Journal of Chromatography, 419 (1987) 414–420
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3716

Note

Determination of ciprofloxacin and its 7-ethylenediamine metabolite in human serum and urine by high-performance liquid chromatography

WALID M. AWNI*, JAY CLARKSON and DAVID R.P. GUAY

Pharmacokinetics Laboratory, The Drug Evaluation Unit, Hennepin County Medical Center, 701 Park Avenue South, Minneapolis, MN 55415 (U.S.A.) and*
College of Pharmacy, University of Minnesota, Minneapolis, MN 55455 (U.S.A.)

(First received January 13th, 1987; revised manuscript received March 11th, 1987)

Ciprofloxacin [1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperzinyl)-3-quinoline carboxylic acid, Fig. 1] is a new second-generation quinoline carboxylic acid antimicrobial agent with high in vitro activity against a wide range of gram-negative and gram-positive organisms. Ciprofloxacin is thought to act by DNA gyrase inhibition, similar to nalidixic acid and norfloxacin [1].

Several high-performance liquid chromatographic (HPLC) methods have been reported for the analysis of ciprofloxacin in biological fluids [2–10]. Some of these methods have utilized UV detection [3,4,7,8], while others have utilized fluorescence detection [2,4–6,9,10]. Most of these methods have not included internal standards [3–6, 8–10]. Only one method has included the determination of ciprofloxacin metabolite concentrations [6].

The subject of this report is an optimized HPLC method for the determination of ciprofloxacin and its 7-ethylenediamine metabolite in human serum and urine using fluorescence detection. This method involves a simple one-step extraction procedure for ciprofloxacin and its 7-ethylenediamine metabolite from urine and serum, utilizes an internal standard, and is highly sensitive with a limit of sensitivity of 1 ng/ml for ciprofloxacin in urine or serum. This method has been used extensively in the pharmacokinetic assessment of ciprofloxacin in humans.

0378-4347/87/$03.50

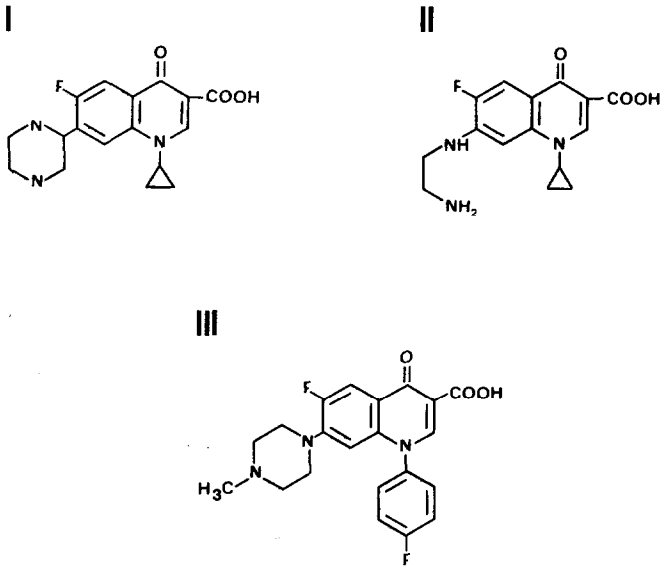

Fig. 1. Chemical structures of ciprofloxacin (I), 7-ethylenediamine metabolite of ciprofloxacin (II) and difloxacin, internal standard (III).

EXPERIMENTAL

Instrumentation

Isocratic liquid chromatographic separation was accomplished using a Model 600 gradient pump and controller (Waters Assoc., Milford, MA, U.S.A.); Model 712 WISP autosampler (Waters); Kratos Model 980 fluorescence detector (Kratos Analytical, Ramsey, NJ, U.S.A.) with excitation wavelength set at 278 nm and emission wavelength at 470 nm; Model 4290 plotting integrator (Spectra-Physics, San Jose, CA, U.S.A.). Separation was carried out at room temperature on a 30 cm×3.9 mm prepacked μBondapak C_{18} column, 5 μm particle size (Waters).

Chemicals

Water was purified by reverse osmosis. Acetonitrile and methanol were HPLC grade (Burdick & Jackson Labs., Muskegon, MI, U.S.A.). Tetrabutyl ammonium hydroxide was purchased from Sigma (St. Louis, MO, U.S.A.). Phosphoric acid was purchased from Mallinckrodt (Paris, KY, U.S.A.). Lyophilized ciprofloxacin (Bay 09867,I) and 7-ethylenediamine ciprofloxacin metabolite M-1 (R3964,II) were a gift from Miles Pharmaceuticals (West Haven, CT, U.S.A., Fig. 1). Difloxacin (A-56619, internal standard,III, Fig. 1) was a gift from Abbott Labs. (Abbott Park, IL, U.S.A.).

