Journal of Chromatography, 491 (1989) 379–387
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4743

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE DETERMINATION OF THREE NEW FLUOROQUINOLONES, FLEROXACIN, TEMAFLOXACIN AND A-64730, IN BIOLOGICAL FLUIDS

C. KOECHLIN*, F. JEHL, L. LINGER and H. MONTEIL

Institute of Bacteriology, Faculty of Medicine, 3, Rue Koeberlé, 67000 Strasbourg (France)

(First received October 6th, 1988; revised manuscript received February 3rd, 1989)

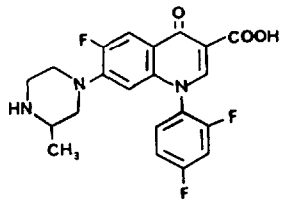
SUMMARY

High-performance liquid chromatographic procedures have been developed for the measurement of three new fluoroquinolones, fleroxacin, temafloxacin and A-64730, in serum, urine and bile. The sample treatment consists of a two-step chemical extraction. The three molecules are chromatographed on a C_{18} reversed-phase analytical column with spectrofluorimetric detection. At a signal-to-noise ratio of 4, the detection limits in serum are 2.5, 10 and 20 ng/ml, for fleroxacin, temafloxacin and A-64730, respectively. The calibration curves are rectilinear between these detection limits and 20 μg/ml. The intra- and inter-assay coefficients of variation are in the ranges 0.8–5.4 and 2.2–7.6%, respectively. These simple and reliable assay procedures will be of great interest for further pharmacokinetic studies and drug monitoring in hospital use.

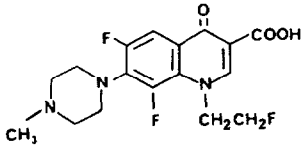
INTRODUCTION

Fleroxacin, temafloxacin and A-64730 are three trifluorinated quinolones that have recently been synthesized. They possess a broad spectrum of antimicrobial activity against both Gram-negative and Gram-positive bacteria. Fleroxacin (Ro 23-6240; AM-833, Fig. 1) generally has antibacterial activity similar to that of ofloxacin and norfloxacin but less than that of ciprofloxacin [1–3]. However, one of the most interesting characteristics of this new therapeutic agent is its long elimination half-life of 9–11 h in humans [4,5]. Temafloxacin (A-63004, Fig. 1) is as active as ciprofloxacin in vitro against staphylococci and streptococci, but exhibits lower in vitro activity against

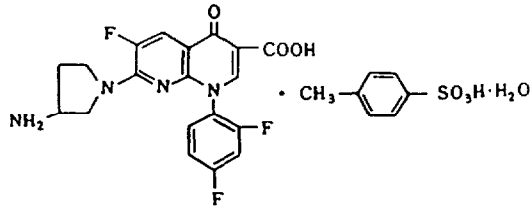
0378-4347/89/$03.50



Temafloxacin



Fleroxacin



A-64730 (III)

Fig. 1. Structures of fleroxacin, temafloxacin and A-64730 (III).

Pseudomonas aeruginosa [6]. The generally better activity obtained in vivo with this antibiotic is probably due to its pharmacokinetic parameters (same elimination half-life as ciprofloxacin, but seric peak six times higher in mice) [6]. Lastly, A-64730 (T-3262; III, Fig. 1) has excellent in vitro activity against Gram-positive bacteria and anaerobic species in contrast to the other quinolones [7–9]. No data are currently available on its pharmacokinetics.

This paper describes high-performance liquid chromatographic (HPLC) assay procedures in serum, urine and bile for the three fluoroquinolones which are, to our knowledge, the first published for temafloxacin and III. Our aim was to allow monitoring of these new drugs and further pharmacokinetic studies, which are of particular interest for fluoroquinolones since (i) good pharmacokinetics can in part compensate for a low activity against bacteria (see above for temafloxacin) and (ii) owing to their pharmacokinetic properties these compounds are generally very suitable for oral administration and could be used to treat infections that in the past could be treated only parenterally. We have developed assay methods in urine and bile in order to allow further investigations on the urinary and biliary elimination of the three fluoroquinolones, since such molecules are excreted by both pathways [10–12].

