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High performance liquid chromatographic determination of ciprofloxacin in human plasma and urine and its application to pharmacokinetic studies

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Abstract

A simple, highly sensitive and selective reversed-phase HPLC assay with UV detection for the determination of ciprofloxacin in human plasma and urine was developed and validated. The drug and internal standard, ofloxacin, were extracted from plasma or urine with isopropanol:methylene chloride (1:9, v v) and back extracted with orthophosphoric acid. Separation was achieved on a C18 reversed-phase column with a mobile phase of acetonitrile:methanol:0.1M sodium dihydrogen orthophosphate:triethylamine:glacial acetic acid (8:10:83:1:2, v v) and UV detection at 278 nm. The chromatograms were free from interference at the retention times of ciprofloxacin and the internal standard while both compounds eluted as completely resolved peaks. The calibration curves were linear ($r^2 \geq 0.996$) over concentration ranges of 0.01 to 3.0 µg ml in plasma and 0.05 to 5.0 µg ml in urine. The method was reproducible with coefficients of variation <6% for both inter-and intra-day assays while the limits of quantitation were 10 ng/ml in plasma and 50 ng ml in urine. Recoveries for ciprofloxacin and internal standard were >98% in both plasma and urine. The method was successfully applied to estimate the pharmacokinetics of ciprofloxacin after oral administration of a 500 mg dose of ciprofloxacin tablets to 12 healthy volunteers.

Keywords: Ciprofloxacin; Reversed-phase HPLC; Pharmacokinetics; Ofloxacin

Introduction

Ciprofloxacin [1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline carboxylic acid] is a first generation fluoroquinolone with an extensive antimicrobial spectrum. It has excellent *in vitro* activity against a broad spectrum of gram-negative and gram-positive organisms including those resistant to β-lactam antibiotics and aminoglycosides (Davis *et al.*, 1996). The mechanism of action of ciprofloxacin is the inhibition of DNA gyrase, an enzyme critical to bacterial chromosome replication (Hooper and Wolfson, 1993).

Following oral administration, ciprofloxacin is rapidly absorbed from the gastro-intestinal tract and is extensively distributed into body tissues and fluids at concentrations greater or equal to twelve times that observed in the serum (Davis *et al.*, 1996; Bergogne-Bérézin *et al.*, 1986). Highest concentrations of the drug are generally

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attained in the bile, liver, gallbladder, uterus, endometrium, ovaries and fallopian tubes, seminal fluid, prostatic tissue and fluid, tonsils, lung tissues, including bronchial mucosa, bronchial epithelial lining fluids, and alveolar macrophages (Baldwin *et al.*, 1993). The low plasma concentrations require a sensitive quantitative method to determine the drug levels.

Several HPLC methods have been reported in literature for the determination of ciprofloxacin in biological fluids. A few of these methods use UV detection (Zhai *et al.*, 1995; Kamberi *et al.*, 1998; Manceau *et al.*, 1999; Zhu *et al.*, 1999; Maya *et al.*, 2001) while others involved the use of expensive fluorescence detection, not often available in most laboratories [Nix *et al.*, 1985; Shah *et al.*, 1995; Birmingham *et al.*, 1999; Morlet *et al.*, 2000; Montgomery *et al.*, 2001; Zotou *et al.*, 2002; Imre *et al.*, 2003; Idowu and Peggins, 2004). Some of these methods are not sufficiently specific or sensitive while others involved laborious and complicated extraction techniques or expensive instrumentation. Moreover, some did not use an internal standard while others involved the use of two or more mobile phases or of elevated column temperatures.

Therefore, the objective of this study was to develop and validate a HPLC method in order to study the pharmacokinetics of orally administered ciprofloxacin in human subjects.

Experimental

Chemicals and reagents. Ciprofloxacin (Reference standard; Batch: R-0123-03) was kindly supplied by Bayer AG (Wuppertal, Germany). Ofloxacin, used as internal standard, was kindly supplied by Nigerian German Chemicals (Ota, Nigeria). HPLC grade acetonitrile and methanol were purchased from Sigma-Aldrich (England). Acetic acid, methylene chloride, iso-propanol, orthophosphoric acid (85%), sodium hydroxide, sodium dihydrogen orthophosphate and triethylamine were obtained from BDH Chemicals Ltd (Poole, England). All chemicals and solvents were of analytical grade or higher. Glassware and extraction tubes were cleaned by soaking overnight in detergent solution, followed by washing and rinsing with distilled water, 0.085% H_3PO_4, methanol, acetone and then oven dried before being used.