Stock solutions

Stock solutions containing 200, 20 and 2 μg/ml of lyophilized ciprofloxacin were prepared in water. Aliquots of stock drug solution were stored in polypro-

pylene tubes at −70°C until used. The internal standard stock solutions containing 200, 20 and 2 μg/ml were prepared in water. Aliquots were prepared and stored as above. A stock solution of ciprofloxacin metabolite (II) was received from the supplier as an aqueous 100 μg/ml solution. This solution was diluted further to 10 μg/ml and divided into aliquots for storage as above.

Serum quality control samples of 2.0 and 0.2 μg/ml were made by diluting 100 μl of 200 μg/ml stock and 10 μl of 200 μg/ml stock ciprofloxacin to a final volume of 10 ml in pooled human serum. Urine quality control samples of 10 and 1 μg/ml were made by diluting 500 and 50 μl of 200 μg/ml stock ciprofloxacin solution to a final volume of 10 ml with 1:100 diluted urine.

Sample preparation

A standard curve including concentrations of 0, 0.04, 0.1, 0.2, 0.4, 1.0 and 2.0 μg/ml in human serum was prepared each day. Serum samples were stored at −70°C. The extraction procedure was performed with all solutions at room temperature. Each sample was thoroughly mixed and 500 μl of serum were transferred to a 12×75 mm glass tube containing 500 μl phosphate buffer (pH 7.4) and 10 μl of internal standard (20 ng/μl). Dichloromethane (2 ml) was then added. The sample was vortexed for 15–30 s, and then centrifuged at 4200 *g* for 7 min. The upper aqueous layer was discarded and the organic layer was evaporated under a nitrogen stream. Samples were reconstituted with 200 μl of mobile phase, vortexed and transferred to polypropylene low-volume inserts and injections of 20 μl were made using the autosampler.

In urine, a standard curve including concentrations of 0, 0.4, 1.0, 2.0, 5.0, 10.0 and 20.0 μg/ml was prepared daily. Urine samples were diluted 1:99 by mixing 10-μl aliquots of urine with 990 μl of mobile phase. After vortexing, aliquots were transferred to low-volume inserts and 10-μl injections were made by autosampler.

Mobile phase

Potassium dihydrogenphosphate (5.444 g) was added to a 1.0-l graduated cylinder and dissolved in approximately 500 ml of water. Tetrabutyl ammonium hydroxide (8.50 ml) was added and the mixture was diluted with water to yield 1.0 l of solution. This was filtered through a 0.22-μm Nylon-66 membrane filter (AMF, Meriden, CT, U.S.A.) and 1.0 l of water was filtered and added to the same flask. The pH was adjusted to 3.00 ± 0.03 with concentrated phosphoric acid (85%). Methanol (346 ml) and acetonitrile (123 ml) were added and the entire solution was degassed for about 10 min. The solution was also sparged with helium throughout the chromatographic run.

Chromatographic conditions

The final composition of the mobile phase was 14% (v/v) methanol, 5% (v/v) acetonitrile, 0.3% (w/v) tetrabutylammonium hydroxide in 0.02 *M* potassium dihydrogenphosphate, adjusted with phosphoric acid to pH 3.0. The flow-rate was set at 1.7 ml/min. Fluorescence detector excitation wavelength was set at 278 nm and emission wavelength at 470 nm. The chart speed setting was 0.5 cm/min and the range was set at 0.1.

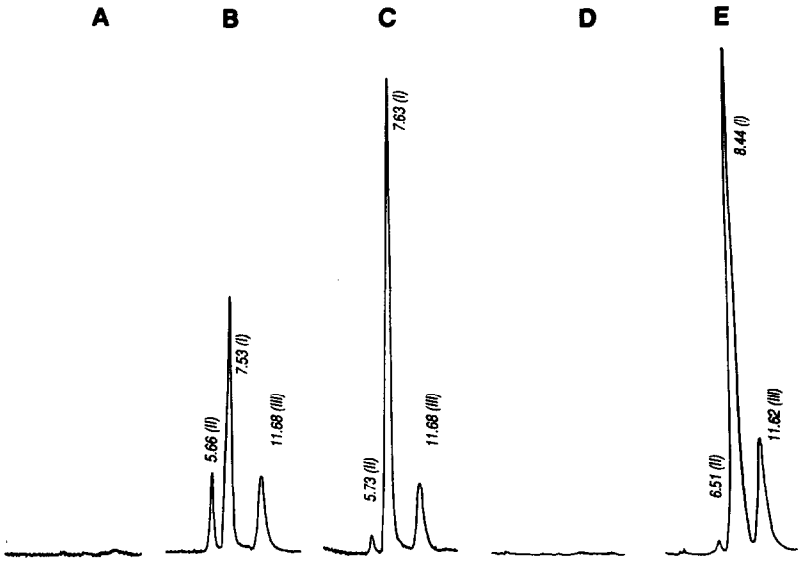

Fig. 2. Chromatograms of (A) blank serum; (B) spiked serum containing 2 μg/ml ciprofloxacin (I), 1 μg/ml of the metabolite of ciprofloxacin (II) and internal standard (III); (C) patient serum containing 5.83 μg/ml I and 0.167 μg/ml II; (D) blank urine; (E) patient urine containing 17 μg/ml I and 0.120 μg/ml II.

