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Short Communication

Improved high-performance liquid chromatographic determination of ciprofloxacin and its metabolites in human specimens

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ABSTRACT

A simple approach to the quantitation of ciprofloxacin and its three metabolites, M1 (desethylene-ciprofloxacin), M2 (sulfo-ciprofloxacin) and M3 (oxo-ciprofloxacin), in human serum, urine, saliva and sputum is described. This assay allows the parent drug and its metabolites to elute and be resolved in a single chromatogram at 280 nm using a linear gradient. The procedure involved liquid–liquid extraction. Separation was achieved on a C_{18} reversed-phase column. The limit of detection of ciprofloxacin is 0.05 μg/ml and that of its three metabolites is 0.25 μg/ml. This method is sufficiently sensitive for pharmacokinetic studies.

INTRODUCTION

Ciprofloxacin, 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolone carboxylic acid, is a relatively new quinolone carboxylic acid derivative with an extended antibacterial spectrum. Currently, ciprofloxacin is used in a wide variety of infections, notably, of the urinary tract, respiratory tract and gastrointestinal tract, as well as skin and soft tissue infections. The drug is thought to act by DNA gyrase inhibition, a mechanism similar to its parent compound, nalidixic acid.

There have been numerous papers reporting the high-performance liquid chromatographic (HPLC) separation of ciprofloxacin using both ultraviolet (UV) and fluorescence detection. However, when it comes to the resolution of ciprofloxacin and its metabolites, only a few reports have been published to date. With the exception of the study by Krol *et al.* [1] and Myers and Blumer [2] the current methods have not been able to simultaneously separate ciprofloxacin and its three identified metabolites, M1 (desethylene-ciprofloxacin), M2 (sulfo-ciprofloxacin) and M3 (oxo-ciprofloxacin). The latter's work, in contrast to that of Krol *et al.* [1], did not incorporate an internal standard (I.S.) which is crucial as their method of sample preparation involved more than one extraction step. The lack of internal standard is also reflected in some of the other published methods [3,4]. The other limita-

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tions with some of the present methods include the use of two different mobile phases to separate the metabolites [3,4] and the tedious sample preparation involved [1,4]. Additionally, a majority of these methods [2–6] utilise expensive fluorescence detection, a facility which is not commonly available in every laboratory. Chan *et al.* [7] used a UV detection method but were unable to elute M2.

This report describes a reversed-phase HPLC method which simultaneously elutes ciprofloxacin, M1, M2 and M3 on a gradient using UV detection. The method has been used in pharmacokinetic studies in patients with cystic fibrosis (unpublished results).

EXPERIMENTAL

Chemicals

Ciprofloxacin, M1, M2 and M3 were obtained from Bayer Pharmaceuticals (Wuppertal-Elberfeld, Germany). Tinidazole, used as an I.S., was obtained from Pfizer Pharmaceuticals (Sydney, Australia). All solvents were of HPLC grade. Acetonitrile, dichloromethane and methanol were from Mallinckrodt (Sydney, Australia). Orthophosphoric acid (85%) and 2-propanol were acquired from Ajax Chemicals (Sydney, Australia). Potassium dihydrogen orthophosphate (KH_2PO_4) was obtained from BDH Chemicals and *d,l*-dithiothreitol from Sigma (Sydney, Australia).

Instrumentation

The chromatographic equipment was a Waters system comprising of a Model 680 automated gradient controller which controlled two Model 6000A solvent delivery pumps, a Model U6K injector and a Model 440 fixed-wavelength UV detector (Waters Assoc., Sydney, Australia). The output of the detector was monitored by an Omniscribe B-5000 strip chart recorder (Houston Instruments, Alltech, Sydney, Australia). The stationary phase consisted of a 5-μm C_{18} reversed-phase Brownlee column, Spheri-5 OD-5A (Activon Scientific Products, Sydney, Australia), with dimensions of 25 cm × 4.6 I.D. mm. The column was guarded by a pre-column (Guard-Pak) filled with a μBondapak C_{18} cartridge (Waters Assoc.).

Stock solutions

Stock solutions containing 1 mg/ml lyophilized ciprofloxacin and metabolites were prepared in methanol and ultrasonicated for 10 min. The stock solutions were diluted 1:10 with methanol to give a working solution of 100 μg/ml.

Tinidazole, the I.S., was also made up as a stock solution of 1 mg/ml in methanol. A dilution of 1:100 was made of the stock using distilled water to obtain a working solution of 10 μg/ml for the serum, saliva and sputum assays. For the urine assay, a dilution of 1:20 was made of the stock solution to obtain a final working solution of 50 μg/ml.