EXPERIMENTAL

Chemicals and standard solutions

Fleroxacin was provided by Produits Roche (Neuilly, France); temafloxacin and III were obtained from Abbott Labs. (Abbott Park, IL, U.S.A.). Stock solutions of fleroxacin and III of 1 mg/ml were prepared as recommended by the manufacturers by first adding the minimum solution volume of 0.1 *M* sodium hydroxide (to fleroxacin) or glacial acetic acid (to III) and then adding the appropriate volume of distilled water. Stock solutions of temafloxacin are made in water, as recommended, by heating the flask in a boiling water-bath until dissolution. Acetonitrile, dichloromethane, glacial acetic acid (all HPLC grade), tetrabutylammonium bromide, sodium dihydrogenphosphate monohydrate and sodium hydroxide were from E. Merck (Darmstadt, F.R.G.). Water was obtained daily from a Milli-Ro–Milli-Q system (Millipore, Velizy, France). Standards were prepared by adding the corresponding volumes of the stock solutions to the drug-free biological fluids (serum, urine and bile). The standards were stored frozen at −80 °C.

Sample handling

First step. This step was common to the three compounds. An aliquot (500 µl) of human or animal fluid (serum, urine Hiluted1:20 or bile diluted 1:10) was added to 3.2 ml of dichloromethane in a 6-ml screw-capped glass tube. After mixing on a vortex mixer, the tubes were gently shaken for 10 min by rotation (20 rpm) and then centrifuged for 10 min at 1000 *g*. The upper aqueous layer was aspirated off, and 3 ml of the lower organic phase were transferred to a second screw-capped glass tube.

Second step. Fleroxacin and temafloxacin were back-extracted from the organic phase, using 200 µl of 0.1 *M* sodium hydroxide, by rotation (20 rpm) for respective times of 30 and 10 min. Centrifugation at 1000 *g* for 10 min resulted in phase separation.

Compound III was back-extracted using 200 µl of acetic acid at pH 2.5 by rotation (20 rpm) for 10 min. Centrifugation at 1000 *g* for 10 min resulted in phase separation.

A 20-µl volume of the upper aqueous layer was then injected into the analytical column.

Recovery study

Ten samples of serum, urine and bile were spiked with 0.5, 5, 20 and 10 µg/ml of each antibiotic. They were assayed, and the resulting peak heights were compared with the peaks resulting from aqueous solutions at the same concentrations.

Chromatography

The isocratic liquid chromatograph was constituted from the following components: a Model 302 solvent-delivery module (Gilson, Villiers-Le-Bel, France); a sample-injection valve equipped with a 20-μl loop (Gilson); a Model RF 535 spectrofluorimeter (Shimadzu, Kyoto, Japan) operated at excitation wavelengths of 277, 275 and 265 nm and emission wavelengths of 445, 450 and 433 nm for fleroxacin, temafloxacin and III, respectively; a Model 740 recording data processor (Millipore, Waters Division, Milford, MA, U.S.A.). Separations were performed at ambient temperature on a 150 mm $\times$ 4.6 mm I.D. C_{18} reversed-phase analytical column, with 5 μm particle size (Ultrasphere®, Beckman).

The mobile phases were 5 m*M* tetrabutylammonium bromide–10 m*M* sodium dihydrogenphosphate monohydrate, containing 10, 19 and 18% (v/v) acetonitrile for fleroxacin, temafloxacin and III, respectively. Phosphoric acid (14.6 *M*) was then added to adjust the pH to 2. The mobile phases were filtered through a 0.45-μm membrane, and the flow-rate was set at 2.0 ml/min. The range setting depended on the concentrations of the drugs to be measured.

