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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF CIPROFLOXACIN AND ITS METABOLITES IN SERUM, URINE AND SPUTUM

CAROLYN M. MYERS and JEFFREY L. BLUMER*

Division of Pediatric Pharmacology and Critical Care, Rainbow Babies and Childrens Hospital, 2101 Adelbert Road, and Departments of Pediatrics and Pharmacology, Case Western Reserve University School of Medicine, Cleveland, OH 44106 (U.S.A.)*

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SUMMARY

A versatile and sensitive method requiring no internal standard was developed for quantitating ciprofloxacin in serum, urine and sputum by high-performance liquid chromatography with fluorescence detection. Acetonitrile and chloroform were employed to remove protein and lipophilic substances from an aqueous, ciprofloxacin-containing sample layer. The proportions of acetonitrile and 0.1 *M* potassium phosphate, pH 2.5, in the mobile phase were varied to suit the purpose of the assay. For the routine determination of ciprofloxacin pharmacokinetics, isocratic 19% acetonitrile was used. A gradient from 15 to 35% acetonitrile was chosen to show the appearance of metabolites which formed during the biodisposition of ciprofloxacin. In the latter case urine samples were diluted for assay and protein was precipitated from serum samples with trichloroacetic acid. Four fluorescent metabolites were observed in all patient specimens, and with tandem ultraviolet detection two additional ultraviolet-absorbing metabolites were readily found in urine specimens.

INTRODUCTION

Empiric therapy for potentially serious infections often requires the use of broad-spectrum parenteral antimicrobial agents. The resultant hospitalization may prove costly to both the patient and society. This negative impact may be compounded when chronic infection by recalcitrant organisms necessitates repeated hospitalizations for intravenous therapy. Thus, efforts directed toward the development of orally bioavailable broad-spectrum agents have intensified in recent years.

One strategy employed in the quest for broad-spectrum oral agents was to synthetically modify agents whose effectiveness was pharmacokinetically rather than pharmacodynamically limited. The first quinolone, nalidixic acid, was introduced

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into medical practice in the early 1960s. Subsequent structural modifications have yielded a series of substances which show a broad spectrum of antimicrobial activity and low incidence of resistance. Among the group of quinolones synthesized to date [1–4], ciprofloxacin remains the most potent agent against both gram-positive and gram-negative pathogens. Its antimicrobial activity has none of the deficiencies apparent with the broad-spectrum β-lactam agents [5–7].

In order to study the pharmacokinetics of ciprofloxacin in the blood and urine of cystic fibrosis patients, a high-performance liquid chromatographic (HPLC) assay using fluorescence detection described herein was developed. During the analyses of patient specimens, the widespread presence of several previously unidentified fluorescent species was observed. Therefore the original method was modified to study three of these fluorescent metabolites as well as two primarily ultraviolet (UV)-absorbing metabolites and one fluorescent metabolite which had been identified previously [8].

EXPERIMENTAL

Reagents

Ciprofloxacin and three metabolites (Fig. 1) were provided by Miles Pharmaceuticals (West Haven, CT, U.S.A.). Potassium hydroxide and HPLC-grade phosphoric acid (85%) were purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.). Glass-distilled acetonitrile and methylene chloride were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

HPLC equipment and conditions

Analyses were performed on a Varian Instruments Model 5020 liquid chromatograph (Varian Assoc., Palo Alto, CA, U.S.A.) equipped with an automated Valco Model C6U column switching valve with a 50-μl loop. A guard column (4 cm $\times$ 4 mm), filled with Vydac 40-μm pellicular reversed-phase packing, was placed between the injector and the column. Chromatography was performed on a 30 cm $\times$ 4 mm Micro Pak MCH 10 reversed-phase column (10 μm particle size). A temperature of 40°C was maintained with a column heater. The mobile phase