Chromatographic conditions. The analyses were performed under isocratic condition on a Cecil 1100 series liquid chromatographic system (Cecil Instruments, Cambridge, England) equipped with a CE 1100 delivery pump, a system purge, a computerised system controller and a CE 1200 variable wavelength UV-VIS detector. Injection was through a model 7125 Rheodyne manual injector (Cotati, CA, USA). The column was a Hypersil ODS C_{18} analytical column (5 µm particle size and 250 x 4.6 mm i.d.) reversed phase stainless steel column purchase from Alltech Associates Inc. (Deerfield, IL, USA).

The HPLC system was equilibrated with the mobile phase consisting of a mixture of acetonitrile, methanol, sodium dihydrogen orthophosphate (0.1 M), triethylamine and glacial acetic acid (8:10:83:1:2, $^{v}/_{v}$) adjusted to pH 3.0 with a dilute solution of orthophosphoric acid. The chromatography was performed at ambient temperature at a flow rate of 1.7 ml/min and the eluent was monitored by UV absorbance at 278 nm. Quantitation of ciprofloxacin was obtained by plotting the peak area ratios of ciprofloxacin to internal standard as a function of concentration.

Standard solutions. Stock solutions of ciprofloxacin (1 mg/ml) and internal standard (100 mg/ml) were prepared in a 20:1 ($^{v}/_{v}$) mixture of water and 0.1M NaOH. Working standards of ciprofloxacin were prepared from the stock by sequential dilution with water to yield final concentrations of 20 and 50 µg/ml. Calibration standards (n = 6) were obtained

by adding varying amounts of the working standard solution of ciprofloxacin to drug-free plasma or urine to achieve a concentration range of 0.01 to 3.0 µg/ml for plasma and 0.05 to 5.0 µg/ml for urine.

Quality control samples were prepared by adding known amounts of ciprofloxacin to drug-free plasma or urine to give three concentrations of 0.25, 0.5 and 1.0 µg/ml for plasma and 1.0, 2.0 and 3.0 µg/ml for urine. The three concentration points represent the low, middle and high portions of the standard curve respectively.

All the samples were protected from light and stored at – 20 °C until analysed.

Extraction procedure. To 500 µl of plasma, blank plasma or plasma spiked with varying amounts of ciprofloxacin working standard solution was added 10 µl of internal standard solution followed by the addition of phosphate buffer (pH 7.4) to give final volumes of 1 ml in each glass tube. The mixture was extracted with 3 ml of a mixture of isopropanol:methylene chloride (1:9, v/v); vortexed for 1 min and centrifuged for 10 min at 10,000 rpm. The organic layer was transferred into a clean extraction tube and back extracted into 1 ml of 0.085% orthophosphoric acid. Approximately 400 µl of the aqueous phase was transferred to a brown vial tube of which 20 µl was injected unto the HPLC column. The methodology for the extraction of ciprofloxacin from the urine samples was similar to that from plasma samples except that blank or urine samples from volunteers were diluted 1:1000 with water before use.

Stability. Drug-free plasma and urine were spiked with known amounts of ciprofloxacin and stored at - 20 °C. The samples (n = 4) were used to assess the stability of ciprofloxacin over a period of one month.

Precision and accuracy. Precision and accuracy data were obtained by analysing aliquots of three quality control samples of ciprofloxacin in both plasma and urine. Intra-day reproducibility was determined by repeated analysis of the quality control samples (n = 5) on the same day while inter-day reproducibility was determined by repeated analysis of the quality control samples on three different days (n = 5).

Specificity and selectivity. The specificity of the method was determined by comparing the chromatograms obtained from the samples containing ciprofloxacin and internal standard with those obtained from blank samples. The selectivity was assessed by injecting different concentrations of drugs that are usually co-administered with ciprofloxacin.

Recovery. Recoveries of ciprofloxacin from spiked samples were determined by comparing the peak-area ratios of extracted plasma or urine spiked with ciprofloxacin standards with those obtained by direct injections of known concentrations of ciprofloxacin (n = 5).

Limit of quantification. The limit of quantification (LOQ) in plasma and urine samples is the lowest concentration from which it is possible to quantify the drug with a CV $\leq$20%.

