Journal of Chromatography, 339 (1985) 347—357
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2500

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR DETERMINATION OF CIPROFLOXACIN IN BIOLOGICAL FLUIDS

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(First received August 31st, 1984; revised manuscript received November 21st, 1984)

SUMMARY

A simple and precise high-performance liquid chromatographic procedure has been developed for the determination in biological fluids of ciprofloxacin, a new, with extended antibacterial spectrum, quinoline carboxylic acid. The work-up procedure involves a chemical extraction step followed by isocratic chromatography on a reversed-phase analytical column, with ultraviolet detection. The detection limit for blood levels is 10 ng/ml. The calibration curve is linear from this detection limit to 10 μg/ml. The statistical analysis of the correlation made between this assay and an agar diffusion procedure during a pharmacokinetic study suggests the existence of one or more active metabolites which could be mainly excreted in the bile.

INTRODUCTION

Quinoline carboxylic acids such as nalidixic and pipemidic acids have been available for years. The narrow spectrum of these antibiotics, and the low

0378-4347/85/$03.30

concentrations obtained in serum limit their use essentially against Gram-negative enteric bacilli encountered in urinary tract infections [1].

Enoxacin [2], AT 2266, a new pyridone carboxylic anti-pseudomonal agent [3], ofloxacin [4], norfloxacin [5—8], pefloxacin [9], cinoxacin [10] and ciprofloxacin [11—13] are recently developed quinoline derivatives that are structurally related to nalidixic acid. They show a greater potency and broader antibacterial spectrum which includes Gram-negative and Gram-positive bacteria. Numerous in vitro studies have shown their activity against *Enterobacteriaceae*, *Staphylococcus* spp., *Pseudomonas aeruginosa*, *Haemophilus* spp., *Neisseria* spp., *Bacteroïdes fragilis*, *Acinetobacter calcoaceticum* and *Listeria monocytogenes* [1—13]. Although they have a parallel spectrum of activity, ciprofloxacin seems to be more potent than the other new quinoline derivatives [10, 14—17].

As further pharmacokinetic investigations are needed, particularly on the possibility of treating systemic infections, we have developed a rapid, accurate and specific assay for the measurement of this antibiotic in human serum, bile and urine, by means of reversed-phase high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Chemicals

Ciprofloxacin (Bay 09867) was obtained from Bayer Pharma, France. Stock solutions of 10 mg/ml were prepared in double-distilled water. Acetonitrile, methylene chloride, tetrabutylammonium bromide and phosphoric acid were purchased from E. Merck, Darmstadt, F.R.G. Water was daily double-distilled in quartz.

For the establishment of the calibration curves, serum, urine and bile free of any antibiotic were obtained from patients before any treatment with ciprofloxacin.

Sample treatment

An aliquot (500 µl) of biological fluid (serum, urine diluted 1:20 or bile diluted 1:10) is added to 3.5 ml of methylene chloride in a 6-ml glass screw-capped tube. After mixing for a few seconds on a vortex mixer, the tubes are gently shaken for 10 min by rotation (20 rpm) and then centrifuged for 10 min at 1000 *g*. The upper aqueous layer is discarded by aspiration, and 3 ml of the lower organic phase are transferred to a second glass screw-capped tube. Ciprofloxacin is then back-extracted using 200 µl of phosphoric acid at pH 2 by rotation for 30 min. Centrifugation at 1000 *g* for 10 min, results in phase separation; 20 µl of the upper aqueous phase are then injected into the liquid chromatograph.

Establishment of the standard curves

Sera free of antibiotics were spiked with increasing amounts of ciprofloxacin (10, 20, 50, 100, 200 ng/ml and 0.5, 1, 2, 5, 10 µg/ml). These sera were then submitted to HPLC analysis and peak heights were plotted against the concentrations of drug to check for linearity. The same procedures

were followed for urine and bile, except for the amounts added yielding concentrations ranging from 10 to 200 μg/ml and from 1 to 200 μg/ml, respectively.

Chromatographic conditions

The isocratic liquid chromatograph was constituted from the following units: a 112 solvent delivery module (Beckman, Berkeley, CA, U.S.A.), a 210 sample injection valve equipped with a 20-μl loop (Beckman), a Model 160 selectable-wavelength detector (Beckman) and a Model ICR 1 B recording data processor (Intersmat Instruments, Courtry, France). Separations were performed on a 150 × 4.6 mm, C_{18} reversed-phase analytical column, particle size 5 μm (Ultrasphere, Beckman).