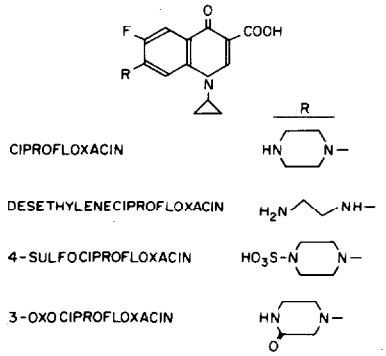

Fig. 1. Structures of ciprofloxacin and three of its metabolites.

was acetonitrile–0.1 *M* potassium phosphate, pH 2.5 (19:81, v/v), and the flow-rate was 0.8 ml/min. All potassium phosphate buffers were prepared by titrating phosphoric acid solutions to the appropriate pH with 45% (w/w) potassium hydroxide. The column was stored in acetonitrile–water (70:30, v/v). The only maintenance required during the analysis of more than 3000 samples was cleaning the guard column inlet frit when pressures increased. The same column was utilized for the gradient method described below.

Peaks were detected with a Varian Fluorichrom fluorometer equipped with a tungsten lamp set at a high gain, low lamp and an attenuation of 5. A 330I interference filter was employed for excitation. For emission the 3-75 and 4-76 filters were used. These two filters eliminate most radiation below 370 nm and in the infrared. The chromatograms were recorded on a Linear Model 291 recorder (Linear Instruments, Irvine, CA, U.S.A.) at 20 cm/h, and peaks were integrated on the Varian CDS 111L.

For analyses of metabolites by HPLC, dual spectrometry was employed. Mobile phase was pumped through a Varian Model 5500 liquid chromatograph equipped with a variable-wavelength UV 200 detector set at 280 nm. A variable-wavelength 2070 spectrofluorometer was set at 330 nm excitation and 440 nm emission. Gain $\times 1$ on the spectrofluorometer was used for urine samples and gain $\times 10$ was used for serum samples. Peaks were integrated on a DS 604 computer and printed on a Hewlett-Packard ThinkJet.

Sample preparation

Serum, urine and sputum specimens were routinely stored at $-70\,^{\circ}C$. Serum samples (1 ml) were processed for chromatography by the addition of 20 μl phosphoric acid and 2 ml acetonitrile. The mixture was vortexed for 20 s and centrifuged for 3 min at 2500 *g*. The protein precipitate was discarded, and the entire supernatant solution was added to 4 ml methylene chloride in a conical-shaped centrifuge tube. The tube was vortexed for 20 s and centrifuged for 1 min at 2500 *g*. In this step the methylene chloride extracted the acetonitrile away from the ciprofloxacin-containing aqueous layer. Most of the organic bottom layer was then discarded. Following a brief centrifugation the upper aqueous layer was ready for injection. Between injections the syringe and loop were rinsed twice with 0.85% sodium chloride. This effectively eliminated the carry-over problems which have been described by Nix et al. [9].

Sputum sample volumes were first estimated in graduated, conical-shaped centrifuge tubes and diluted with an equal volume of 0.3 *M* potassium phosphate, pH 1.5. The sample was then subjected to sonication up to a total of 20 s in 5-s bursts depending upon the viscosity of the sputum. The total volume of the sonicated mixture was measured, and a 1-ml portion was taken for analysis. If necessary the sample was brought to 1 ml using the potassium phosphate buffer, pH 1.5. Phosphoric acid (20 μl/ml of sample), adjusted to the amount of actual sputum in the 1-ml sample, was added and the sample subsequently processed as serum.

Room temperature urine samples were vigorously mixed prior to a 20- to 500-fold dilution with water. To 0.1 ml of these dilutions were added 0.9 ml of 0.3 *M* potassium phosphate, pH 1.5, 1 ml of acetonitrile, 4 ml of methylene chloride and

1 ml of hexane. The mixtures were vortexed for 20 s and centrifuged for 1 min at 2500 *g*. Most of the bottom layer was discarded. After brief recentrifugation the upper layer was ready for injection.

Standards

Ciprofloxacin standards in pooled human serum at a concentration of 1.00 μg/ml were prepared in 1-ml aliquots and frozen at −70°C. It was observed initially that serum gave more reproducible results from day to day than standards in 0.1 *M* potassium phosphate, pH 7.5, so a serum matrix was used for preparing all standards. Weber et al. [10] have commented upon the instability of ciprofloxacin in aqueous solution with repeated freezing and thawing.