Calibration graphs

A standard curve of concentrations between 0.10 and 10 μg/ml in drug-free control serum (Commonwealth Serum Labs., Sydney, Australia) or drug-free control urine (Bio-Rad Labs., Sydney, Australia) or distilled water was prepared each day. The working solution was used to spike the drug-free matrices. Distilled water was used to make up the calibrators for the quantitation of drug concentrations in sputum and saliva. Extraction of standards was performed as described below. The ratio of the peak height of the drug to that of the I.S. was plotted against the concentration of the drug.

Sample preparation

All patients specimens were stored at − 20°C until assayed. Serum, saliva and sputum were processed in the same way except that the sputum samples required pretreatment with 0.4 m*M* dithiothreitol (1:1, v/v) [8] for 10 min in the ultrasonicator to break the disulfide bonds. The extraction procedure is essentially derived from that of Vallée *et al.* [8] and is described briefly here. The initial deproteinization step involved adding 100 μl of a working solution of I.S. and an equal volume of acetonitrile to the matrix. Normally 1 ml of the specimen was used. In the case of saliva or sputum, where the quantity may be

insufficient, the volumes of solvents used in the extraction steps were scaled down accordingly. The specimen–acetonitrile mixture was vortex-mixed for a few seconds and centrifuged at 3000 *g* for 5 min. The supernatant was then added to a 5-ml mixture of dichloromethane–2-propanol (90:10) in a glass tube. The mixture was vortex-mixed and then left on a mixer for 10 min. It was centrifuged at 3000 *g* for 5 min. The top aqueous layer was aspirated and the bottom organic layer was transferred to a new glass tube. This was blown down with nitrogen gas in a 40°C water bath and reconstituted in 50 μl of mobile phase B (see below). The injection volume was between 10 to 20 μl.

With the urine samples, a dilution between 1:2 and 1:128 was made accordingly with distilled water. To 50 μl of the diluted urine in a microfuge tube, 25 μl of working solution of tinidazole were added. The mixture was vortex-mixed and 10–20 μl of it were injected directly onto the column.

Mobile phase

A 0.1 *M* solution of potassium dihydrogen orthophosphate (13.61 g in 1 l of distilled water), KH_2PO_4, was adjusted to pH 2.6 with concentrated orthophosphoric acid. Pump A delivered the KH_2PO_4 buffer (solvent A) while pump B delivered a 1:1 mixture of KH_2PO_4 buffer and acetonitrile (solvent B). All solvents were filtered and degassed before use.

Chromatographic conditions

Separation of ciprofloxacin and its three metabolites was achieved at room temperature using a gradient system. The initial composition of the mobile phase consisted of a mixture of 70% solvent A and 30% solvent B at zero time which changed linearly over 20 min to 50% solvent A and 50% solvent B, after which it was brought back to the initial composition within 5 min. Although all the compounds were eluted by about 17 min, the gradient was programmed to run for 25 min to ensure complete equilibration to the initial condition. The UV detector was set at 280 nm and sensitivity was set at 0.02 absorbance units full scale (a.u.f.s.). The chart speed setting was 0.25 cm/min.

RESULTS AND DISCUSSION

Fig. 1 shows representative chromatograms of the four different specimens taken from a patient prior to administration of ciprofloxacin. The figure also shows sample chromatograms of serum, urine and water standards. The retention times of ciprofloxacin, M1, M2, M3 and tinidazole were approximately 8.2, 6.4, 10.0, 16.8 and 5.2 min, respectively. There were no interfering peaks in any of the patients' specimens. Given that gradient conditions were utilised to separate the compounds, the baseline was relatively free from drift.

The linearity of the calibration curves was verified from 0.1 to 20 μg/ml for ciprofloxacin and 0.25 to 20 μg/ml for each of its metabolties in serum, urine and water. The correlation coefficients between the peak-height ratio of the drug to the I.S. and concentration were ≥ 0.999 for the parent drug and between 0.993 and 0.998 for the metabolites. The limits of detection determined at a signal-to-noise ratio of 3 were 0.05, 0.25, 0.25 and 0.25 μg/ml for ciprofloxacin, M1, M2 and M3, respectively. The within-day coefficients of variation (C.V.) for ten aliquots were ≤ 5, 8, 8 and 6% for ciprofloxacin, M1, M2, and M3, respectively, measured at mid concentration of the standard curves for serum, urine and water. Day-to-day reproducibility of at least ten aliquots yielded C.V.s of between 10 to 12% for all the compounds.

Contrary to the findings of Jehl *et al.* [7], the present technique was able to measure concentrations of metabolites using UV spectroscopy. Whilst UV detection is usually less sensitive than fluorometric methods, the present method is able to measure concentrations of ciprofloxacin and its metabolites in patients with cystic fibrosis after single oral doses of 500 and 750 mg (unpublished results). The range of concentrations of ciprofloxacin, M1, M2 and M3 have been found to fall well within the range of detection and linearity of the method.

