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Reversed-phase high-performance liquid chromatographic determination of enoxacin and 4-oxo-enoxacin in human plasma and prostatic tissue
Application to a pharmacokinetic study

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Abstract

A simple high-performance liquid chromatographic method has been developed for the simultaneous determination of enoxacin and 4-oxo-enoxacin in plasma and prostatic tissue. The work-up procedure involves a liquid–liquid extraction step followed by isocratic chromatography on a reversed-phase analytical column, with ultraviolet absorbance detection (λ=340 nm). Using a mobile phase of 20.9% (v/v) acetonitrile buffer (pH 2.1), adequate retention time and separation among the analytes has been obtained using tetrabutylammonium hydroxide included in the eluent. Retention times are 5.2 min for enoxacin, 6.8 min for pefloxacin and 12 min for 4-oxo-enoxacin. For plasma and prostatic tissue, the precision of the assay was below 9%. The percent recovery from the nominal values for accuracy ranged from 94 to 108%. The limits of quantitation were 20 ng/ml for plasma and 50 ng/g for tissue (precision <18%). The detection limits were 10 ng/ml and 25 ng/g, respectively. The calibration curves were linear from 20 to 1000 ng/ml for plasma and from 50 to 2500 ng/g for tissue. In plasma, the extraction recoveries averaged 52% for enoxacin and 63% for 4-oxo-enoxacin. In prostatic tissue, they were 57 and 76% for the two analytes, respectively. This method has been employed for the determination of enoxacin and 4-oxo-enoxacin in plasma and prostatic tissue samples from patients following repeated oral administration of enoxacin (400 mg twice a day for four days).

Keywords: Enoxacin; Oxo-enoxacin; Fluoroquinolones; Antibiotics; Pefloxacin

1. Introduction

Enoxacin, is a synthetic antibacterial drug of the fluoroquinolone class. Like other fluoroquinolones, enoxacin is rapidly bactericidal against a broad spectrum of bacteria [1]. Enoxacin shows rapid concentration-dependent bacterial killing (similar to that of the aminoglycosides) with a long postantibiotic effect and activity against organisms in their stationary phase [1]. The antibacterial activity of enoxacin is related to its ability to inhibit the A-subunit of bacterial DNA-gyrase [1]. Enoxacin is effective in the treatments of (i) infections in the upper and lower respiratory tract caused by susceptible organisms, (ii) infections at all levels of the urinary tract, (iii) sexually transmitted diseases, (iv) infections of the gastrointestinal tract, (v) osteomyelitis caused by susceptible strains of staphylococci and Gram-negative bacteria, (vi) endocarditis and meningitis [1]. Enoxacin is likely to prove useful in

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the prophylaxis of Gram-negative rod bacteraemia in neutropenic patients [1]. Enoxacin is formulated for oral and intravenous administrations. This drug is minimally bound to serum proteins and has a large volume of distribution (about 200 l); it has a serum elimination half-life of about 5 h, justifying the usual 12-h dosage intervals [2–4]. Total clearance averaged 480 ml/min. Approximately 60% of enoxacin is excreted unchanged in the urine and another 10–15% is excreted as an active metabolite (4-oxo-enoxacin) [4]. Other pathways of minor importance are N-formylation, N-acetylation, the cleavage of the piperazine ring [formation of 7-(2-aminoethylamino) metabolite] and the total breakdown of the piperazine ring (formation of 7-amino metabolite), each of them representing less than 1% of the administered dose [5]. The metabolic biotransformation occurs primarily at the piperazinyl ring in the C_7 position. Enoxacin has been reported to have an oral bioavailability of about 77–90% [2,4].

Some methods have been described for the determination of enoxacin in biological samples [6–9].

The present paper describes an isocratic reversed-phase high-performance liquid chromatography (HPLC) method for the separation and measurement of enoxacin and its metabolite, the 4-oxo-enoxacin, in human plasma and prostatic tissue. Another fluoroquinolone, pefloxacin, was used as internal standard (I.S.). Fluoroquinolones are zwitterions with the pK_a values of ~6 and ~8 for the two amino groups [8]. At acidic pH, these compounds will possess a net positive charge. In order to reduce the retention times of the solutes while retaining the separation among them, tetrabutylammonium hydroxide (TBA) was included in the eluent [10]. This method was validated with respect to accuracy, precision, selectivity and limits of quantitation (LOQs) and of detection (LODs) according to Good Laboratory Practice Guidelines [11–13]. Moreover, stability tests under various conditions have been performed.

2. Experimental

2.1. Chemicals and reagents

Enoxacin (1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-piperazin-1-yl-1,8-naphthyridine-3-carboxylic acid) and 4-oxo-enoxacin were supplied by the Research Institute Pierre Fabre (Castres, France). These drugs were stored in a refrigerator (about 4°C). The I.S. (pefloxacin) was a gift from Roger Bellon Laboratories (Paris, France) and was stored at room temperature (20°C). The structures of enoxacin, 4-oxo-enoxacin and the I.S. are shown in Fig. 1.

Acetonitrile, methylene chloride and isopropyl alcohol and dipotassium hydrogenphosphate and potassium dihydrogenphosphate were obtained from Carlo Erba (Val de Reuil, France). All solvents were HPLC-grade and used without further purification. Citric acid, ammonium perchlorate, TBA (40%, w/v) were all analytical-grade and obtained from Merck (Darmstadt, Germany), Fluka (Buchs, Switzerland) and Sigma (St. Louis, MO, USA), respectively.