Establishment of detection limit and calibration curve

The limit of quantitation in serum, urine and bile was defined as the lowest concentration of antibiotic resulting in a signal-to-noise ratio of 4.

Drug-free serum samples were spiked with increasing amounts of fluoroquinolones, resulting in the following concentrations: detection limit (see Table II), 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10 and 20 μg/ml. These samples were then chromatographed and peak heights were plotted against the concentrations of fluoroquinolone to check for linearity. Each standard was assayed in triplicate.

Precision

Drug-free serum, urine and bile were spiked with known amounts of antibiotic.

Intra-assay reproducibility. Ten aliquots of a serum containing 0.5 μg/ml and ten aliquots of a serum containing 5 μg/ml fluoroquinolone were randomly distributed in different series of assays, on the same day. The concentrations of drug tested with bile and urine for the within-day reproducibility were 10 and 20 μg/ml, respectively.

Inter-assay reproducibility. Ten aliquots of the same biological fluids used for the within-day study were assayed one by one during ten days, using each time the calibration curve of the day. For both intra-assay and inter-assay reproducibilities, precision was evaluated by calculating the coefficients of variation (C.V.).

Selectivity

Interference studies were carried out with many substances that could be co-administered with quinolones: β-lactam antibiotics (penicillins, cephalosporins, clavulanic acid, imipenem and aztreonam), aminoglycosides (gentamicin, tobramycin, netilmicin and amikacin), vancomycin, teicoplanin, rifampicin, fosfomycin and other therapeutic agents (analgesics, salicylate, phenobarbital, carbamazepine, phenytoin, primidone, valproic acid, theophylline, digitoxin, furosemide, quinidine, digoxin, procainamide, lidocaine). Potential interferences from related drugs, including ofloxacin, pefloxacin, norfloxacin, ciprofloxacin and difloxacin, were also examined.

RESULTS

Recovery study

The results of recovery studies are reported in Table I. The best values were obtained with fleroxacin and temafloxacin, for which extraction recoveries from serum, urine and bile were close to 100%. The recovery values for III were lower, but owing to the 2.5-fold concentration allowed by the extraction procedures described, satisfactory quantitation limits could be reached (20 ng/ml in serum).

TABLE I

EXTRACTION RECOVERIES

Antibiotic	Recovery ($n=10$) (%)		
	Serum	Urine	Bile
Fleroxacin	99.6 ± 1.6	100.0 ± 2.0	100.0 ± 3.0
Temafloxacin	96.0 ± 2.0	82.8 ± 4.3	95.2 ± 4.2
III	56.8 ± 2.8	40.8 ± 2.9	44.4 ± 2.2

TABLE II

DETECTION LIMITS AT A SIGNAL-TO-NOISE RATIO OF 4

Antibiotic	Body fluid	Detection limit (ng/ml)
Fleroxacin	Serum	2.5
	Urine	50
	Bile	25
Temafloxacin	Serum	10
	Urine	200
	Bile	100
III	Serum	20
	Urine	500
	Bile	250

Detection limits and linearity

Detection limits are indicated in Table II. They were established in serum at 2.5 ng/ml for fleroxacin, 10 ng/ml for temafloxacin and 20 ng/ml for III at a signal-to-noise ratio of 4. For the urinary and biliary levels these values were increased on account of the dilutions effected (1:20 and 1:10, respectively). Regression correlation coefficients revealed high linearity, with values exceeding 0.9995 for the three compounds in the three biological fluids.

Precision

The results are shown in Table III. The intra-assay reproducibility was characterized by C.V. ranging from 0.8% (assay of 20 μg/ml temafloxacin in urine) to 5.4% (assay of 5 μg/ml III in serum). The inter-assay C.V. ranged from 2.2% (assay of 20 μg/ml fleroxacin in urine) to 7.6% (assay of 0.5 μg/ml III in serum).