Calculations

No internal standard was needed because of the direct method of sample preparation. Good coefficients of variation supported the use of an external standard. Corrections for matrix differences were applied where appropriate; the HPLC ciprofloxacin peak areas from spiked sputum and urine were, respectively, 0.899 (±0.035) and 0.906 (±0.033) of that from spiked serum.

Fluorometry

Fluorescence spectra were run on a Farrand Mark I spectrophotometer.

Mass spectrometry

Mass spectral determinations were performed on a Kratos MS 30 high-resolution spectrophotometer.

RESULTS

Ciprofloxacin assay

The analysis was linear to at least 10.00 μg/ml. Duplicate serum samples at 0.02, 0.05, 0.10, 0.30, 0.80, 2.00, 5.00 and 10.00 μg/ml yielded a correlation coefficient of 0.99992 between peak area and serum concentration. The mean absolute deviation from a straight line was 0.033 μg/ml, and the limit of detectability was 0.02 μg/ml. Since peak heights yielded a correlation coefficient of 0.99985 and a mean absolute deviation from a straight line of 0.044 μg/ml, peak heights could be used for some small peaks that failed to be measured by integration.

Fluorescence was sensitive to the matrix of the injected sample. For example, serum, serum–0.85% sodium chloride (50:50), sputum and urine were processed and then spiked with identical amounts of ciprofloxacin. These samples gave fluorescent peak areas relative to ciprofloxacin–spiked 0.3 *M* potassium phosphate, pH 1.5, of 1.11, 1.15, 1.02 and 1.02, respectively. Therefore recovery of ciprofloxacin from each matrix was determined relative to the same matrix which was first processed and then spiked. Recoveries of 1.00 μg/ml ciprofloxacin from serum, sputum and urine were 98, 96 and 96%, respectively.

The within-day coefficients of variation at 1.00 μg/ml ($n=16$) for serum, sputum and urine were 2.8, 2.8 and 1.2%, respectively. At 0.10 μg/ml ($n=16$) these

within-day coefficients of variation were 9.7, 6.4 and 4.9%, respectively. The between-day coefficient of variation in serum at 1.00 μg/ml ($n=16$) was 4.5%.

Ciprofloxacin stability was tested in serum, urine and 0.3 *M* potassium phosphate, pH 1.5. At 1.00 μg/ml it was completely stable in these mixtures over twenty weeks at 4, −20 or −70 °C.

Applicability to clinical studies

Drugs which might interfere with the ciprofloxacin assay were tested. None of the following showed interfering fluorescent peaks under the assay conditions outlined above: acetaminophen, carbenicillin, caffeine, ceftazidime, chloramphenicol, cimetidine, phenobarbital, phenytoin, sulfamethoxazole, theobromine, theophylline, ticarcillin and diazepam. Salicylic acid did exhibit fluorescence but its recovery in this assay was only 4% and it eluted 4.5 min later than ciprofloxacin.

The interference of endogenous substances with ciprofloxacin fluorescence was not observed. Serum and urine pre-drug specimens showed no peak at the elution time of ciprofloxacin in 35 pharmacokinetic studies. There was evidence of a very small peak in two sputum pre-drug specimens but in both cases the patients had taken ciprofloxacin ten days earlier. It is possible that the peak was actually ciprofloxacin which had not been completely cleared from the lungs.

Ciprofloxacin metabolites detected by fluorescence

Although there were no substances which interfered with the ciprofloxacin peak, chromatography of serum, sputum and urine from patients who received ciprofloxacin showed the presence of new fluorescent peaks. Figs. 2 and 3 illustrate the chromatography of both pre-drug and on-therapy specimens. Four fluorescent metabolites (I, II, IV and V) could be detected in serum, sputum and urine. Peaks I and II were present in all specimens while peaks IV and V showed a marginal detectability in serum and particularly sputum. Chromatography of pre-drug specimens in the routine method often showed fluorescent material at the position of peak I.