Method validation. Quantitation of ciprofloxacin was achieved from calibration curves in the range of 0.01 - 3.0 µg/ml for plasma and 0.05 - 5.0 µg/ml for urine. Calibration curves were generated by least-squares linear regression analysis. The calibration curves were obtained by plotting the peak-area ratios of ciprofloxacin to internal standard versus ciprofloxacin concentrations in spiked samples. A standard curve was generated for each run and unknown ciprofloxacin concentrations in plasma and urine samples were calculated by interpolation using these calibration curves. Quality controls were performed in duplicate for each batch of samples.

Pharmacokinetic study. The HPLC method developed was used to investigate the plasma profile of ciprofloxacin after a single oral dose, under fasting condition, in healthy volunteers. Twelve healthy adult male volunteers aged between 19 and 44 years (mean ± SD, 26 ± 7 years) and weighing between 50 and 68 kg (59 ± 5 kg) were recruited into the study. Each subject was ascertained to be in good health by results of a detailed medical history, clinical examination and routine laboratory tests. The subjects, all non-smokers, were not on concomitant medications and were asked to abstain from any form of drugs at least 14 days prior to the commencement of the study and during the duration of the study. In addition, the subjects were instructed to abstain from alcoholic drinks, cigarettes smoking, beverages and any dairy products before, and during the period of study. Following written informed consent, each volunteer received a 500 mg oral dose of Ciproxin® tablets (Gemini Pharmaceuticals, Lagos, Nigeria) with 250 ml of water. Blood (5 ml) and urine samples were collected prior to, and at predetermined time intervals of 0.5, 1, 2, 3, 4, 6, 8, 12, and 24h for blood, and 0-2, 2-4, 4-8, 8-12, and 12-24h for urine samples, after drug administration. Blood samples were collected in heparinized tubes following which plasma was separated, by centrifuging at 10,000 rpm for 10 min. All samples were stored frozen at -20 °C until assayed. Approval for the study was obtained from the Ethical Committee of Obafemi Awolowo University, Ile-Ife, Nigeria.

Pharmacokinetic calculations. Pharmaco kinetic parameters were calculated by a non-compartment method. The peak plasma concentration (C_{max}) and the time to reach C_{max} (t_{max}) were obtained directly from the experimental data. The terminal log-linear phase of the plasma ciprofloxacin concentration was identified visually for each subject and the elimination rate constant (k_{el}) was determined by a linear regression analysis of the log-linear portion of the plasma-concentration-time curve. The elimination half-life ($t_{½}$) was calculated from the equation $t_{½} = \ln 2/k_{el}$. The area under the plasma concentration-time curve up to the last quantified data point (AUC_{0-24}) was calculated with the use of the linear trapezoidal rule and the $AUC_{0-\infty}$ was calculated with extrapolation to infinity by dividing the last measured concentration by k_{el}. The renal clearance was calculated as the amount of ciprofloxacin excreted in urine from 0 to 24 hours (A_e) divided by the AUC_{0-24}.

Results and Discussion

Typical chromatograms of blank plasma, spiked plasma and actual sample obtained from the pharmacokinetic study are shown in Fig 1. Similar chromatograms from urine are shown in Fig 2. The approximate retention times of the internal standard, ofloxacin, and ciprofloxacin were 8.3 and 10.1 min, respectively. Under the described chromatographic conditions ciprofloxacin was well resolved from the internal standard. The overall chromatographic run time was 12 min. The specificity of the assay was established with different sources of plasma and urine. No interfering peaks appeared at the retention times of the compounds of interests in either plasma or urine. Also, no interference occurred with some commonly co-administered drugs such as amodiaquine, pyrimethamine, indomethacin, chloroquine and paracetamol.

The assay exhibited a continuous and reproducible linear relationship between the ratios of the peak areas of ciprofloxacin and that of the IS with the corresponding concentrations of ciprofloxacin over the range of 0.01 - 3.0 μg/ml in plasma and 0.05 - 5.0 μg/ml in urine. The regression equation typical for the calibration curves in both plasma and urine are presented with their correlation coefficients in Table 1.

Table 1: Regression analysis equation for ciprofloxacin.

Sample	Regression equation $y = (m \pm SD)x + (c \pm SD)$	*Correlation coefficient*
Plasma	$y = 0.0016 \pm 0.0001x + 0.1345 \pm 0.01155$	0.997
Urine	$Y = 0.0014 \pm 0.0001x + 0.1528 \pm 0.0135$	**0.996**

y = peak-area ratio of ciprofloxacin to internal standard; x = concentration of ciprofloxacin in mg/ml, c = intercept; m = slope; SD = standard deviation

Table 2: Inter-day accuracy (recovery) and precision for ciprofloxacin in quality control samples in human plasma and urine (n = 5).