In conclusion, this study has shown that HPLC separation of ciprofloxacin and its three metabolites can be accomplished using UV detec-

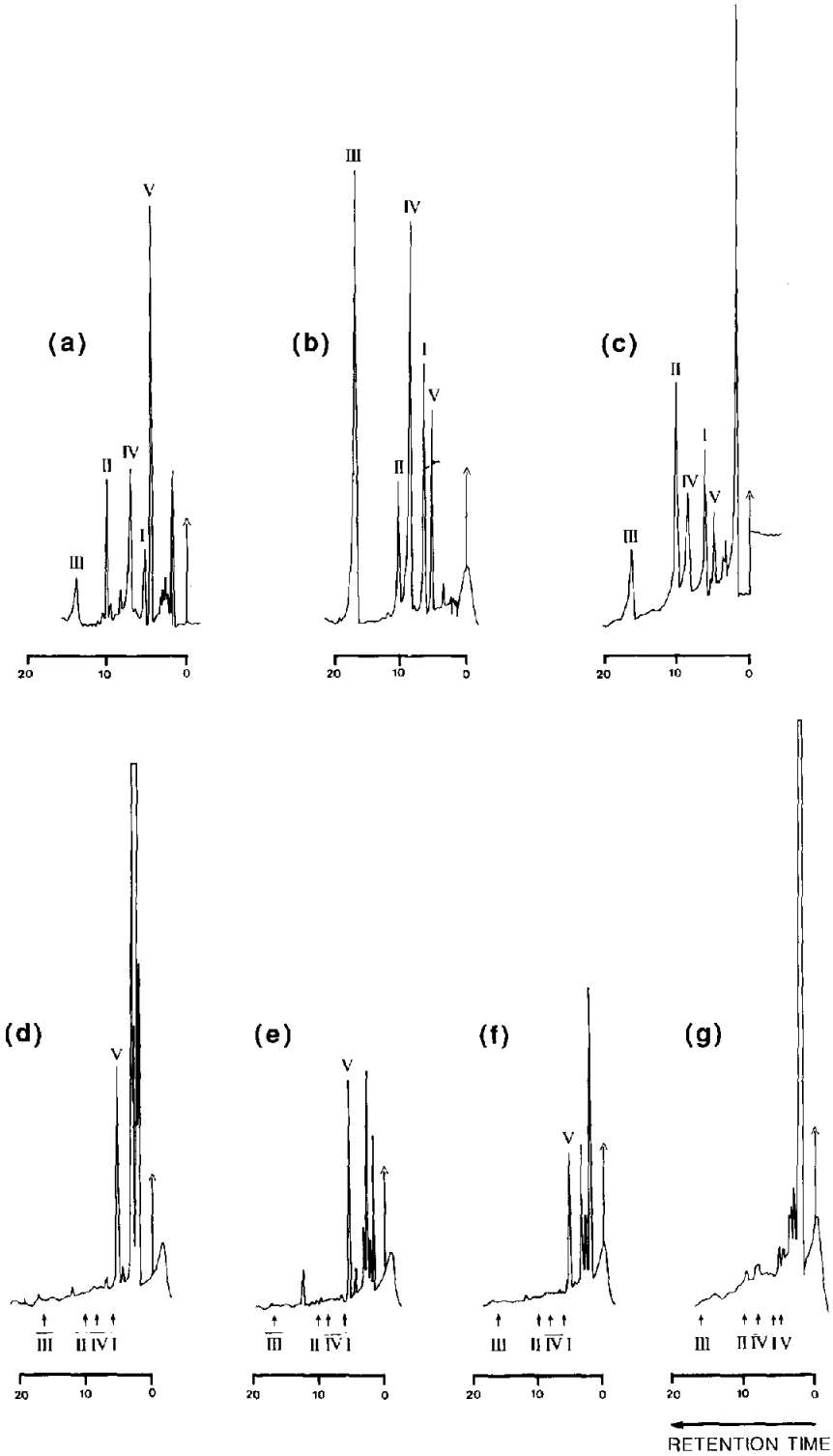

Fig. 1. Representative chromatograms of (a) serum standard (0.25 μg/ml), (b) water standard (2.5 μg/ml), (c) urine standard (20 μg/ml), (d) blank saliva with I.S. added, (e) blank serum with I.S. added, (f) blank sputum with I.S. added and (g) blank urine without I.S. Peaks: I = M1; II = M2; III = M3; IV = ciprofloxacin; V = tinidazole (I.S.).

tion, and thus is applicable to pharmacokinetic studies.

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