The four fluorescent metabolites were isolated in small quantities by preparative HPLC. Urine was extracted without dilution and injected at a mobile phase flow-rate of 0.4 ml/min. In order to confirm the identity of the isolated metabolite peaks collected in the column effluent, they were reinjected onto the HPLC column under routine assay conditions. Each peak retained its distinctive elution time. The capacity factors for these peaks are shown in Table I. Fluorescence spectra were also examined. Ciprofloxacin showed double excitation peaks at 280 and 328 nm and an emission peak at 446 nm (Fig. 4). The spectra of the metabolites were quite similar to one another (Table I). They all exhibited about a 10-nm bathochromic shift of the 328-nm ciprofloxacin excitation peak. In addition metabolites I and II showed at least a 17-nm hypsochromic shift of the ciprofloxacin emission peak while metabolites IV and V showed a 12-nm hypsochromic shift.

A metabolite of ciprofloxacin, desethyleneciprofloxacin, which has been isolated from urine, characterized and named M-1 [8], showed the same HPLC elution time and fluorescence spectra as peak I (Table I). Desethyleneciproflox-

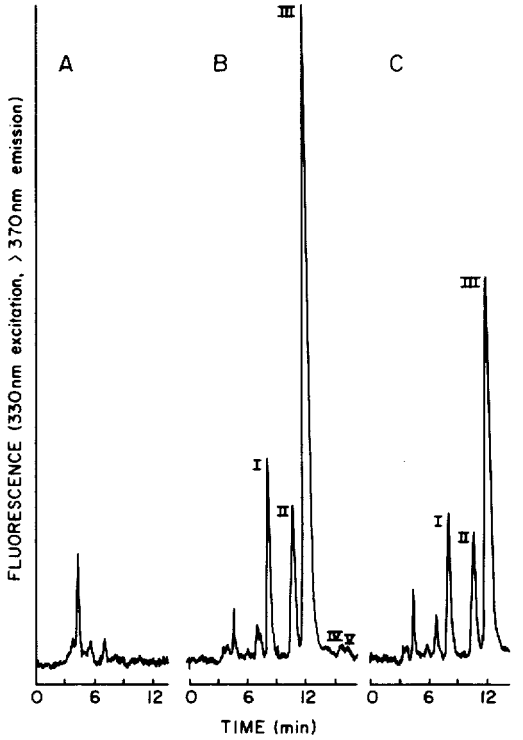

Fig. 2. Isocratic HPLC of ciprofloxacin (III) and metabolites (I, II, IV and V) in patient serum. (A) Serum blank; (B) 1.21 μg/ml ciprofloxacin at 30 min post-dose; (C) 0.69 μg/ml at 8 h.

acin results from a cleavage of the 1-piperazinyl ring to an N-ethyleneamine group. Peak I was desalted by HPLC in 0.1 *M* formic acid–acetonitrile (8:2). After evaporation a mass spectral determination by methane chemical ionization at a source temperature of 200 °C showed a fragmentation pattern consistent with the above structure (Fig. 5). The mass of the molecular ion was within 0.003 mass units of that theoretically calculated for desethyleneciprofloxacin. Unfortunately the unknown peak II which was processed in the same manner behaved as a salt and did not give a mass spectrum. Krol [11] has suggested that peak II may result from further metabolism of the UV-absorbing metabolite, oxociprofloxacin.