The mobile phase consisted of acetonitrile—0.005 *M* tetrabutylammonium bromide (10:90). Phosphoric acid (14.6 *M*) was then added to adjust the pH to 2. The mobile phase was filtered through a 0.22-μm membrane. The flow-rate was set at 2 ml/min and the eluent monitored at 254 nm. The range setting of the spectrophotometer depended on the concentration of drug measured.

Precision

Serum, urine and bile free of antibiotic were spiked with known amounts of ciprofloxacin for the study of reproducibility.

Within-day reproducibility. Ten aliquots of a serum containing 0.5 μg/ml and ten aliquots of a serum containing 1.5 μg/ml ciprofloxacin were randomly distributed in different series of assays, on the same day, during a pharmacokinetic study. The concentrations of drug tested with bile and urine for the within-day reproducibility were 10 and 20 μg/ml, respectively.

Between-day reproducibility. Ten aliquots of the same biological fluids as those 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 within-day and between-day reproducibilities, precision was evaluated by calculating the coefficients of variation.

Microbiological assay procedure

The quantitative determination of microbiologically active drug in plasma, bile and urine was made by an agar diffusion assay. The reference strain used was *Escherichia coli* ICB No. 4004 (Bayer, Wuppertal, F.R.G.). Isotonic sensitest agar (Oxoïd) medium was prepared according to the manufacturer's instructions and poured onto 20 cm × 20 cm assay plates (about 80 ml per plate). The final agar thickness was 0.2 cm on the plates. After cooling the agar, 2-mm diameter wells were punched. The arrangement of the punched holes and the positioning of the different samples were randomized. An exactly measured volume of 5 μl of sample was dispended into each well. Serum standard concentration (5, 2.5, 1.2, 0.6, 0.3 μg/ml) are obtained by diluting the stock solution with serum free of any antibiotic. For urine and bile standard concentrations (10, 5, 2.5, 1.2, 0.6 μg/ml) the dilutions were made with buffered saline at pH 7.2. Each sample was run in triplicate. Results were read after an overnight incubation at 37°C. Inhibition zones were measured with the aid of a light projector.

Statistical analysis

The comparison between the two methods was made by orthogonal regression analysis with all of the data for each of the three series of body fluids, i.e. serum, urine and bile. All the samples were obtained during a pharmacokinetic study. For each of these body fluids, the mean difference of all paired values was compared with a difference of zero, using Student's t test for the comparison of two means. An α value of 0.05 was retained as the threshold point for statistical significance.

RESULTS

Typical chromatograms resulting from the analysis of various body fluids are shown in Figs. 1 and 2. Ciprofloxacin appears as a well resolved peak, the retention time of which is 2.4 min. During our pharmacokinetic study, no interference owing to endogenous substances or to co-administered drugs could be detected. These clinical specimens were known to contain one or more of the following drugs: analgesics, salicylate, phenobarbital, carbamazepine,

