and vary between 3 and 7 h, depending on the subject and the dose. Approximately 50% of the ingested dose is excreted in 24 h in the urine, 40% as parent drug and 10% as 4-oxo metabolite. Table I shows some pharmacokinetic parameters of enoxacin and its metabolite in the subjects A and B.

Protein binding

Plasma protein binding for enoxacin and its 4-oxo metabolite was ca. 55% as shown in Table II.

Urine

Urinary concentrations ranged from 0.5 to 1500 μg/ml for enoxacin and from 1 to 500 μg/ml for its metabolite. The renal clearance constant, the

TABLE I

SOME PHARMACOKINETIC PARAMETERS OF ENOXACIN IN HUMANS

Subject	Dose (mg)	Maximum plasma concentration (μg/ml)		Excreted in urine in percentage of the dose	
		Enoxacin	4-Oxo	Enoxacin	4-Oxo
A	400	1.8 (1.5)*	0.24 (1.5)	45.3	13.8
A	600	2.3 (1.5)	0.24 (1.5)	38.1	10.0
B	400	3.6 (1.0)	0.53 (1.0)	40.0	16.3
B	600	3.5 (1.0)	0.85 (1.0)	30.2	18.0

*The values between parentheses indicate the time (h) at which the maximum plasma concentration is reached.

TABLE II

HALF-LIFE AND PROTEIN BINDING OF ENOXACIN AND ITS METABOLITE IN HUMANS

Subject	Half-life (h)		Protein binding		Dose (mg)
	Enoxacin	4-Oxo	Enoxacin	4-Oxo	
A	5.3	5.3	55.4 ± 3.1	n.d.*	400
A	7.0	7.0	58.4 ± 6.3	54.2 ± 3.8	600
B	4.0	4.0	52.0 ± 3.0	n.d.*	400
B	3.0	3.0	55.1 ± 5.3	55.2 ± 8.1	600

*Not measurable, concentration too low.

proportionality constant between the renal excretion rate (μg/min) and the plasma concentration (μg/ml), was 214 ± 21 ml/min for the parent drug and 404 ± 51 ml/min for the metabolite in the two volunteers. After deglucuronidation of urine and plasma, no glucuronide of enoxacin or its metabolite could be detected.

DISCUSSION

This HPLC method enables the measurement of plasma, urine and saliva concentrations of enoxacin and its 4-oxo metabolite in humans and can be used for the determination in patients. The method of Nakamura et al. [2] requires a relatively large sample volume of 1 ml, but is able to detect four other metabolites. The presence of these metabolites seems of less clinical importance, as each of them accounts for 1% or less of the administered dose.

Similar HPLC methods and metabolic profiles have been reported for a structural analogue of enoxacin, i.e. norfloxacin [3, 4].

Enoxacin is mainly eliminated by renal excretion with a high renal clearance (240 ml/min). The metabolite 4-oxo-enoxacin shows an even higher renal clearance of 400 ml/min. This means that both compounds are excreted by glomerular filtration and active tubular secretion. Approximately 50% of the dose is recovered from the urine, which corresponds to the low bioavailability of 50% reported by Eandi et al. [3]. The elimination of enoxacin depends totally on the renal clearance. Therefore, in patients with impaired renal function, the elimination will be slower and the half-life longer.

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