Body fluid	Concentration spiked (µg/ml)	Mean concentration found (µg/ml)	Recovery (%)	Coefficient of variation (%)
Plasma	0.10	0.098	98.0	3.4
	1.0	0.98	98.4	1.31
	2.50	2.47	98.8	3.95
Urine	0.50	0.49	98.1	1.52
	1.50	1.48	98.7	1.98
	4.0	3.97	99.3	0.10

Table 3: Intra-day accuracy (recovery) and precision for ciprofloxacin in quality control samples in human plasma and urine (n = 5).

Body fluid	Concentration added (µg/ml)	Mean concentration found (µg/ml)	Recovery (%)	Coefficient of variation (%)
Plasma	0.10	0.099	99.1	0.60
	1.0	0.99	99.3	1.61
	2.50	2.46	98.4	4.02
Urine	0.50	0.504	100.8	1.44
	1.50	1.48	98.7	2.16
	4.0	3.97	99.3	0.15

Table 4: Pharmacokinetic parameters for ciprofloxacin after a single oral administration to twelve subjects.

Pharmacokinetic parameter	Mean ± SD	Range
C_{max} (mg/ml)	1.21 ± 0.43	0.54 – 2.13
AUC_{0-24} (mg-h/ml)	5.83 ± 2.23	2.13 – 9.31
$AUC_{0-\infty}$ (mg-h/ml)	6.17 ± 3.26	3.53 – 10.38
T_{max} (h)	1.33 ± 0.96	0.5 – 4.0
t½ (h)	5.20 ± 1.63	2.86 – 15.57
Clr (L/h)	17.43 ± 10.97	3.97 – 46.99

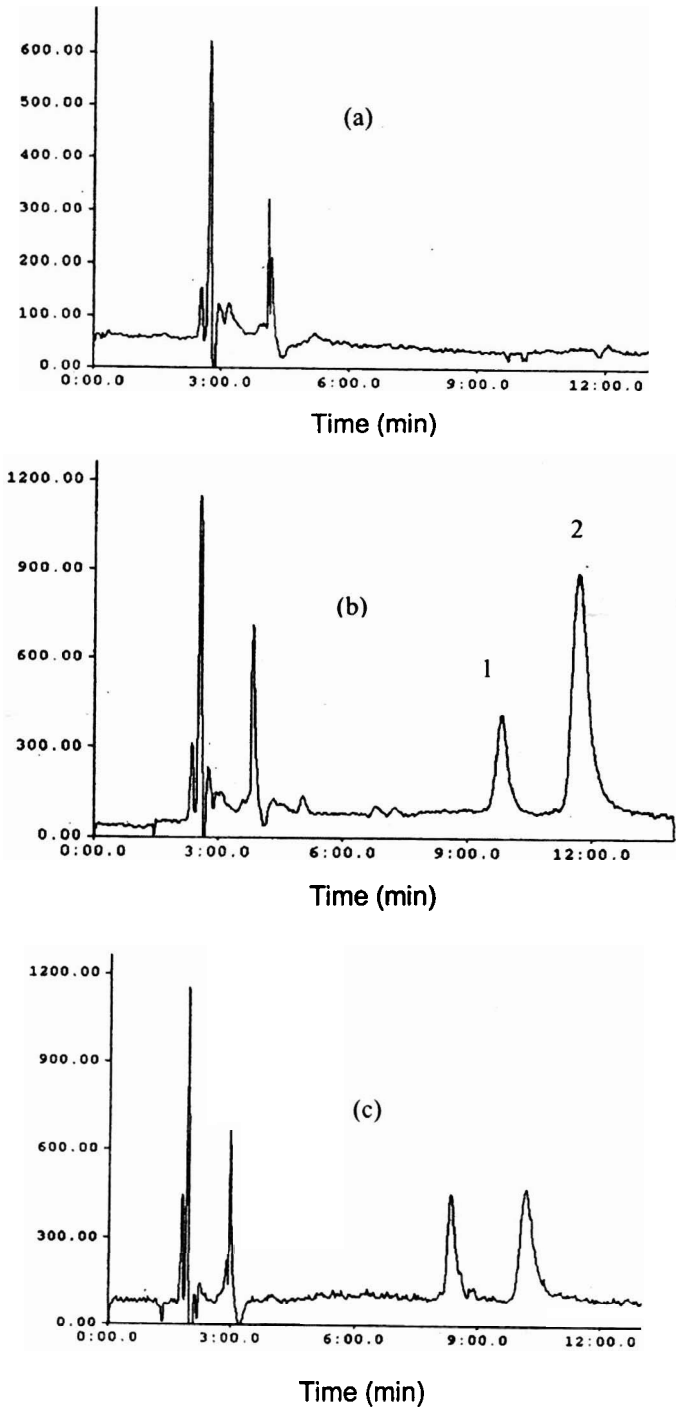

Figure 1: Chromatograms of (a) blank plasma, (b) blank plasma spiked with IS (1) and ciprofloxacin (2), and (c) human plasma 2.0 h after oral administration of a single 500 mg dose of ciprofloxacin