RESULTS AND DISCUSSION

Chromatograms resulting from the analysis of blank serum, patients' serum, blank urine and patients' urine are shown in Fig. 2. Retention times for the M-1 metabolite, ciprofloxacin and internal standard are approximately 5.7, 7.6 and 11.7 min, respectively. No interfering peaks have been detected in patients serum or urine. Ciprofloxacin concentrations as low as 1 ng/ml in serum or urine, at 5:1 signal-to-noise ratio, were easily measurable using the method described here.

Extraction efficiency of the assay procedure

Duplicate samples with high and low concentrations of ciprofloxacin (2.0 and 0.2 μg/ml, respectively) in human serum were extracted with and without the initial addition of internal standard. The extraction was carried out as usual for all samples. Before reconstituting, internal standard was added to the dried samples containing only ciprofloxacin. These were dried down again and then all samples were reconstituted as usual. Comparison of peak-height ratios from the samples with extracted versus unextracted internal standard indicated an extraction efficiency of 61% at the 2 μg/ml concentration and 70% at the 0.2 μg/ml concentration of ciprofloxacin.

Precision

Serum. To assess the within-day precision, the mean, standard deviation (S.D.) and coefficient of variation (C.V.) of the peak-height ratio over the entire range of concentrations of the standard curve were estimated by running quadruplicate

TABLE I

INTRA-DAY AND INTER-DAY CIPROFLOXACIN PRECISION IN SERUM AND URINE

S.D. = standard deviation; C.V. = coefficient of variation.

Ciprofloxacin concentration (μg/ml)	Peak-height ratio					
	Within-day ($n=4$)			Day-to-day ($n=3$)		
	Mean	S.D.	C.V. (%)	Mean	S.D.	C.V. (%)
Serum						
2.0	3.27	0.250	7.7	4.28	0.068	1.6
1.0	1.53	0.073	4.8	-	-	-
0.4	0.60	0.039	6.4	0.67	0.038	5.7
0.2	0.316	0.024	7.6	0.41	0.022	5.5
0.1	0.133	0.009	6.8	0.197	0.021	10.7
0.04	-	-	-	0.081	0.002	2.6
Urine						
20.0	5.76	0.187	3.3	5.36	0.187	3.5
10.0	2.94	0.041	1.4	2.56	0.205	8.0
5.0	1.47	0.015	1.0	1.305	0.120	9.2
2.0	0.521	0.069	13.2	0.466	0.034	7.3
1.0	0.351	0.009	2.7	0.214	0.029	13.4
0.4	0.163	0.016	9.8	0.090	0.017	18.9

standard curves on the same day (Table I). The C.V. at each concentration was lower than 8%.

The day-to-day precision in serum was determined by comparing the peak-height ratios for three standard curves extracted and injected within one month. The mean, S.D. and C.V. of the peak-height ratios were calculated at each standard concentration (Table I). Coefficients of variation were less than 11% at all drug concentrations.

Urine. Table I also illustrates the within-day precision of the standard curve. The mean, S.D. and C.V. of the peak-height ratios were calculated from a quadruplicate standard curve which was run on the same day. Although the C.V. of one of the standards was 13.2%, the C.V. of all other standards were less than 10%. The day-to-day precision data in urine is also presented in Table I.

Stability of the drug

Ten injections of 50 ng of both ciprofloxacin and internal standard were made over a 4-h period. The peak-height ratios showed no indication of breakdown of the drug and internal standard (mean = 1.216, C.V. = 0.58%). The drug was also found to be stable in serum for up to 48 h at room temperature.