Chromatograms and selectivity

Typical chromatograms resulting from HPLC analysis of the three fluoroquinolones in spiked human serum, spiked human urine and spiked human bile are shown in Fig. 2. The chromatograms depicted in Fig. 3 are representative for the analysis of serum samples from rabbits treated with fleroxacin intraperitoneally (Fig. 3a and b) or temafloxacin intraperitoneally (Fig. 3c and d), and from a pig receiving III intravenously (Fig. 3e and f). In all cases, fleroxacin, temafloxacin and III eluted at respective retention times of 1.8, 1.9 and 2.0 min as well resolved peaks. During specificity studies, all chromatograms were always carefully checked for skewed, shouldering or tailing peaks.

TABLE III

PRECISION

Antibiotic	Body fluid	Concentration (μg/ml)	Coefficient of variation (%)	
			Intra-assay ($n=10$)	Inter-assay ($n=10$)
Fleroxacin	Serum	0.5	5.0	7.4
		5	2.3	4.9
	Urine	20	1.9	2.2
	Bile	10	2.7	3.7
Temafloxacin	Serum	0.5	3.6	6.0
		5	2.1	4.5
	Urine	20	0.8	2.8
	Bile	10	2.2	2.5
III	Serum	0.5	4.2	7.6
		5	5.4	6.6
	Urine	20	2.6	6.4
	Bile	10	3.2	5.2

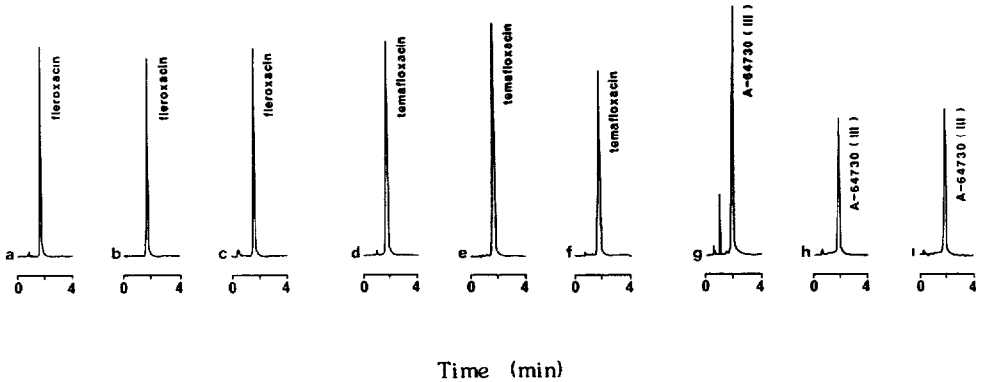


Fig. 2. Chromatograms of extracts of human (a) serum, (b) urine and (c) bile supplemented with 5, 20 and 10 μg/ml fleroxacin, respectively, (d) serum, (e) urine and (f) bile supplemented with 5, 20 and 10 μg/ml temafloxacin, respectively, and (g) serum, (h) urine and (i) bile supplemented with 5, 20 and 10 μg/ml III, respectively. Chart-speed, 0.5 cm/min; range 0.01 a.u.f.s.; HPLC conditions as described in the text.