In order to study the appearance of the fluorescent metabolites in more detail, a gradient assay was developed. Acetonitrile was placed in one reservoir and 0.1 *M* potassium phosphate, pH 2.5, in the second reservoir. The gradient was run from 15 to 27% acetonitrile over 30 min. For this study it was necessary to use an alternative method of sample preparation since the routine method was designed to eliminate late-eluting peaks. Peaks IV and V showed particularly poor recovery in the routine method. Consequently the protein in a 0.5-ml serum sample was precipitated with 30 μl of 70% trichloroacetic acid (TCA) (w/v). The resulting supernatant was analyzed by HPLC. Standards were prepared by spiking sera with known amounts of ciprofloxacin and desethyleneciprofloxacin. Since all the fluorescent metabolites exhibited recoveries from serum of approximately 50%

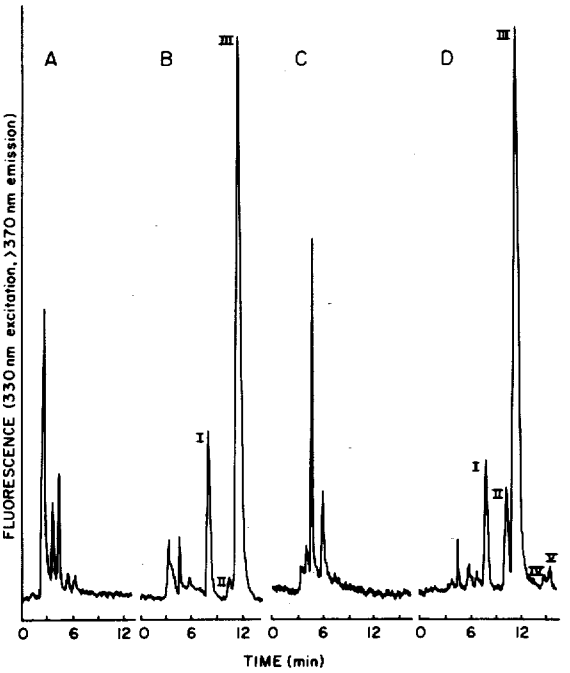

Fig. 3. Isocratic HPLC of ciprofloxacin (III) and metabolites (I, II, IV and V) in patient sputum and urine. (A) Sputum blank; (B) 1:5 dilution of sputum at a diluted concentration of 1.36 μg/ml ciprofloxacin; (C) 1:200 dilution of blank urine; (D) 1:500 dilution of urine at a diluted concentration of 1.26 μg/ml ciprofloxacin.

TABLE I

FLUOROMETRIC AND CHROMATOGRAPHIC IDENTIFICATION OF SUBSTANCES ISOLATED FROM URINE AFTER A DOSE OF CIPROFLOXACIN; COMPARISON WITH KNOWN SUBSTANCES

Three to eleven spectra are included in each peak determination; values are mean ± S.D.

Substance	Fluorescence (nm)			HPLC (k')*
	Excitation	Peaks	Emission	
Peak I	278 ± 1.2	342 ± 0.7	429 ± 1.7	1.70
Peak II	277 ± 2.6	341 ± 1.5	428 ± 0.5	2.60
Peak III	280 ± 2.8	328 ± 1.6	446 ± 1.4	2.96
Peak IV	277 ± 2.5	339 ± 2.1	434 ± 1.3	4.34
Peak V	276 ± 2.1	338 ± 0	434 ± 1.3	4.66
Desethylene-ciprofloxacin	279 ± 2.1	342 ± 1.9	430 ± 2.3	1.69
Ciprofloxacin	281 ± 1.4	328 ± 0.8	449 ± 2.2	2.93

*Capacity factor or $k' = (t_P - t_M)/t_M$, where t_P is the elution time of the peak and t_M is the elution time of the solvent front in the routine, isocratic method.