The phosphate buffer solution (pH 7.4) consisted of dipotassium hydrogenphosphate (28.2 g) and potassium dihydrogenphosphate (5.17 g) in distilled water (1 l).

For the validation of the method human plasma was obtained from pooled blood samples collected from healthy volunteers. Coagulation was prevented by adding EDTA–sodium salt then, the blood was centrifuged at 2000 *g* for 10 min. Prostatic tissue was obtained from patients hospitalized for prostatic

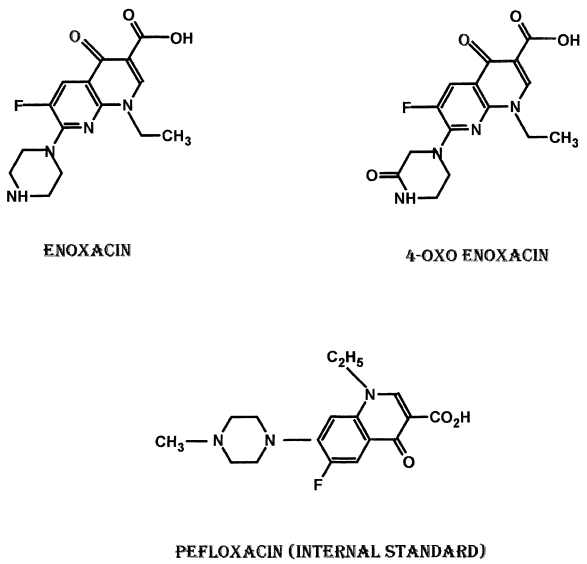

Fig. 1. Structural formulae of enoxacin, 4-oxo-enoxacin and pefloxacin.

surgery. Prostatic tissue and plasma samples were stored at −30°C before use.

2.2. Stock solutions and standards

Stock solutions of enoxacin and 4-oxo-enoxacin were prepared by dissolving 25 mg of each compound in 25 ml of 0.008 *M* sodium hydroxide. These solutions could be stored at −20°C for over one month with no evidence of decomposition. Working solutions were obtained by dilution of the stock solutions with distilled water in the ratio 1:40, 1:100, 1:400 and 1:1000, extemporaneously. Stock solution of pefloxacin (3.2 g/l) was prepared in distilled water. This solution could be stored at 4°C for over six months. Working solution (8 mg/l) was obtained by dilution with purified water 400-fold, extemporaneously. Specific volumes of the working solutions containing enoxacin and 4-oxo-enoxacin were used to spike the plasma (range 20–1000 ng/ml) or prostatic tissue (range 50–2500 ng/g) samples prior to extraction.

An unextracted working aqueous solution containing the two drugs to be analysed at a concentration of 300 ng/ml and the I.S. at a concentration of 320 ng/ml was prepared daily to check the resolution of the chromatographic system.

2.3. Chromatographic system and conditions

The apparatus used for the HPLC analysis was a Hewlett-Packard multisolvent delivery system HP 1100 (Hewlett-Packard, Les Ulis, France) equipped with an autosampler, an oven and a variable-wavelength UV detector (Model HP 1050). Data acquisition and treatment was performed with an IBM computer using the ChemStation G2170 AA. Separation was carried out at 25°C on a C_{18} Nucleosil column (250×4 mm I.D., 5 μm particle size; Merck). A guard column (LiChrospher 100 RP-18, 4×4 mm I.D., 5 μm particle size; Merck) was placed just before the inlet of the analytical column to reduce contamination of the analytical column. The mobile phase was an acetonitrile–salt solution (pH 2.1) and prepared as follows. Citric acid (18.1 g) and ammonium perchlorate (4.1 g) were dissolved in distilled water (about 300 ml), and 209 ml of acetonitrile was added. The mixture was diluted to yield 1 l of solution, then 3 ml of TBA was added. This mixture was filtered through a 0.45-μm HV filter (Millipore, Bedford, MA, USA) prior to use and vacuum deaerated throughout the chromatographic run. A flow-rate of 0.9 ml/min which corresponds to a pressure of about 1.50 MPa was employed with UV detection at 340 nm, which provided optimal absorbance for all two analytes.

2.4. Sample processing

In a 10-ml screw-capped glass centrifuge tube, 0.5 ml plasma, 20 μl of I.S. (pefloxacin, 8 mg/l) and 0.5 ml of phosphate buffer were added and vortex-mixed briefly. A 5 ml volume of methylene chloride–isopropanol (4:1, v/v) was added to the sample and vortex-mixed for 30 s. The tubes were gently shaken for 15 min in an electric shaker and then centrifuged for 10 min at 1500 *g*. The lower layer was separated using phase separators silicone treated filter paper Whatman 1 PS (Prolabo, Paris, France) and the aqueous phase was discarded. The organic phase was transferred to a fresh tube and evaporated under N_2 at 25°C. A 100-μl aliquot of mobile phase was added to the tubes and 20 μl of the sample was injected into the column.

Prostatic tissues were powdered under liquid nitrogen. An aliquot of 200 mg was weighed in a 10-ml screw-capped glass centrifuge tube then 20 μl of I.S. solution was added. The mixture was vortex-mixed briefly, then incubated at 4°C for 2 h, to allow a steady-state tissue protein binding. The assay procedure completed as for plasma samples described above.