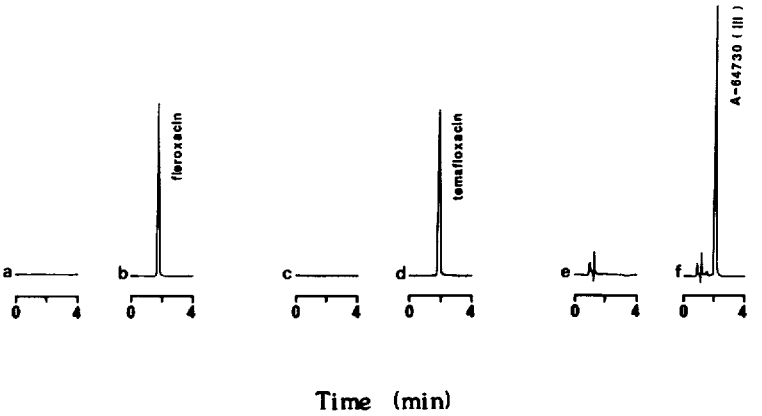


Fig. 3. Chromatograms of extracts of (a) control serum ($t=0$), (b) serum from a rabbit treated with 15 mg/kg fleroxacin intraperitoneally ($t=2.5$ h), (c) control serum ($t=0$), (d) serum from a rabbit treated with 15 mg/kg temafloxacin intraperitoneally ($t=2.5$ h), (e) control serum ($t=0$) and (f) serum from pig to which 10 mg/kg III was administered intravenously ($t=1$ h). Chart-speed, 0.5 cm/min; range 0.01 a.u.f.s.; HPLC conditions as described in the text.

No interferences could be observed with any substances that might be co-administered with fluoroquinolones (see Experimental for the list of drugs tested). Furthermore, no interfering endogenous peaks could be detected in the elution zone of the three compounds. Although there were rare interferences between fluoroquinolones (temafloxacin and III, fleroxacin and ofloxacin), this is not a drawback since two fluoroquinolones are never used together in treatment.

DISCUSSION

The development of HPLC assays generally consists of three steps: (i) biological sample handling procedures; (ii) choice of stationary phase and development of mobile phases; (iii) establishment of the chromatographic conditions. Our aim, throughout these steps, was to optimize the procedures without losing the reliability required for pharmacokinetic studies or routine hospital use.

For fluoroquinolones, various approaches to sample treatment have been proposed. Although ultrafiltration is sometimes preferred, owing to its simplicity and precision [13], most authors have developed extraction procedures for quinoline carboxylic acids. Generally, dichloromethane or chloroform was employed to extract them [4,13–18]. These procedures offer advantages in terms of concentration of the analyte (i.e. lower detection limit), reduced background from endogenous components resulting in clean chromatograms and lower assay cost. We described here a simple extraction method for fleroxacin, temafloxacin and III, which allows the sample handling of the three quinolones in parallel. The good reproducibilities of the assays in the biological fluids in conjunction with the proven linearity indicate that an internal standard to overcome sample-to-sample variation is not necessary.

The second step during HPLC assay development relates to the stationary and mobile phases. Reversed-phase chromatography on C_{18} silica, with addition to the mobile phase of tetrabutylammonium bromide, tetraethylammonium bromide or sodium dodecyl sulphate as ion-pairing reagent, has already been used successfully for many quinolones [13,14,16,19,20], including fleroxacin [4]. In our case, this procedure also allowed us to obtain reproducible results and excellent resolution of the three fluoroquinolones. Furthermore, all three could be chromatographed with only one type of mobile phase (which can be applied with slight modifications to other fluoroquinolones), a mixture of tetrabutylammonium bromide, sodium dihydrogenphosphate monohydrate and acetonitrile in various ratios. Thus, the column could be rapidly equilibrated when different quinolones had to be analysed successively. This implies time-saving and limits the number of chemicals required.

Among the chromatographic conditions to optimize, the selection of a detection procedure is important. For fluoroquinolones, essentially two types of detection can be used: UV spectrophotometry and fluorescence. The choice between these two possibilities must be in accordance with the fluoroquinolone of interest [10,15]. In particular it depends on the value of the molar extinction coefficient (UV) compared with the relative fluorescence efficiency (fluorescence detection) of the fluoroquinolone, the chromatographic conditions used in the two cases and the mobile phases. In practice, the difference is expressed in terms of detection limits. During the assay development we compared the advantages of the two detection modes. For fleroxacin and temafloxacin, al-

though both methods can be used under our conditions, fluorescence detection is the more suitable. For III, the two detectors are comparable although UV detection gave a slightly better limit of quantitation (10 versus 20 ng/ml with fluorimetry).