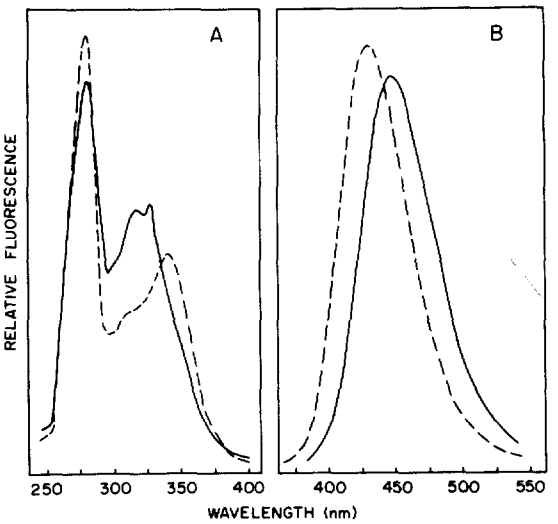

Fig. 4. Fluorescence spectra of ciprofloxacin and peak II isolated by HPLC from urine. (A) Excitation spectra of ciprofloxacin (—) at an emission wavelength of 445 nm and peak II (- - -) at an emission wavelength of 430 nm. (B) Emission spectra of ciprofloxacin (—) at an excitation wavelength of 280 nm and peak II (- - -) at an excitation wavelength of 275 nm.

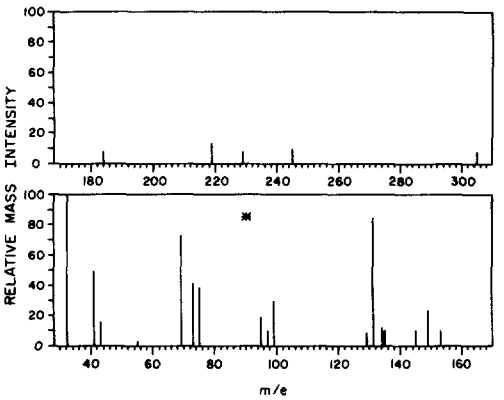

Fig. 5. Mass spectrum of peak I (desethyleneciprofloxacin). (*) Relative mass intensity of ions with $m/e > 90$ are shown at five times the actual intensity.

in this TCA preparation and since the fluorescence spectra of the four metabolites were so similar to one another (Table I), the desethyleneciprofloxacin standard was used to determine estimated concentrations of peaks II, IV and V. Fig. 6 is a plot of changing serum ciprofloxacin and metabolite concentrations over time in a patient who was given a single 8.6 mg/kg dose of ciprofloxacin. This plot shows that the maximum serum concentrations of these fluorescent molecules appeared sequentially: ciprofloxacin was followed by peak I, then peak II and lastly peaks IV and V. Pre-drug specimens showed no peaks eluting at the same time as the fluorescent metabolites in this gradient method.

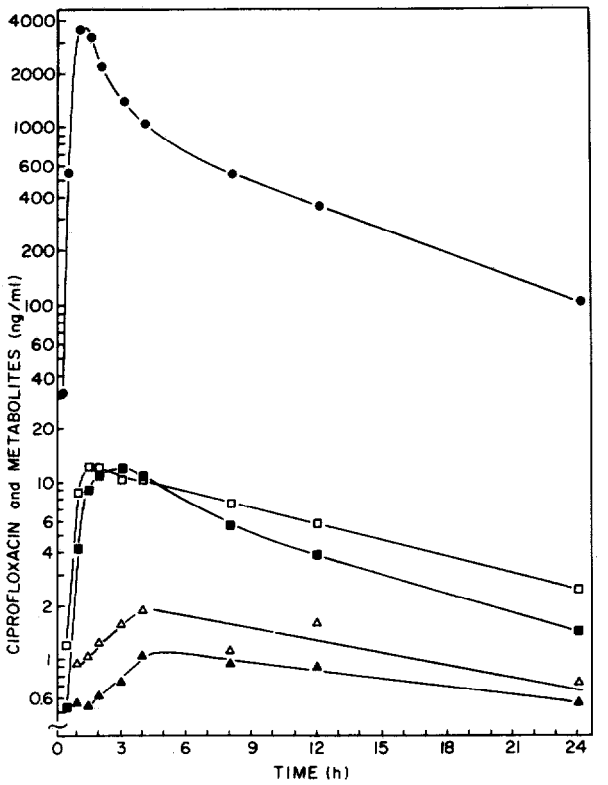

Fig. 6. Pharmacokinetics of ciprofloxacin and its fluorescent metabolites in serum following a single 8.6 mg/kg dose. (●) Ciprofloxacin; (□) peak I; (■) peak II; (▲) peak IV; (△) peak V.

