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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF NORFLOXACIN IN HUMAN TISSUES AND PLASMA WITH FLUORESCENCE DETECTION

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SUMMARY

A high-performance liquid chromatographic method with fluorimetric detection is described for the quantitative determination of norfloxacin in renal and prostatic tissues and in plasma. It consists of tissue pretreatment, purification by solid-state extraction and separation and quantification by high-performance liquid chromatography on a C_8 reversed-phase column. Analytical recoveries ranged from 95.2 to 97.6%. Within-day and between-day precision were assessed by analysing serum containing 50 and 500 ng/ml norfloxacin. At each concentration, the within-day precision was $\leqslant 3.6\%$ (coefficient of variation; $n=10$) and the day-to-day precision was $\leqslant 5.3\%$ ($n=10$). The limit of detection was 1 ng/ml.

INTRODUCTION

Norfloxacin [1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinidine carboxylic acid] is a new synthetic broad-spectrum antibacterial agent that is structurally related to nalidixic and oxolinic acids [1]. Norfloxacin exhibits antibacterial activity against both gram-positive and gram-negative bacteria and is superior to other nalidixic acid analogues, in that it shows a greater activity against *Pseudomonas aeruginosa* than gentamicin [2, 3].

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The in vitro activity of norfloxacin against *Neisseria gonorrhoee* is comparable with that of rosaxacin [3]. Norfloxacin is moderately well absorbed after oral administration in percentages ranging from 15 to 30%, depending on the animal species [4, 5]. The potential usefulness of norfloxacin in the treatment of urinary tract infections, especially in prostate infections, justifies intensive studies of its tissue concentrations and pharmacokinetics. Norfloxacin and some of its metabolites have been previously investigated by high-performance liquid chromatography (HPLC) in different biological fluids [6, 7]. Such research suggests the analysis of the drug by liquid–liquid extraction and subsequent chromatographic determination using a spectrophotometric detector. The extraction procedure is slow and elaborate, and for tissues higher sensitivity is necessary, because of the low concentrations. The introduction of solid-phase extraction and the use of liquid chromatography with fluorescence detection provide the solution to these problems. The analytical procedure involves pretreatment of the tissues, purification of the obtained samples by a solid-phase extraction and quantitation by HPLC with fluorimetric detection.

EXPERIMENTAL

Principle

The renal and prostatic tissues were homogenized in methanol. The extracts were purified by adsorption on a liquid–solid column. The organic phase was evaporated and reconstituted with the mobile phase, and a portion of this solution was injected into the chromatograph. The concentration of the drug in the specimen was calculated by comparing the peak areas produced by norfloxacin in the sample with that of a standard.

High-performance liquid chromatography

A Series 3 liquid chromatograph equipped with a Model LS-3 fluorescence detector and a Rheodyne Model 7125 injector with a 20-μl loop (all from Perkin-Elmer, Norwalk, CT, U.S.A.) were used, with a Partisil PXS C_8 column (25 cm$\times$4.6 mm I.D., 10 μm average particle size) (Whatman, Clifton, NJ, U.S.A.). The mobile phase was acetonitrile–methanol–phosphate buffer (pH 2.5) (19:3:78, v/v/v) at a flow-rate of 1.2 ml/min. Norfloxacin was monitored by setting the excitation and emission wavelengths of the detector at 300 and 420 nm, respectively, with 10-nm slit-widths. The experiments were performed at room temperature.

Reagents and materials

All chemicals used were of reagent grade. Chromatography-grade methanol and acetonitrile were obtained from Carlo Erba (Milan, Italy). Phosphate buffer, (pH 2.5) was prepared by dissolving 2.72 g potassium dihydrogen phosphate in 1 l of freshly distilled water and adjusting the pH to 2.5 with phosphoric acid. Carbopack B 80–120 mesh was obtained from Supelco (Bellefonte, PA, U.S.A.).

Standards

Stock norfloxacin standard (Merck Sharp & Dohme, West Point, PA, U.S.A.) was prepared in 0.05 *M* sodium hydroxide and was stable at 4°C. The working standards were prepared by diluting the stock solution with methanol.

Collection of specimens

Prostatic specimens. Eight subjects with a prostatic adenoma underwent transvesical prostatectomy. They were treated with 400 mg of norfloxacin 12 h before the operation and with a further 400 mg ca. 90 min prior to collection. The adenoma was washed many times with physiological solution, and sterile surgical instruments were used to obtain a sample of uncontaminated tissue. This method avoids any contamination from the urine. At the same time, a blood sample was collected. The serum was frozen together with the prostatic tissue.

Renal specimens. Seven subjects, three males and four females, underwent a urological operation and with good renal functionality. The patients were treated with 400 mg of norfloxacin 12 h before the operation and with a further 400 mg ca. 90 min prior to collection of a sample of renal parenchyma. A blood sample was collected at the same time. The serum was frozen together with the renal tissue.