To allow pharmacokinetic investigations and monitoring of fluoroquinolones, it is essential to ensure a selective and accurate assay procedure, such as HPLC, for two reasons: (i) fluoroquinolones are often used in association with other antimicrobial agents and HPLC allows a reliable assay even in this case; (ii) as fluoroquinolones are generally metabolized, it is necessary to assay specifically the parent compounds and each possible active (or non-active) metabolite. Whereas the two metabolites of fleroxacin (the N-demethyl- and N-oxide derivatives) were identified and measured in human serum and urine by Weidekamm et al. [4], actually only parent compounds are available for temafloxacin and III. But when their metabolites are identified and synthesized, it will be important to develop their assay by HPLC.

REFERENCES

1 A.M. Clarke and S.J.V. Zemcov, Eur. J. Clin. Microbiol., 6 (1987) 161–164.
2 K. Machka and I. Braveny, Eur. J. Clin. Microbiol., 6 (1987) 482–485.
3 N. Manek, J.M. Andrews and R. Wise, Antimicrob. Agents Chemother., 30 (1986) 330–332.
4 E. Weidekamm, R. Portmann, K. Suter, C. Partos, D. Dell and P.W. Lücker, Antimicrob. Agents Chemother., 31 (1987) 1909–1914.
5 R. Wise, B. Kirkpatrick, J. Ashby and D.J. Griggs, Antimicrob. Agents Chemother., 31 (1987) 161–163.
6 D.J. Hardy, R.N. Swanson, D.M. Hensey, N.R. Ramer, R.R. Bower, C.W. Hanson, D.T.W. Chu and P.B. Fernandes, Antimicrob. Agents Chemother., 31 (1987) 1768–1774.
7 A.M. Espinoza, N.X. Chin, A. Novelli and H.C. Neu, Antimicrob. Agents Chemother., 32 (1988) 663–670.
8 P.B. Fernandes, D.T.W. Chu, R.N. Swanson, N.R. Ramer, C.W. Hanson, R.R. Bower, J.M. Stamm and D.J. Hardy, Antimicrob. Agents Chemother., 32 (1988) 27–32.
9 K. Fujimaki, T. Noumi, I. Saikawa, M. Inoue and S. Mitsuhashi, Antimicrob. Agents Chemother., 32 (1988) 827–833.
10 J.M. Brogard, F. Jehl, H. Monteil, M. Adloff, J.F. Blicklé and P. Levy, Antimicrob. Agents Chemother., 28 (1985) 311–314.
11 H. Lode, G. Höffken, C. Prinzing, P. Glatzel, R. Wiley, P. Olschewski, B. Sievers, D. Reimnitz, K. Borner and P. Koeppe, Drugs, 34 (1987) 21–25.
12 L.O. White, Quinolones Bull., 3 (1987) 1–4.
13 G.R. Granneman and L.T. Sennello, J. Chromatogr., 413 (1987) 199–206.
14 A.J. Groeneveld and J. Brouwers, Pharm. Weekbl., 8 (1986) 79–84.
15 L.I. Harrison, D. Schuppan, S.R. Rohlfing, A.R. Hansen, C.S. Hansen, M L. Funk, S.H. Collins and R.E. Ober, Antimicrob. Agents Chemother., 25 (1984) 301–305.
16 F. Jehl, C. Gallion, J. Debs, J.M. Brogard, H. Monteil and R. Minck, J. Chromatogr., 339 (1985) 347–357.
17 G. Montay and J.P. Tassel, J. Chromatogr., 339 (1985) 214–218.
18 R. Nakamura, T. Yamaguchi, Y. Sekine and Hashimoto, J. Chromatogr., 278 (1983) 321–328.
19 K.H. Lehr and P. Damm, J. Chromatogr., 425 (1988) 153–161.
20 L.O. White, A.P. MacGowan, A.M. Lovering, D.S. Reeves and I.G. Mackay, Drugs, 34 (1987) 56–61.