UV-absorbing metabolites

Other ciprofloxacin metabolites have been identified previously [8]. Two of these which exhibit a poor fluorescence could be identified by HPLC using simultaneous UV and fluorescence detection and extending the gradient from 15 to 35% over 50 min. Fig. 7 shows chromatograms of pre-drug and on-therapy urine analyzed in this way. Prior to injection urine was diluted twenty-fold with 0.3 *M* potassium phosphate, pH 1.5. The elution times of the UV-absorbing peaks at 31 and 45 min (Fig. 7B) corresponded to sulfociprofloxacin and oxociprofloxacin, respectively (Fig. 1). Several late-eluting fluorescent peaks were also observed. The four fluorescent metabolites, the two UV-absorbing metabolites and ciprofloxacin comprised, respectively, 0.20, 1.13 and 3.52% of the dose in this 8–16 h post-dose urine sample. The total amount of the six urinary metabolites from this cystic fibrosis patient who was given a single 10.2 mg/kg dose of ciprofloxacin (750 mg total) was 12.6% of the ciprofloxacin dose. Unchanged ciprofloxacin represented 41.2% of the dose. Chromatographic analysis of urine from three cystic fibrosis and two normal subjects given 750 mg of ciprofloxacin yielded mean urinary recoveries of fluorescent metabolites, UV-absorbing metabolites and ciprofloxacin of 1.3, 9.9 and 47% of the dose, respectively. Preliminary data have suggested that the point of maximum urinary excretion of the UV-absorbing metabolites was slightly earlier than that of the fluorescent metabolites.