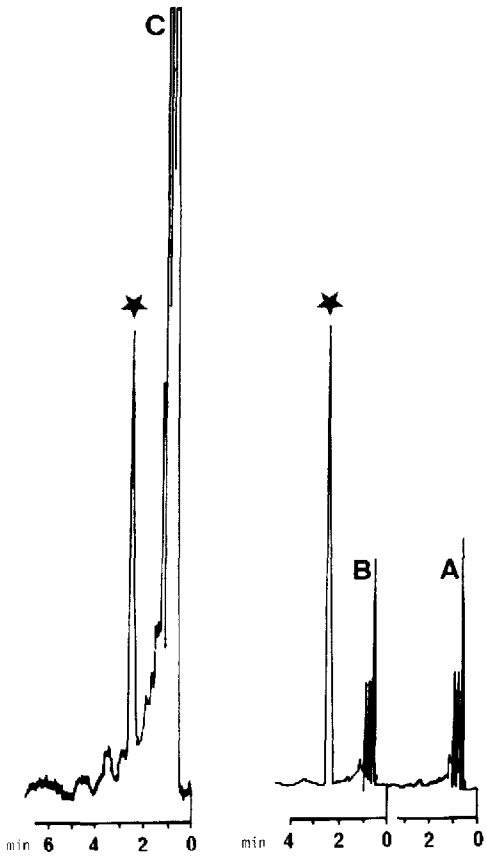

Fig. 1. HPLC profiles of: (A) an extracted human serum before any treatment with ciprofloxacin, at 0.05 a.u.f.s.; (B) an extracted human serum containing 1 μg/ml ciprofloxacin (⋆) at 0.05 a.u.f.s.; (C) an extracted human serum containing 150 ng/ml ciprofloxacin (⋆) at 0.005 a.u.f.s. Detector wavelength: 254 nm; chart speed: 0.5 cm/min.

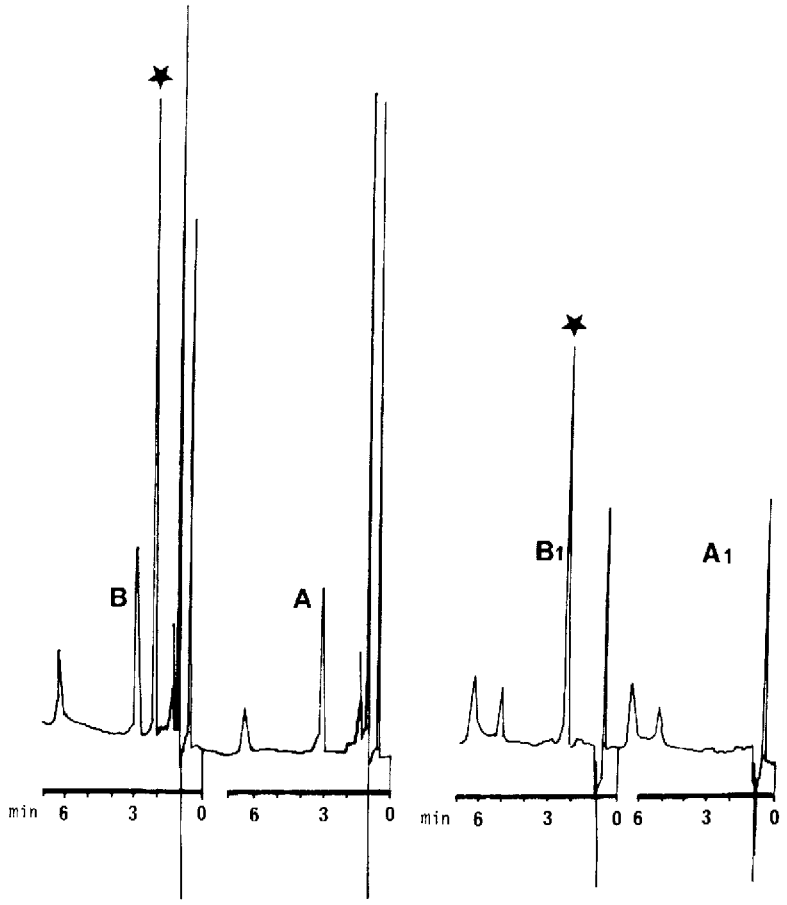

Fig. 2. HPLC profiles of: (A1) human bile before any administration of ciprofloxacin, at 0.05 a.u.f.s.; (B1) human bile from a patient after oral absorption of 500 mg of ciprofloxacin (concentration of this 1:10 diluted bile = 0.9 μg/ml), at 0.05 a.u.f.s. (⋆); (A) human urine before any administration of ciprofloxacin, at 0.05 a.u.f.s.; (B) human urine from a patient after oral absorption of 500 mg of ciprofloxacin (concentration of this 1:20 diluted bile = 1.15 μg/ml), at 0.05 a.u.f.s. (⋆). Detector wavelength: 254 nm; chart-speed: 0.5 cm/min.

phenytoin, primidone, valproic acid, digoxin, quinidine, procainamide, lidocaine, theophylline, digitoxin and furosemide. The limit of detection of our procedure was 10 ng/ml for blood levels, resulting in a signal-to-noise ratio of 4:1. For the urinary and the biliary levels, the detection limits were, respectively, 500 and 200 ng/ml. A profile of drug concentration versus time is depicted in Fig. 3 for two patients after an orally administered dose of 500 mg of ciprofloxacin.