2.5. Instrument calibration

Calibration standards in plasma were prepared using concentrations of 20, 80, 143, 333, 500 and 1000 ng/ml for enoxacin and 4-oxo-enoxacin. In the prostatic tissue, the following concentrations were prepared: 50, 100, 150, 312.5, 625, 1250 and 2500 ng/g; then after vortex-mixing, the mixture was incubated at 4°C for 2 h before treatment to yield a steady-state tissue protein binding. The volume added was always smaller than or equal to 2% of total volume of the samples, so that the integrity of the biological samples was maintained.

2.6. Data analysis

From recorded peak areas the ratios of the drug to I.S. were calculated. Unweighted least squares linear regression of the peak-areas as a function of the theoretical concentrations was applied to each standard curve. The resulting slopes and intercepts were used to obtain concentration values for that day's quality control samples and unknown samples.

The linearity of the method was confirmed by comparing the slopes and the intercepts of linear calibration curves with zero, and the correlation coefficients with 1. Moreover, the Kolmogorov–Smirnov test was used to compare the distribution of the residuals (difference between nominal and back-calculated concentrations) to the expected one [$N(0,1)$].

2.7. Specificity

The specificity of the method was verified against endogenous compounds due to the matrices. Several blank plasma and prostatic tissues from different healthy subjects and patients were tested for the absence of interfering compounds. The retention times of endogenous compounds in the matrix were compared with that of enoxacin, 4-oxo-enoxacin and I.S.

To investigate the possibility of interference by other antibiotics known amounts of ciprofloxacin, ofloxacin, vancomycin, amikacin, tobramycin, rifampicin, roxythromycin, fosfomycin were added to blank plasma samples. These samples were treated according to the described sample preparation. In addition for drugs that were metabolized in the body, plasma samples from treated patients were checked for interference.

2.8. Precision and accuracy

Both inter-day and intra-day reproducibilities of the assay were tested. Three concentrations of enoxacin and 4-oxo-enoxacin in plasma and tissue were included in this study, the first high (800 ng/ml, 2000 ng/g), the second middle (400 ng/ml, 1000 ng/g) and the third low (50 ng/ml, 125 ng/g). Several aliquots of each samples were tested the same day to determine the intra-day reproducibility. Aliquots of the same sample were tested once a day during different days to determine inter-day reproducibility.

The accuracy was expressed as error percent [(mean of measured−mean of added)/mean of added]×100, while the precision was given by the inter-day and intra-day relative standard deviations (R.S.D.s).

2.9. Determination of the limits of quantitation (LOQ) and detection (LOD)

The LOQ was defined as the lowest antibiotic concentrations which can be determined with an accuracy and precision $<20\%$ on a day-to-day basis [10,11]. To determine the analytical error in the LOQ, spiked samples were used.

The LOD was defined as the sample concentrations resulting in a peak area of three times the noise level.

2.10. Recovery study

Five samples of blank plasma were spiked with 80, 143, 333, 500 and 1000 ng/ml of enoxacin and 4-oxo-enoxacin and seven samples of prostatic tissue were spiked with 50, 100, 150, 312.5, 625, 1250 and 2500 ng/g of the two drugs. They were assayed and the resulting peak areas were compared with peaks resulting from aqueous solutions at the same concentrations.

The extraction efficiency was also determined for the I.S.

2.11. Stability of enoxacin and 4-oxo-enoxacin in plasma and prostatic tissue

For stability studies, quality control (QC) concentrations in plasma and tissue representing the low (50 ng/ml, 125 ng/g), middle (400 ng/ml, 1000 ng/g) and high (800 ng/ml, 2000 ng/g) concentrations were used. QC samples were analyzed immediately after preparation (reference values) and after storage. Each determination was performed in triplicate. Concentrations of each analyte were determined against a calibration curve.

The short-term stability of enoxacin and its metabolite in human plasma and prostatic tissue was

assessed at 1, 2, 4 and 6 h after bench-top storage at both ordinary laboratory conditions (20°C) and at 4°C.

The protocol was repeated after 1, 2, 4 and 6 h, and 15, 30, 60 and 90 days storage at −30°C and −80°C. Prior to their analyses, samples were brought to room temperature and thoroughly vortex-mixed.

The freeze–thaw stability was also determined. Spiked samples were analyzed on a daily basis after repeated freezing–thawing cycles at −30°C on three consecutive days.

The stability of enoxacin and 4-oxo-enoxacin in reconstituted extracts was inspected during 20 h at room temperature (20°C) and over a period of four days at 4°C.

2.12. Pharmacokinetic study

Enoxacin (400 mg twice a day) was given orally to 20 patients over a period of four days. These patients were hospitalized for surgery as part of treatment for prostatic adenoma (suprapubic prostatectomy), or for prostatic cancer (bilateral pulpectomy). The patients were enrolled in the study after giving written informed consent. The study protocol was approved by the Regional ''Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale''. Blood samples were collected before administration and 0.5, 1, 2, 3, 4, 8 and 12 h after the administration on days 1 and 4. Additionally, blood samples were drawn just prior the first doses on days 2–3 and at 16, 24 and 48 h post-dose on day 4. The pharmacokinetic parameters were obtained using the SIPHAR computed program [14].