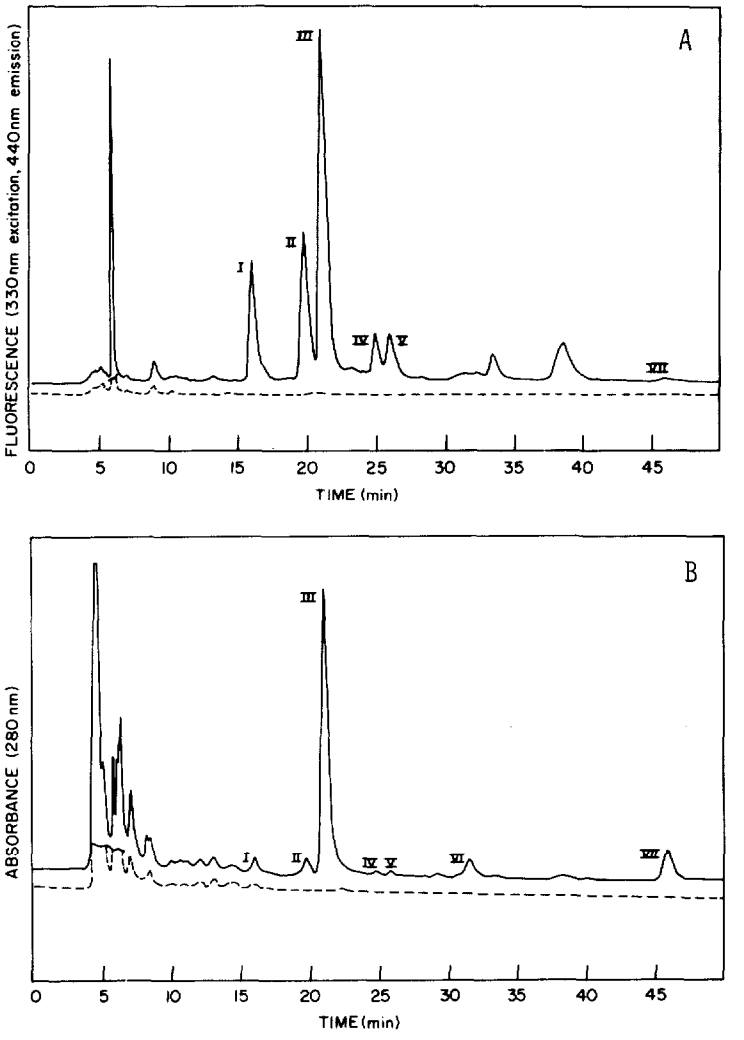

Fig. 7. Gradient HPLC using dual spectrometry on 1:20 dilutions of urine before drug administration (- - -) and 8–12 h after a 10.2 mg/kg dose of ciprofloxacin (—): ciprofloxacin (III), fluorescent metabolites (I, II, IV, V) and UV-absorbing metabolites (VI, sulfociprofloxacin; VII, oxociprofloxacin). (A) Fluorescence detection at 330 nm excitation and 440 nm emission. (B) Ultraviolet detection at 280 nm.

It is difficult to analyze the UV-absorbing metabolites in serum due to a combination of a low extinction coefficient and low extraction recovery. Nevertheless there was an indication that their concentration relative to ciprofloxacin may approach that in urine: the peak serum concentration of sulfociprofloxacin in one cystic fibrosis subject was 10% of the peak ciprofloxacin concentration.

DISCUSSION

The method above is a versatile, sensitive procedure and caused negligible column deterioration. A gradient modification of the method allowed the determi-

nation of several fluorescent metabolites and their appearance relative to one another. The UV-absorbing urinary metabolites could also be identified in the gradient method. In contrast to our gradient method, the polymer column method of Krol et al. [12] resulted in the elution of the sulfo metabolite before peak I and ciprofloxacin.

None of the currently published methods clearly show the widespread presence of fluorescent species generated from the oral ingestion of ciprofloxacin [8–10, 12–18]. A serum chromatogram in the report of Weber et al. (ref. 10, Fig. 2, panel 6) suggested the presence of a peak corresponding to peak II but the peak was small and not mentioned in the text. Gau et al. [8] and Krol et al. [12] identified the early-eluting M-1 (peak I) in their chromatographic systems and quantitated it using UV detection. In our hands our gradient system using dual spectrometry suggested that low concentrations of peak I might be overestimated by absorbance measurements on account of variable, coeluting UV-absorbing material. Hoffken et al. [13] quantitated two metabolites (M1 and M2) from urine (assuming a fluorescent response identical to ciprofloxacin) but did not illustrate or describe the chromatographic location of their metabolites. Other methods in the literature show only chromatograms of ciprofloxacin-spiked matrixes [9,14] or show no chromatograms at all [15, 16]. The concentrations of the metabolites are too low to be detected in routine methods employing UV spectroscopy [18].

It is interesting that norfloxacin, a close relative of ciprofloxacin, possesses a piperazinyl ring which is particularly subject to metabolic alteration [19]. Metabolites which could be identified by both fluorescence and ultraviolet spectroscopy were products of piperazinyl ring cleavage, i.e. oxidation with cleavage of the ring and degradation of the ring to an amine or N-ethyleneamine. The metabolites which retained an intact ring showed minimal fluorescence. This pattern is also emerging with ciprofloxacin. Desethyleneciprofloxacin possesses a degraded piperazinyl ring while sulfociprofloxacin and oxociprofloxacin have an intact piperazinyl ring. Peaks II, IV and V are probably products of piperazinyl ring cleavage.

Some metabolites of ciprofloxacin have also been examined by Beermann et al. [20]. They examined both urine and feces after oral doses of ^{14}C-labeled ciprofloxacin. In urine 44.7% of the label was recovered as unchanged ciprofloxacin, and 11.3% was recovered as desethyleneciprofloxacin, sulfociprofloxacin and oxociprofloxacin. These values are very similar to the results presented above. Most of the remaining label was found in the feces and showed a similar ratio of ciprofloxacin to metabolites. Intravenous drug administration resulted in somewhat less metabolic conversion and lower fecal recovery.

The variety of fluorescent and UV-absorbing metabolites can be easily separated by the gradient method outlined above. Quantitatively, the UV-absorbing metabolites are the most important and can account for about 12% of the dose in the urine. While the fluorescent metabolites identified here account for less than 2% of the dose, their role in the biodisposition of ciprofloxacin can now be elucidated.

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