Recovery study

Ten sera spiked with known amounts of ciprofloxacin were chromatographed as described in Materials and methods, and the resulting peak heights were compared with the peaks resulting from aqueous solutions of the same amounts. The double extraction described led to a concentration of ciprofloxacin of 175 ± 6% (n = 10).

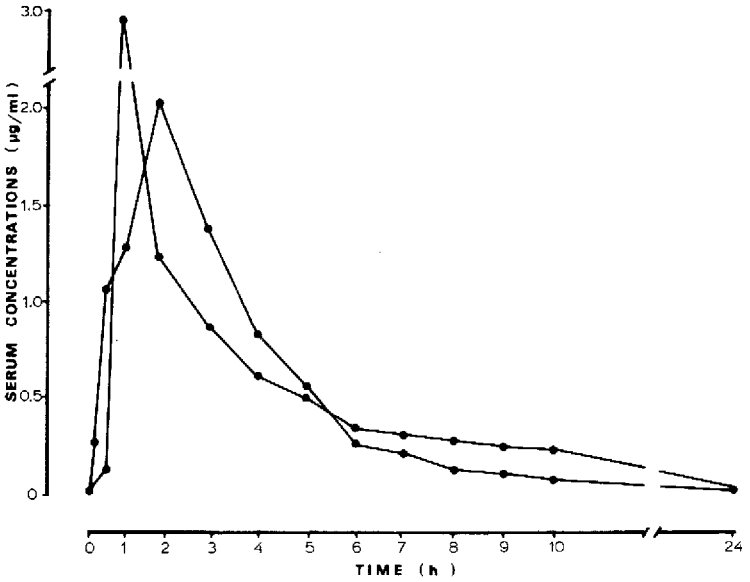

Fig. 3. Profile of drug concentration versus time in two patients after an orally administered dose of 500 mg of ciprofloxacin. Assays were done by HPLC.

Linearity

The study of the linearity was carried out with concentrations ranging from 10 ng/ml to 10 µg/ml. Three detector sensitivity ranges were employed: in the first step, the serum concentrations ranged from 10 to 200 ng/ml and these sera were chromatographed at 0.001 absorbance units full scale (a.u.f.s.) sensitivity range; in the second step, concentrations ranged from 0.2 to 2 µg/ml and the detector was set at 0.01 a.u.f.s.; in the last step, concentrations ranged from 2 to 10 µg/ml and the detector was set at 0.05 a.u.f.s. The analytical procedure looked linear whatever the range setting of the spectrophotometer. The three lines corresponding to the three steps above parallel very well. The sample containing 200 ng/ml has been assayed at 0.001 and 0.01 a.u.f.s., and the sample

TABLE I

PRECISION OF THE CHROMATOGRAPHIC ASSAY

Sample	Concentration (µg/ml)	Coefficient of variation (n = 10) (%)
Within-day reproducibility		
Serum	0.5	5.2
	1.5	4.3
Urine	20	8.2
Bile	10	8.6
Between-day reproducibility		
Serum	0.5	7.2
	1.5	6.5
Urine	20	10.2
Bile	10	10.8

spiked with 2 μg/ml ciprofloxacin has been chromatographed at 0.01 and 0.05 a.u.f.s. In both cases, the product of the peak height and the sensitivity range is constant. The correlation coefficient obtained between the peak heights and the ciprofloxacin concentrations for the three steps together was $r = 0.997$.

Precision

The results of replicate analyses of spiked serum are given in Table I. The coefficients of variation range from 4.3% for serum to 8.6% for bile for the within-day reproducibility, and from 6.5% to 10.8% for the between-day reproducibility.

Accuracy

Our chromatographic procedure has been compared with a reference method, i.e. the microbiological assay as described in Materials and methods. The degree of correlation between both assays should indicate the accuracy of the chromatographic procedure.

Serum. Analysis of the orthogonal linear regression for a series of 135 samples resulted in a correlation coefficient of $r = 0.932$. It resulted in the following equation (Fig. 4): y (= agar diffusion) = $1.02x$ (= HPLC) + 0.05. For the paired values of ciprofloxacin concentrations, the mean difference (agar diffusion — HPLC) compared to a difference of zero did not yield to a statistically significant value: $\epsilon = 0.789$ and $\alpha = 0.43$ (threshold point for significance: $\alpha = 0.05$). Thus, the concentrations as measured by both assays are statistically identical.