3. Results

3.1. Chromatography

In order to reduce the retention times of the analytes while retaining the separation among them, and to obtain an acceptable asymmetry coefficient, TBA was included in the eluent. The capacity factor and the asymmetry coefficient as a function of the TBA concentration (0, 1, 2, 3 and 4 ml added to 1000 ml of eluent) is shown in Fig. 2; each determination is the mean value from three replicates.

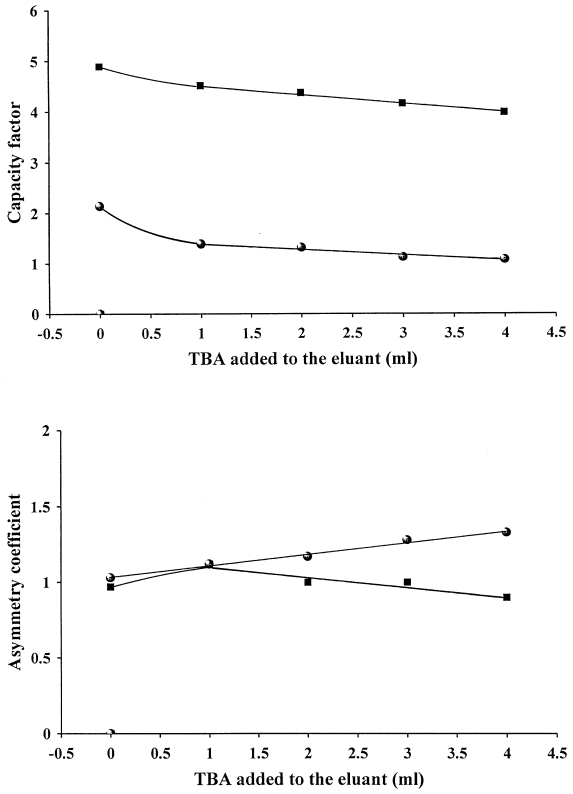

Fig. 2. Plot showing variation of capacity factor and asymmetry coefficient with mobile phase TBA concentration (0, 1, 2, 3 and 4 ml added to 1000 ml of eluent). Enoxacin (●), 4-oxo-enoxacin (■).

The retention of all the fluoroquinolones decrease as mobile phase TBA increases, and peak sharpening as a consequence of decrease in retention time was also observed. In addition, the asymmetry coefficients were modified (Fig. 2). Thus, in the presence of TBA at 3:1000 ml we obtained a good compromise between the retention time and the asymmetry coefficient.

Under the chromatographic conditions used, the number of theoretical plates was approximately 7500. The pre-column was exchanged every 100 sample runs and the column was replaced when the number of theoretical plates had decreased below 6000. Representative chromatograms are shown in Fig. 3 for plasma and in Fig. 4 for prostatic tissue. There was clear resolution of the compounds of interest ($\alpha_{1,2}=1.7$, $\alpha_{2,3}=2.3$) which had retention times of 5.2 min for enoxacin, 6.8 min for pefloxacin