Linearity of the standard curve

Ciprofloxacin standard curves were linear in the range 0.02–20 μg/ml in serum and in the range 0.4–20 μg/ml in urine. The reproducibility of the slope of the

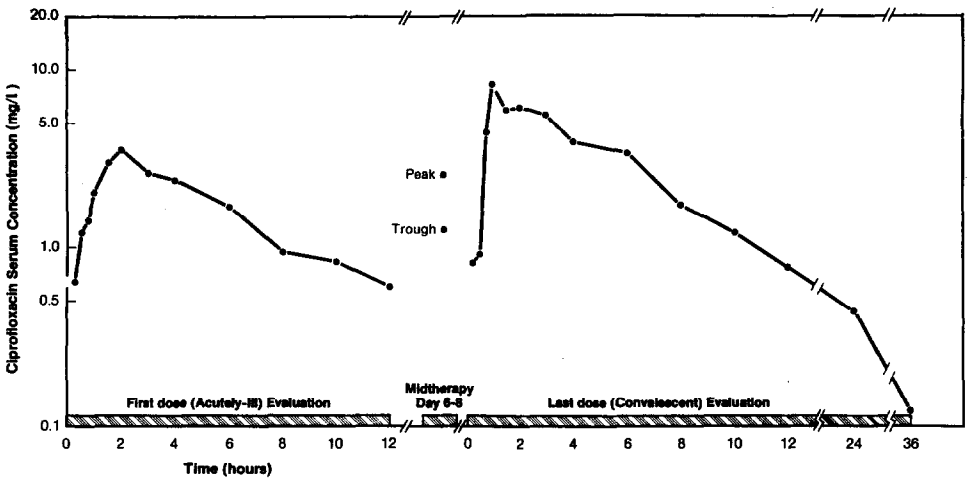

Fig. 3. Representative ciprofloxacin serum concentration versus time curve for an elderly patient with pneumonia receiving 750 mg ciprofloxacin orally every 12 h for fourteen days.

standard curve in serum and urine during a two-month period was also determined. The mean slope of the serum standard curve, C.V. and correlation coefficient were 1.977, 11% and unity, respectively. The mean slope of the urine standard curve, C.V. and correlation coefficient were 0.266, 3.3% and unity, respectively.

Recovery data

Recovery data were used to determine the accuracy of the analytical method. Recovery was calculated as the percentage ratio of measured/spiked concentration. The mean recovery at the different concentrations of the standard curve was 101.4% with a C.V. 6.2% for ciprofloxacin in serum. In urine, the mean recoveries at the low and high quality controls were determined to be 97.8 and 98.9% with 4.7 and 3.7% C.V., respectively.

Pharmacokinetic studies

The method that we are reporting here has been used extensively in the study of ciprofloxacin pharmacokinetics in acutely ill and covalescent elderly patients [11]. Fig. 3 illustrates the serum concentration versus time data of ciprofloxacin after the administration of 750 mg of ciprofloxacin twice daily for fourteen days to a representative patient. Both serum and urine concentration of the drug have been measured using this method.

In summary, a highly sensitive, accurate and reproducible HPLC method for the determination of ciprofloxacin and its 7-ethylenediamine metabolite in human serum and urine is reported here. This method utilizes fluorescence detection (278 nm excitation, 420 nm emission wavelengths) and an internal standard. Ciprofloxacin concentrations as low as 1 ng/ml can be measured in urine or serum using this method. In addition, the extraction procedure is rapid, inexpensive and

simple. This method has been used successfully in pharmacokinetic studies of ciprofloxacin in patients.

ACKNOWLEDGEMENTS

The assistance of G. Krol and D. Webb, Miles Pharmaceuticals, for providing pure analytical standards, and J. Lovick and R. Hickey for secretarial help is gratefully acknowledged. This work was supported in part by a grant-in-aid from Miles Pharmaceuticals.

REFERENCES

1 J.S. Wolfson and D.C. Hooper, Antimicrob. Agents Chemother., 28 (1985) 581.
2 D.E. Nix, J.M. DeVito and J.J. Schentag, Clin. Chem., 31 (1985) 684.
3 F. Jehl, C. Gallion, J. Debs, J.M. Brogard, H. Monteil and R. Minck, J. Chromatogr., 339 (1985) 347.
4 A. Weber, D. Chaffin, A. Smith and K.E. Opheim, Antimicrob. Agents Chemother., 27 (1985) 531.
5 B. Joos, B. Ledergerber, M. Flepp, J.D. Bettex, R. Luthy and W. Siegenthaler, Antimicrob. Agents Chemother., 27 (1985) 353.
6 K. Boner, J. Clin. Chem. Clin. Biochem., 24 (1986) 325.
7 F. Vallee, M. LeBel and M.G. Bergeron, Ther. Drug Monit., 8 (1986) 340.
8 A.J.N. Greenveld and J.R.B. Brouwers, Pharm. Weekbl., 8 (1986) 79.
9 W. Gau, H.J. Plaschke, K. Schmidt and B. Weber, J. Liq. Chromatogr., 8 (1985) 485.
10 C.E. Fasching and L.R. Peterson, J. Liq. Chromatogr., 8 (1985) 555.
11 D.R.P. Guay, W.M. Awni, P.K. Peterson, S. Obaid, R. Breitenbucher and G.R. Matzke, Am. J. Med., 82, Suppl. 4A (1987) 124.