Extraction column

A Carbopack B 80–120 mesh bed was prepared by pouring 200 mg of adsorbent into water. The floating Carbopack B was removed by decantation and the suspension was introduced into a 15 cm × 0.5 cm I.D. glass column with a small flock of glass wool in the bottom. The adsorbent was packed by briefly and gently tapping the column while the water eluted. The adsorbent was suspended in methanol in order to treat the solution of homogenized tissue.

Sample pretreatment and extraction

Tissues. Samples of tissue (50–500 mg) were homogenized for 2 min in 10 ml of methanol by means of a Politron homogenizer. The resulting mixture was then centrifuged at 6000 *g* for 10 min and the supernatant was passed through the Carbopack column. The column was then washed with 10 ml of a solution A (0.02 *M* methanoic acid in methanol) and then with 5 ml of chloroform–solution A (20:80, v/v). Finally, the norfloxacin was eluted with chloroform–solution A (80:20, v/v) to obtain an eluate volume of 8 ml. The sample was evaporated under a stream of nitrogen at 50°C. The residue was dissolved by adding 100 µl of mobile phase: larger volumes (up to 500 µl) were used when larger amounts of norfloxacin were expected. Aliquots of 20 µl of the resulting solution were injected into the chromatograph.

Serum. Samples of 100 µl were diluted with 5 ml of distilled water, and the solution was allowed to percolate through the Carbopack column. The column was washed with 5 ml of distilled water, followed by 10 ml of solution A and 5 ml of chloroform–solution A (20:80, v/v). The elution and analysis of the norfloxacin were then carried out as for tissue samples. The flow-rate of the solvents percolating through the Carbopack column was adjusted to 2 ml/min under the existing experimental conditions.

TABLE I

ANALYTICAL RECOVERIES OF NORFLOXACIN FROM SERUM ($n=6$)

Added (ng/ml)	Found (mean ± S.D.) (ng/ml)	Coefficient of variation (%)	Recovery (%)
10	9.76 ± 0.23	2.5	97.6
50	47.6 ± 1.6	3.4	95.2
100	96.3 ± 3.0	3.1	96.3
500	478 ± 12	2.5	95.6
1000	970 ± 26	2.7	97.0
1500	1451 ± 35	2.4	96.7

RESULTS AND DISCUSSION

Optimization of the method

In order to optimize the chromatographic conditions, the composition, the pH and the flow-rate of the mobile phase were varied, and different mixtures of acetonitrile–phosphate buffer, methanol–phosphate buffer and acetonitrile–methanol–phosphate buffer at different ratios were analysed. The mobile phase composition and the flow-rate were selected for minimum interference from endogenous and exogenous compounds. The retention time for norfloxacin was 5.6 min.

Homogenization and extraction

Evaluation of the recovery from the tissues was virtually impossible because supplementation by known amounts does not represent the true physico chemical situation of the antibiotic in tissues. In order to evaluate the extraction process, different media should be used to find the one that gives the highest efficiency and precision. Hence we tested media such as 0.4 *M* perchloric acid, methanol, methanol–trichloromethane (1:1, v/v). Organic non-polar solvents were not considered because of their limited penetration through the membrane. Perchloric acid and methanol showed comparably high extraction efficiencies for norfloxacin. Methanol was selected because it minimizes the endogenous interferences.

Recovery from the purification column

The recovery from the Carbopack column was analysed in distilled water, methanol or plasma (drug-free) supplemented by known amounts of norfloxacin until the concentrations referred to in Table I were obtained. It is interesting to note that the omission of the acid-washing step, as reported in the purification scheme, yields a low recovery. This phenomenon can be attributed to the formation of chemical complexes between anions and impurities at the Carbopack B surface [8]. However, the complexes are rapidly hydrolysed if the Carbopack B is washed with an acid solution. The analytical recovery of the norfloxacin was the same in water, methanol and plasma, and the average recovery in the concentration range was 96.4%.

TABLE II

PRECISION OF THE ASSAY

	n	Found (mean ± S.D.) (ng/ml)	Coefficient of variation (%)
Within-day	10	47.7 ± 1.6	3.6
		480 ± 12.5	2.6
Day-today	10	46.0 ± 2.4	5.3
		463 ± 23.1	5.0

Linearity

The peak heights and concentrations were linearily related over the range investigated (5–1500 ng/ml).

Sensitivity

The smallest concentration measurable by this method is 1 ng/ml of norfloxacin, at a signal-to-noise ratio of 3:1.

Precision

Within-day precision, day-to-day precision and accuracy of the method were determined by assaying serum supplemented with 50 and 500 ng/ml norfloxacin (Table II).