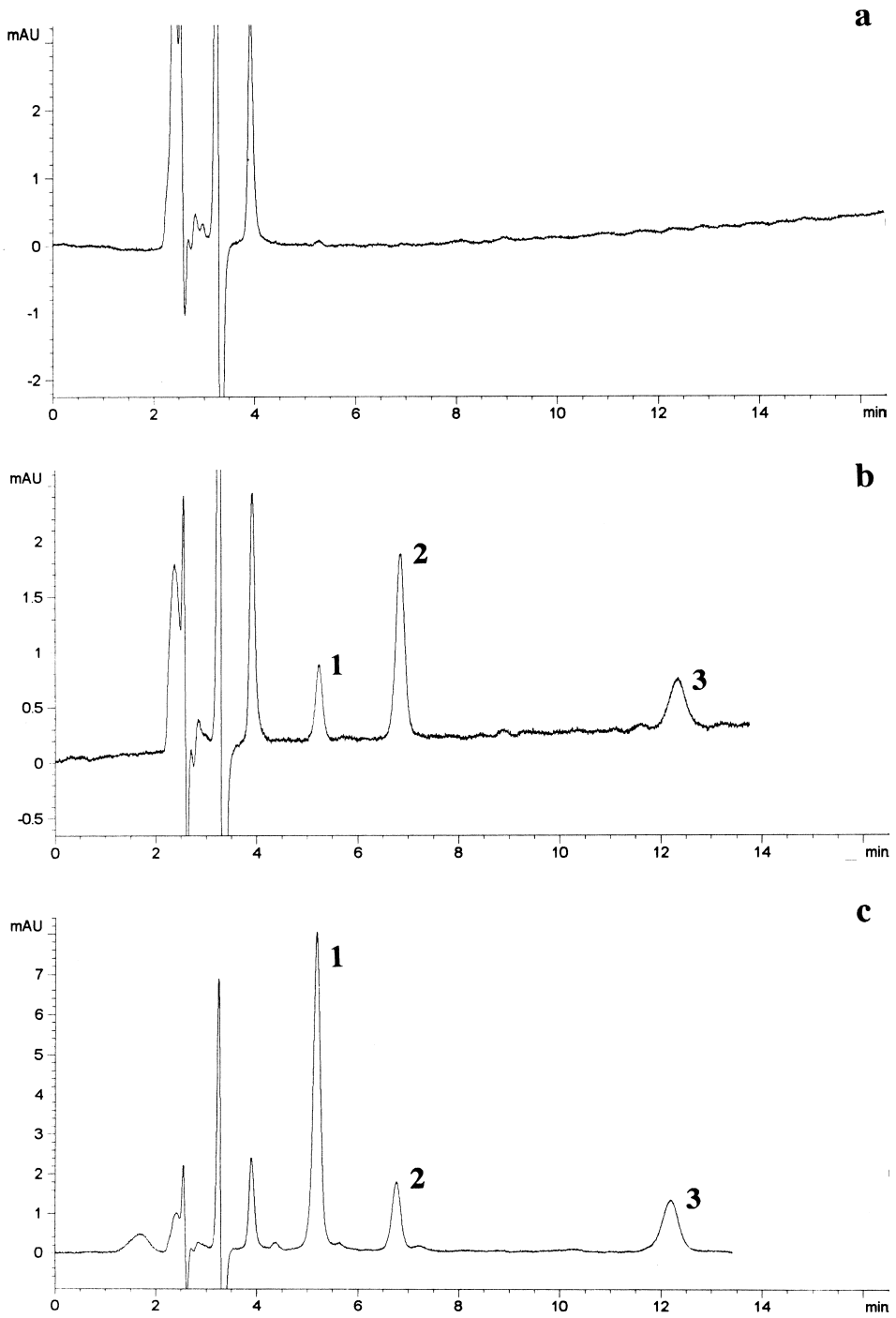

Fig. 3. Chromatograms of blank plasma (a). Plasma spiked with 80 ng/ml of enoxacin and 4-oxo-enoxacin (b). Plasma of a patient treated with 400 mg of enoxacin twice a day (enoxacin: 700 ng/ml; 4-oxo-enoxacin: 240 ng/ml) (c). Peak 1 is enoxacin, peak 2 is pefloxacin (I.S.), peak 3 is 4-oxo-enoxacin. For chromatographic conditions see Section 2.3.