Urine. For the urines, the correlation coefficient resulted from the analysis of 35 samples (Fig. 5): $r = 0.979$. The calculated equation is as follows: y (= agar diffusion) = $0.91x$ (= HPLC) + 16.62. The statistical analysis of the paired values (agar diffusion — HPLC) showed no statistically significant difference: $\epsilon = 1.37$ and $\alpha = 0.18$.

Bile. The analysis of the levels of ciprofloxacin measured by both procedures

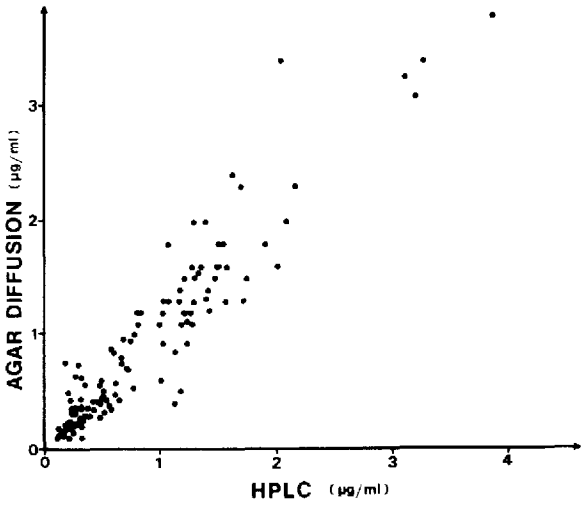

Fig. 4. Correlation of agar diffusion and HPLC results of all the serum samples assayed ($n = 135$); $r = 0.932$. Orthogonal regression equation: $y = 1.02x + 0.05$.

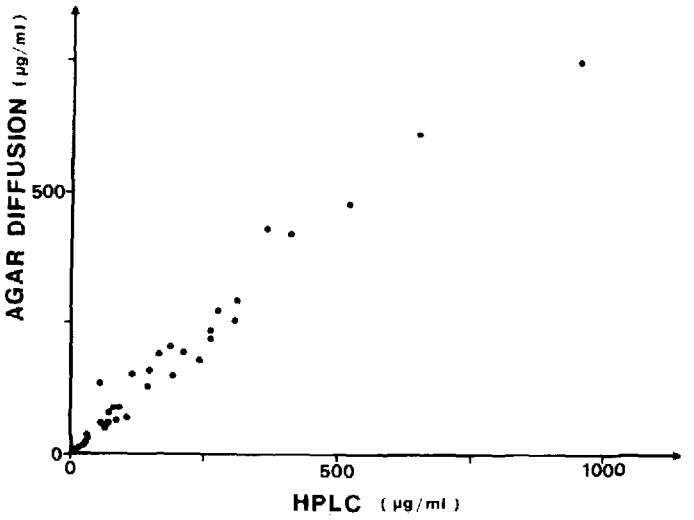

Fig. 5. Correlation of agar diffusion and HPLC results from all the urine samples assayed (n = 35); r = 0.979. Orthogonal regression equation: $y = 0.91x + 16.62$.

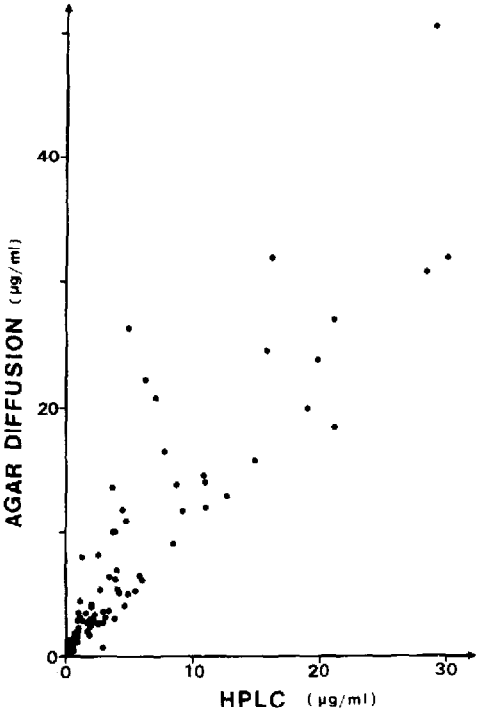

Fig. 6. Correlation of agar diffusion and HPLC results from all the bile samples assayed (n = 86); r = 0.900. Orthogonal regression equation: $y = 1.27x + 1.48$.