Interference

More than twenty different drug-free tissues and plasma samples were processed to ascertain the background peak interference at elution times corresponding to that of norfloxacin. It was found that the background from these samples did not interfere with the analysis.

Clinical applications

Pharmacokinetic studies. Fig. 1 shows a plasma concentration–time plot of norfloxacin following a single oral dose of 400 mg. The maximum drug concentration (C_{max}) occurs 1 h after administration. This behaviour agrees with data reported by other authors [9, 10]. According to those results, the tissues were sampled between 60 and 90 min after administration. Fig. 2 shows chromatograms of (a) drug-free serum and (b) serum of a patient after a single dose of 400 mg of norfloxacin.

Prostatic and renal tissues. The concentrations of norfloxacin in the kidney and prostate (Table III), compared with the in vitro minimal inhibitory concentrations (MIC 90%) [11, 12], indicate that tissue levels become quite high and are efficient in combating the most common bacterial urological infections. Typical chromatograms of prostatic and renal tissues are shown in Figs. 3 and 4, respectively.

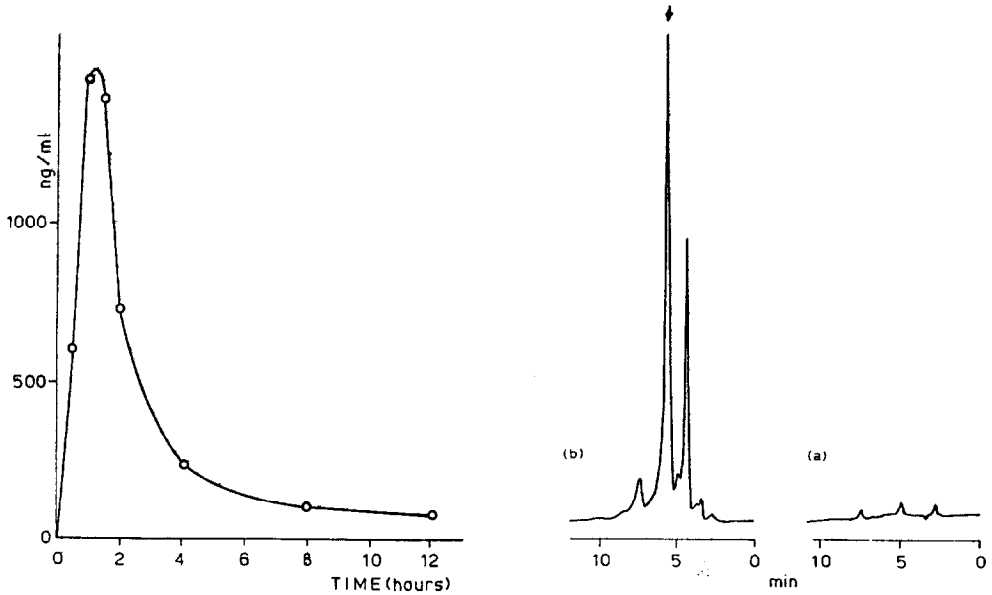

Fig. 1. Plasma concentration–time curve after a single oral dose of 400 mg of norfloxacin.

Fig. 2. Chromatograms of (a) drug-free serum and (b) serum of a patient treated with norfloxacin. The level of norfloxacin was 162 ng/ml.

CONCLUSION

The assay has been developed to quantify the concentration of norfloxacin in renal and prostatic tissues and in plasma. The combination of the purification

TABLE III

CONCENTRATION OF NORFLOXACIN IN TISSUES

Patient	Tissue	Concentration (ng/g of tissue)
1	Prostatic	420
2	Prostatic	169
3	Prostatic	391
4	Prostatic	40
5	Prostatic	210
6	Prostatic	321
7	Prostatic	518
8	Prostatic	793
9	Renal	3665
10	Renal	1012
11	Renal	1689
12	Renal	787
13	Renal	1531
14	Renal	3909
15	Renal	830