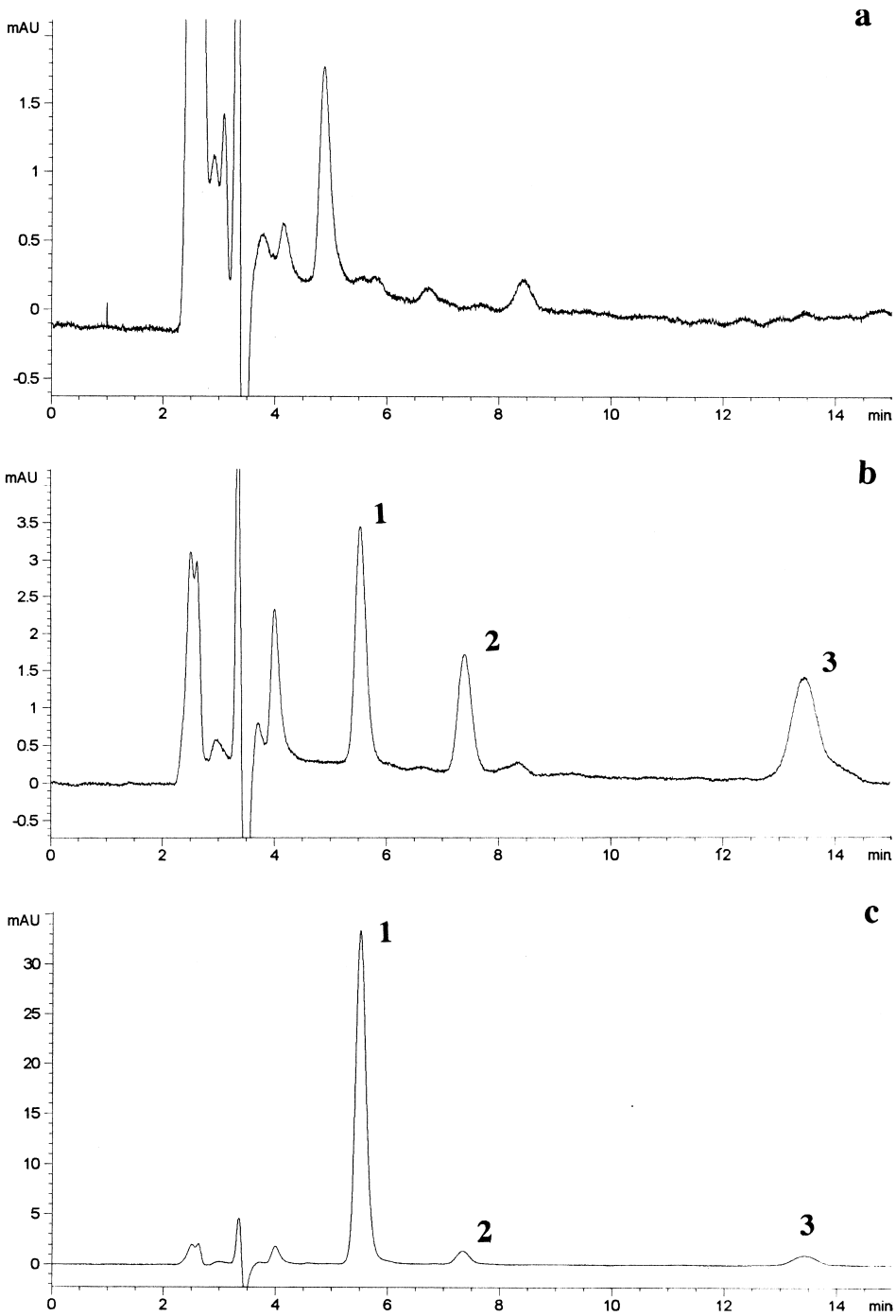

Fig. 4. Chromatograms of blank prostatic tissue (a). Prostatic tissue spiked with 625 ng/g of enoxacin and 4-oxo-enoxacin (b). Prostatic tissue of a patient treated with 400 mg of enoxacin twice a day (enoxacin: 6202 ng/g; 4-oxo-enoxacin: 509 ng/g) (c). Peak 1 is enoxacin, peak 2 is pefloxacin (I.S.), peak 3 is 4-oxo-enoxacin. For chromatographic conditions see Section 2.3.

and 12 min for 4-oxo-enoxacin. The k' values were 1.12, 1.88 and 4.16, respectively. The peak skew was evaluated using the asymmetry coefficient $A_s = b/a$, where b is the distance after the peak maximum and a the distance before the peak maximum, both being measured at 10% of the total peak height. The asymmetry coefficient was 1.28 for enoxacin, 1.0 for pefloxacin and 1.0 for the 4-oxo metabolite.

Extraction and chromatographic analysis of six separate blank plasma and prostatic tissue samples confirmed that there were no endogenous peaks that coeluted with the analytes. A number of commonly administered antimicrobial agents were examined for possible interference with the HPLC method. None of the compounds coeluted with of enoxacin, 4-oxo-enoxacin and pefloxacin.

3.2. Linearity

Peak area ratios of enoxacin and 4-oxo-enoxacin over the I.S. varied linearly with concentration over the range used. The correlation coefficients (r) for calibration curves were equal to or better than 0.996. Intra-assay reproducibility was determined for calibration curves prepared the same day in replicate using the same stock solutions. Inter-assay reproducibility was determined for calibration curves prepared on different days. Results are given Table 1. For each point of calibration standards, the concentrations were back-calculated from the equation of the linear regression curves (experimental concentrations) and the R.S.D.s were computed. Inter-day and intra-day variabilities at concentration of calibration standards are presented in Table 2. A linear regression of the back-calculated concentrations versus the nominal ones provided a unit slope and an intercept equal to 0 (Student t-test).

For each calibration curve, the slope was statistically different from 0, and the intercept was not statistically different from zero. Moreover, the residuals (difference between nominal and back-calculated concentrations) were normally distributed and centered around zero (Kolmogorov–Smirnov test).

3.3. Precision and accuracy

For concentrations of calibration standards, the precision around the mean value ranged from 0.8 to 17.5% for plasma and from 0.2 to 9.3% for prostatic tissue (Table 2).

For the QC samples, the results for accuracy, intra-day and inter-day precision are presented in Table 2. For plasma and prostatic tissue, the precision was characterized by R.S.D.s below 9 and 7%, respectively. The percent recovery from the nominal values for accuracy ranged from 95–104% for plasma and from 97–108% for prostatic tissue (accuracy≤8%).

Table 1
Assay linearity for enoxacin and 4-oxo-enoxacin

	Enoxacin			4-Oxo-enoxacin		
	Correlation coefficient of the linear regression analysis (r±S.D.)	b Slope±S.D.	a Intercept±S.D.	Correlation coefficient of the linear regression analysis (r±S.D.)	b Slope±S.D.	a Intercept±S.D.
Human plasma						
Intra-day reproducibility (n=6)	$0.9996 \pm 3.06 \cdot 10^{-4}$ R.S.D.=0.03%	$5.69 \cdot 10^{-3} \pm 0.25 \cdot 10^{-3}$ R.S.D.=4.4%	0.0076±0.086	$0.9994 \pm 5.72 \cdot 10^{-4}$ R.S.D.=0.06%	$8.63 \cdot 10^{-3} \pm 0.69 \cdot 10^{-3}$ R.S.D.=8.0%	0.135±0.239
Inter-day reproducibility (n=10)	$0.999 \pm 1.10 \cdot 10^{-3}$ R.S.D.=0.11%	$5.93 \cdot 10^{-3} \pm 0.59 \cdot 10^{-3}$ R.S.D.=10%	−0.047±0.136	$0.9991 \pm 1.01 \cdot 10^{-3}$ R.S.D.=0.10%	$8.17 \cdot 10^{-3} \pm 0.65 \cdot 10^{-3}$ R.S.D.=8.0%	0.200±0.215
Prostatic tissue						
Intra-day reproducibility (n=6)	$0.9997 \pm 1.64 \cdot 10^{-3}$ R.S.D.=0.16%	$2.60 \cdot 10^{-3} \pm 0.07 \cdot 10^{-3}$ R.S.D.=2.7%	−0.060±0.027	$0.9994 \pm 3.39 \cdot 10^{-4}$ R.S.D.=0.03%	$2.66 \cdot 10^{-3} \pm 0.074 \cdot 10^{-3}$ R.S.D.=2.8%	−0.0124±0.496
Inter-day reproducibility (n=6)	$0.9999 \pm 4.1 \cdot 10^{-5}$ R.S.D.=0.0041%	$2.63 \cdot 10^{-3} \pm 0.08 \cdot 10^{-3}$ R.S.D.=3.0%	−0.0197±0.01	$0.9997 \pm 1.94 \cdot 10^{-4}$ R.S.D.=0.019%	$2.80 \cdot 10^{-3} \pm 0.048 \cdot 10^{-3}$ R.S.D.=1.7%	$8.28 \cdot 10^{-3} \pm 0.0469$