in bile yielded different data than those obtained for serum or urine. The coefficient of variation calculated with 86 samples was less good: r = 0.900 (Fig. 6). The following equation is obtained: y (= agar diffusion) = $1.27x$ (= HPLC) + 1.48. Among the 86 samples measured, 69 gave higher values when

measured by the agar diffusion assay compared to HPLC. The comparison of the mean difference for the paired values with a difference of zero yielded a statistically highly significant result: ϵ = 5.35 and α = 0.000001. The microbiologically measured levels were higher than those measured chromatographically.

DISCUSSION

High-performance liquid chromatography has already been used for monitoring of quinoline carboxylic acids in biological fluids [9, 18—24]. Up to present, no chromatographic procedure has been described for ciprofloxacin. The assay proposed here is simple, rapid and uses ultraviolet detection for quantitation. Ciprofloxacin shows good absorbance at 254 nm and thus allowed us to avoid using fluorimetric detection which needs more expensive equipment.

Our chromatographic conditions led to chromatograms that appeared "clean" and were similar to those obtained by different authors for nalidixic acid [18], pipemidic acid [21], flumequine, [23], miloxacin [20], rosoxacin [19], norfloxacin [22, 23], and pefloxacin [9]. Among these authors, many used ion-exchange chromatography [18, 20, 22, 24]. Reversed-phase chromatography on a C_{18} bonded silica matrix seemed to us more reproducible [9]. Nevertheless, conventional reversed-phase HPLC never gave us full satisfaction. Indeed, we carried out various assays with "classical" mobile phases generally used in reversed-phase HPLC, e.g. a mixture of water or salt solution with a non-polar organic solvent, and every time it resulted in severe peak tailing of ciprofloxacin, whatever the pH or the ionic strength. Finally, using ion-pairing chromatography with tetrabutylammonium bromide, an excellent resolution of ciprofloxacin was obtained.

Initial attempts to prepare protein-free filtrates by deproteinization with an organic solvent (acetonitrile, methanol) gave good results for many β-lactam antibiotics [25], but caused partial co-precipitation of ciprofloxacin. Cartridge chromatographic techniques (Waters C_{18} Sep-Pak^{TM}) yielded clean chromatograms, but the resulting dilution of the samples lowered the detection limit.

Although no internal standard is used, the coefficients of variation and the linearity are in quite good agreement with those required in hospital routine use or for pharmacokinetic studies, for which the ten times lower detection limit of the HPLC procedure compared to agar diffusion procedure may be very interesting.

Correlation with microbiological assay

Quinoline carboxylic acids are well known to be metabolized in large part [9, 19, 20, 22, 23]. Some of the potential metabolites may present an important antibacterial activity, as is found for norfloxacin, a major metabolite of pefloxacin [9]. When one assays levels in biological fluids using an agar diffusion method, there is a risk of overestimation of drug concentration.

One or more metabolites may exist and could be potentially active. A previous pharmacokinetic study of ciprofloxacin [26] carried out using a microbiological assay revealed that 30% of the dose administered was recovered

as unchanged drug in the urine. This means that metabolism of the drug may occur. We found that the correlation was good between the two assay methods for serum and urine. It is less evident for biliary levels, for which a highly significant difference was found between agar diffusion and HPLC. This difference may be explained by the existence of an active metabolite which could be eliminated in bile. Thus, the agar diffusion test would have assayed the entire antibacterial activity, when HPLC assayed only ciprofloxacin.

To establish the chromatographic condition for assaying the metabolite(s) we need to have them as pure powder. Work is currently being undertaken to obtain these pure substances.

The absence of a significant difference in serum and urine should not exclude the possibility of the existence of a non-active metabolite, or an active metabolite present in these fluids at too low a concentration to be measured by agar diffusion.

Further investigations may be started in the future to determine pharmacokinetics of ciprofloxacin. In this respect, it is important to use a specific and accurate assay technique. The procedure described above may be an interesting alternative.

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