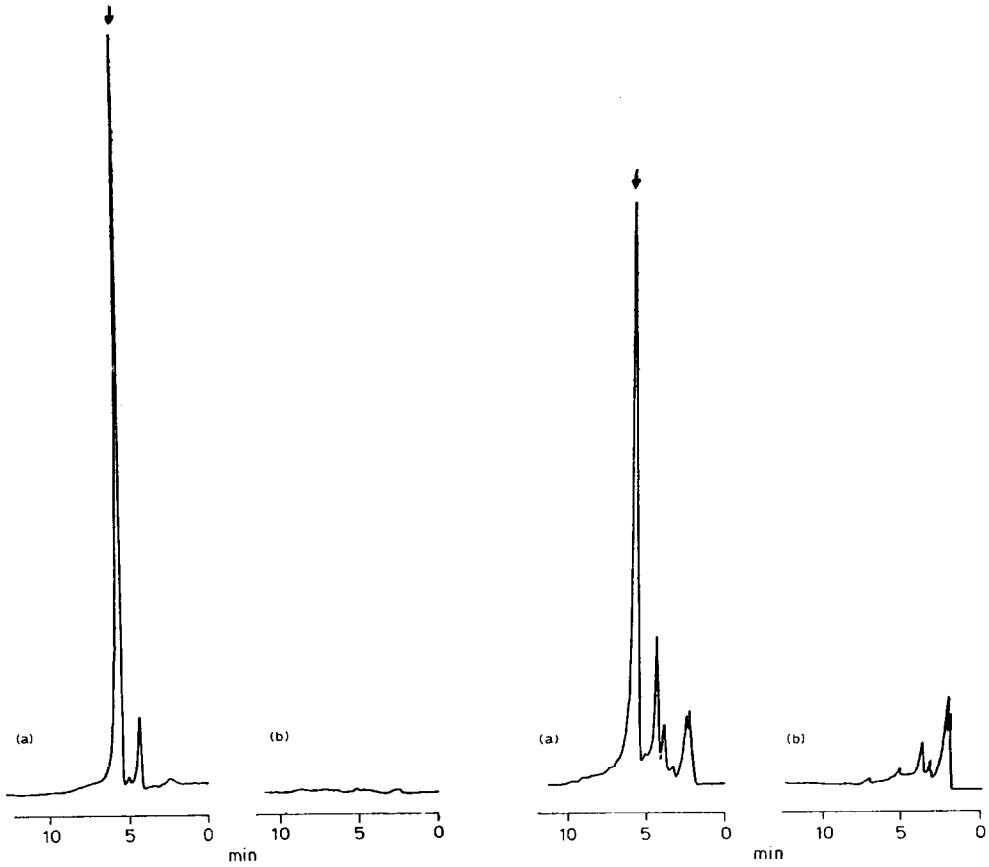

Fig. 3. Representative chromatograms of (a) blank prostatic tissue and (b) prostatic tissue of patient treated with norfloxacin. The level of norfloxacin was 518 ng/g.

Fig. 4. Representative chromatograms of (a) blank renal tissue and (b) renal tissue of patient treated with norfloxacin. The level of norfloxacin was 787 ng/g.

system and quantification by HPLC, using a fluorescence detector, gives adequate sensitivity and sufficient precision and accuracy. The analytical and the pharmacokinetic studies show that the concentrations reach a therapeutic level in both renal and prostatic tissues; in particular the concentration is relatively high in the renal tissues.

REFERENCES

1 H.H. Gadebusch, L.R. Koupal, E. Celozzi, D.L. Shungu, J. Bland, B. Weissberger, B.A. Pelak, E. Fisch, S.K. Chung and J. Huber, Current Chemotherapy, Vol. 1, American Society for Microbiology, New York, 1982, pp. 351–353.
2 K. Hirai, A. Ito, Y. Abe, S. Suzue, T. Irikura, M. Inque and S. Mitsumashi, Antimicrob. Agents Chemother., 19 (1981) 188–189.
3 A. Ito, K. Hirai, M.Inque, H. Koga, S. Suzue, T. Irikura and S. Mitsumashi, Antimicrob. Agents Chemother., 17 (1980) 103–108.

4 S. Murayama, K. Hirai, A. Ito, Y. Abe and T. Irikura, Chemother. (Tokyo), Suppl., 4 (1981) 98-104.
5 Y. Nagatsu, K. Endo and T. Irikura, Chemother. (Tokyo), Suppl., 4 (1981) 105-118.
6 G. Montay, Y. Blain, F. Roquet and A. Le Hir, J. Chromatogr., 272 (1983) 359-365.
7 C. Forchetti, D. Flammini, G. Carlucci, G. Cavicchio, L. Vaggi and M. Bologna, J. Chromatogr., 309 (1984) 177-182.
8 L. Campanella, A. Di Corcia, R. Samperi and A. Gambacorta, Mater. Chem., 7 (1982) 429-438.
9 V.K. Boppana and B.N. Swanson, Antimicrob. Agents Chemother., 21 (1982) 808-810.
10 M. Eandi, I. Viano, F. Di Nola, L. Leone and E. Gennazzani, Eur. J. Clin. Microbiol., 2 (1983) 253-259.
11 M.Y. Khan, R.P. Gruninger, S.M. Nelson and R.E. Klicker, Antimicrob. Agents Chemother., 21 (1982) 848-851.
12 S.W.B. Newsom, Eur. J. Chemother. Antibiot., 3 (1982) 9-14.