Table 2
Intra- and inter-assay reproducibilities of the HPLC analysis for enoxacin and 4-oxo-enoxacin

Theoretical concentration (ng/ml or ng/g)	Enoxacin						4-Oxo-enoxacin					
	Intra-assay reproducibility			Inter-assay reproducibility			I ntra-assay reproducibility			Inter-assay reproducibility		
	Experimental concentration (ng/ml) (mean±S.D.)	R.S.D. (%)	Mean recovery (%)	Experimental concentration (ng/ml) (mean±S.D.)	R.S.D. (%)	Mean recovery (%)	Experimental concentration (ng/ml) (mean±S.D.)	R.S.D. (%)	Mean recovery (%)	Experimental concentration (ng/ml) (mean±S.D.)	R.S.D. (%)	Mean recovery (%)
Human plasma												
20	21.1±3.7	17.5	106	21.1±3.7	17.5	106	23.3±1.94	8.3	117	20.4±1.28	6.3	102
50	49.1±3.96	5.4	98.2	50.7±3.52	6.9	101	49.0±3.64	7.4	98.0	52.0±4.18	8.0	104
80	83.2±3.28	3.9	104	80.7±4.87	6.0	101	84.0±6.88	8.2	105	76.5±5.77	7.5	95.6
143	141±5.3	3.8	98.6	137±7.84	5.7	95.8	143±7.71	5.4	100	141±6.60	4.7	98.6
333	330±9.6	2.9	99.0	315±14.8	4.7	94.6	322±9.86	3.1	96.7	330±10.5	3.2	99.1
400	379±20.3	5.4	94.8	379±29.2	7.7	94.8	398±21.5	5.4	99.5	392±35.4	9.0	98.0
500	491±10.1	2.1	98.2	477±21.0	4.4	95.4	501±20.5	4.1	100	507±18.0	3.6	101
800	812±11.4	1.4	102	805±43.3	5.4	101	772±28.1	3.6	96.5	794±46.3	5.8	99.3
1000	1011±14.4	1.4	101	1012±19.2	1.9	101	1009±8.43	0.8	101	997±15.6	1.6	99.7
Prostatic tissue												
50	49.9±3.96	7.9	99.8	50.3±4.67	9.3	101	51.1±4.67	9.1	102	52.0±2.27	4.4	104
100	95.1±5.88	6.2	95.1	105±6.84	6.5	105	106±9.81	9.3	106	103±5.00	4.8	103
125	135±2.9	2.1	108	130±4.5	3.5	104	133±4.23	3.2	106	123±8.61	7.0	98.4
150	156±9.26	1.5	104	155±3.85	2.5	103	155±9.18	5.9	103	156±6.03	3.9	104
312.5	297±5.64	1.9	95.0	299±7.4	2.5	95.7	304±11.6	3.8	97.3	304±21.4	7.0	97.3
625	613±14.4	2.4	98.1	621±8.3	1.3	99.4	589±29.5	5.0	94.2	626±23.7	3.8	100
1000	1038±31.0	3.0	104	975±31.1	3.2	97.5	1075±56.1	5.2	107	1048±38.4	3.7	105
1250	1242±37.9	3.1	99.4	1248±7.46	0.60	99.8	1273±46.3	3.6	102	1249±38.9	3.1	99.9
2000	2005±99.4	5.0	100	1934±116	6.0	96.7	2057±131	6.4	103	2070±97.2	4.7	103
2500	2509±16.6	0.66	100	2503±4.94	0.20	100	2498±20.1	0.8	99.9	2501±15.1	0.60	100

Calibration standards in plasma: 20, 80, 143, 333, 500 and 1000 ng/ml.
Quality control samples in plasma: 50, 400 and 800 ng/ml.
Calibration standards in prostatic tissue: 50, 100, 150, 312.5, 625, 1250 and 2500 ng/g.
Quality control samples in prostatic tissue: 125, 1000 and 2000 ng/g.

3.4. Recovery

In plasma, the mean recovery ($n=8$) averaged 52.3±3.0% for enoxacin, it was 63.0±3.0% for 4-oxo-enoxacin. In prostatic tissue, recoveries were 56.6±2.0 and 76.4±1.6 for the two analytes ($n=7$), respectively. The extraction efficiency is not statistically different over the range of concentrations studied.

For the I.S., the recovery averaged 68.0±5.3% ($n=6$) in plasma and 73.6±1.2% ($n=7$) in prostatic tissue.

3.5. Limit of quantitation and limit of detection

The LOQ was 20 ng/ml for enoxacin and 4-oxo-enoxacin in plasma (precision<17.5%); it was 50 ng/g in prostatic tissue (precision<10%). The LOD was 10 ng/ml for the two drugs in plasma, it was 25 ng/g in prostatic tissue.

3.6. Stability

When stored at 20°C, 4°C and −30°C for a period of 6 h enoxacin and 4-oxo-enoxacin did not reveal any appreciable degradation, with all samples retaining more than 95% in plasma and 94% in prostatic tissue of their original concentration values (Table 3).

Frozen at −30°C and −80°C enoxacin and its metabolite were stable for three months; compared to

Table 3
Mean percent recoveries after 6 h of storage at 20, 4 and −20°C

Concentration added (ng/ml or ng/g), $n=3$	Enoxacin percent recovery (mean±S.D.)			4-Oxo-enoxacin percent recovery (mean±S.D.)		
	20°C	4°C	−20°C	20°C	4°C	−20°C
Human plasma						
50	96.7±0.4	98.0±1.7	96.2±1.1	95.8±1.3	96.6±0.7	97.6±1.5
400	96.7±1.8	97.3±0.3	97.6±2.3	97.8±0.9	96.7±0.2	97.5±1.5
800	97.6±0.95	97.5±1.1	97.8±0.5	97.3±0.7	98.1±1.0	97.8±1.3
Prostatic tissue						
125	94.3±0.58	99.0±1.7	97.3±2.5	97.0±1.0	98.0±1.7	98.3±0.6
1000	98.3±1.5	97.0±3.0	98.0±1.7	97.6±2.5	97.0±1.7	96.0±0.5
2000	97.7±1.5	99.3±1.2	98.6±1.2	99.3±1.2	98.6±1.2	99.0±1.0

the reference values, no statistical difference appeared. Mean recoveries ranged from 93 to 105%.

At least three freeze–thaw cycles can be tolerated without losses higher than 10%.

Run-time stability at room temperature of processed samples after extraction was determined for each point of calibration standard. After 20 h no significant losses occurred. Plasma and tissue extracts were stable for four days at 4°C.

3.7. Pharmacokinetics

Estimated pharmacokinetic in three patients initially included in this study are given. Enoxacin was rapidly absorbed, as evidenced by the time to the maximum observed enoxacin concentration of 1–2 h. The elimination half-life of enoxacin averaged 5.5 h the first day of treatment, it was 9.6 h the last day. For the metabolite, mean elimination half-lives were 6 and 18 h the first and the last days of treatment, respectively. Using a mean bioavailability of 90%, total clearance and volume of distribution of enoxacin averaged 360 ml/min and 210 l.

Concentrations in prostatic tissue at the time of surgery (about 6 h after the last drug intake) were 6000 ng/g for enoxacin and 500 ng/g for the 4-oxo metabolite. The concentration ratios (tissue/plasma) averaged 2 and 1.3, respectively.

4. Discussion and conclusions

Assay performance of the present method was assessed by all the following criteria: peak shape, endogenous interference, linearity, accuracy, precision, recovery, LOQ, LOD, stability and clinical application. This HPLC method enables the simultaneous measurement of enoxacin and its 4-oxo metabolite in plasma and prostatic tissue. The method of Nakamura et al. [9] is able to detect four other metabolites. However, the presence of these metabolites seems of less clinical importance, as each of them accounted for 1% or less of the administered dose. This method has been validated for concentrations ranging from 20 to 1000 ng/ml and 50 to 2500 ng/g for enoxacin and 4-oxo-enoxacin in plasma and prostatic tissue, respectively. Pefloxacin was regarded as an acceptable I.S. because it exhibits similar extraction properties. The present HPLC method is rapid, sensitive and yields accurate and precise results. The LODs of enoxacin and 4-oxo-enoxacin in plasma (10 ng/ml) were lower than that reported by Vree et al. [6] and Zhai et al. [7] (50 ng/ml); however, the method of Nakamura et al. [9], requiring a relatively large sample volume of 1 ml, reported similar minimum detectable concentrations. In this study, a liquid–liquid extraction was used to extract the two drugs from the biological matrices; we reported a mean recovery of 52% for enoxacin and 63% for the 4-oxo metabolite from plasma, they were 57 and 76% from prostatic tissue, respectively. Using a similar extraction procedure, Zhai et al. [7] reported recoveries for enoxacin ranging from 73 to 80% in human plasma and saliva. Nevertheless, recovery of enoxacin could be widely increased by using chloroform containing 1% ethyl chloroformate

[9]. After direct proteins precipitation with trichloroacetic acid, Vree et al. [6] reported a mean recovery of 82% for enoxacin and 52% for the 4-oxo metabolite. The low recoveries of parent drug and metabolite could be explained by the fact that these drugs are amphoteric compounds which are not extractable when non-polar solvents (e.g., *n*-hexane, ether) are used. The extractability of enoxacin with chloroform is pH dependent (47% is extracted at pH 7.0–8.0 when equal volumes of the organic and aqueous phases are used) [9].

Therefore, the procedure described in this paper is suitable for examining a large number of samples. Stability studies carried out directly in plasma and prostatic tissue indicated that samples were stable for at least three months when stored at −30°C and −80°C. The advantages of the present method are that tissue samples and plasma samples are proceeded the same way. It has been used during a pharmacokinetic study in patients (50 to 75 years) after repeated administrations of enoxacin. During this study tissular concentrations of enoxacin and 4-oxo-enoxacin in prostatic tissue were evaluated. These two drugs penetrate well into prostatic tissue with large difference between patients. This simple HPLC method using liquid–liquid extraction should be of value for monitoring the enoxacin concentration in plasma in patients and for assessing plasma compliance in following prescribed enoxacin regimens. Moreover, it has been also used to determine tissue concentrations of enoxacin and 4-oxo-enoxacin into epididymis. Samples were obtained by direct access to the epididymis through a transverse scrotal incision during prostatic surgery.

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