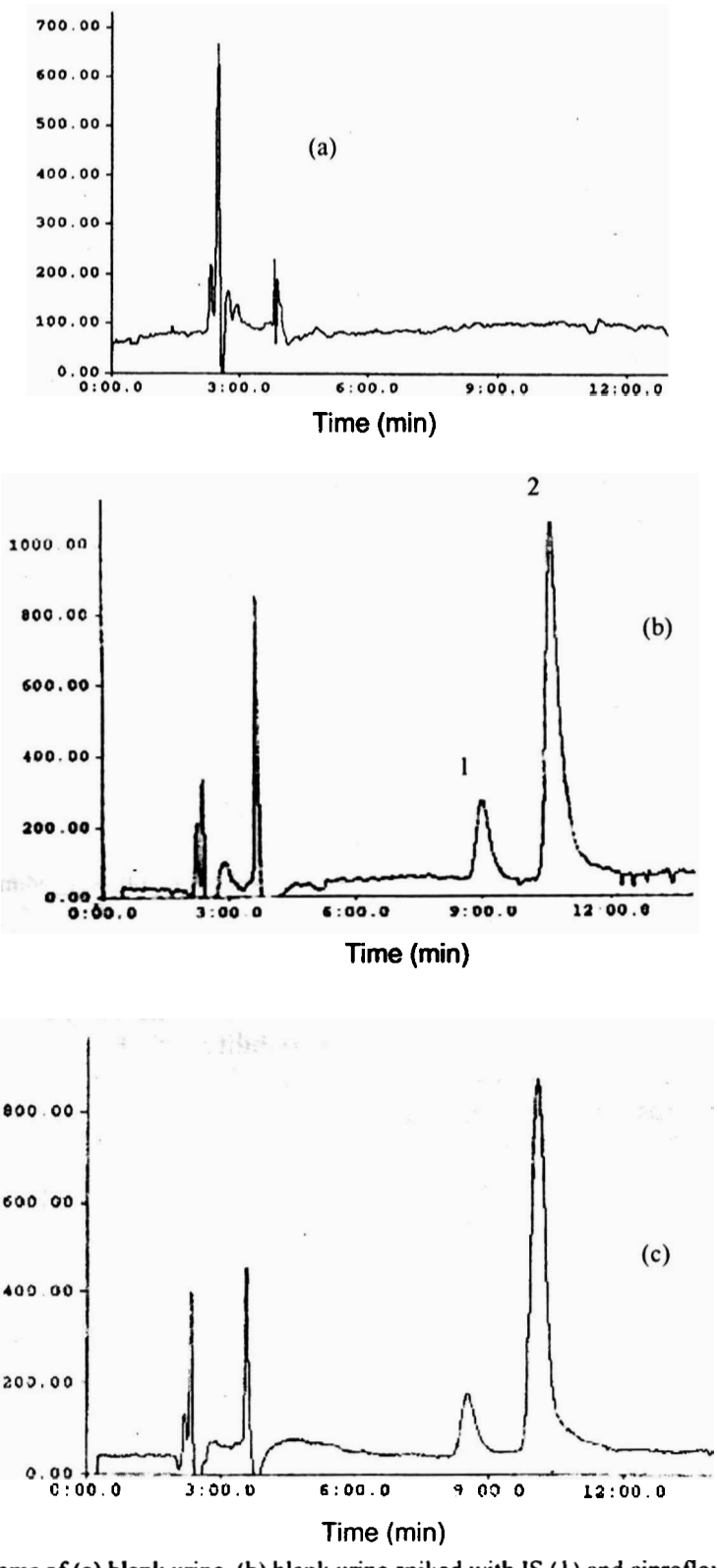

Figure 2: Chromatograms of (a) blank urine, (b) blank urine spiked with IS (1) and ciprofloxacin (2), and (c) urine obtained 4.0 h after oral administration of a single 500 mg dose of ciprofloxacin.

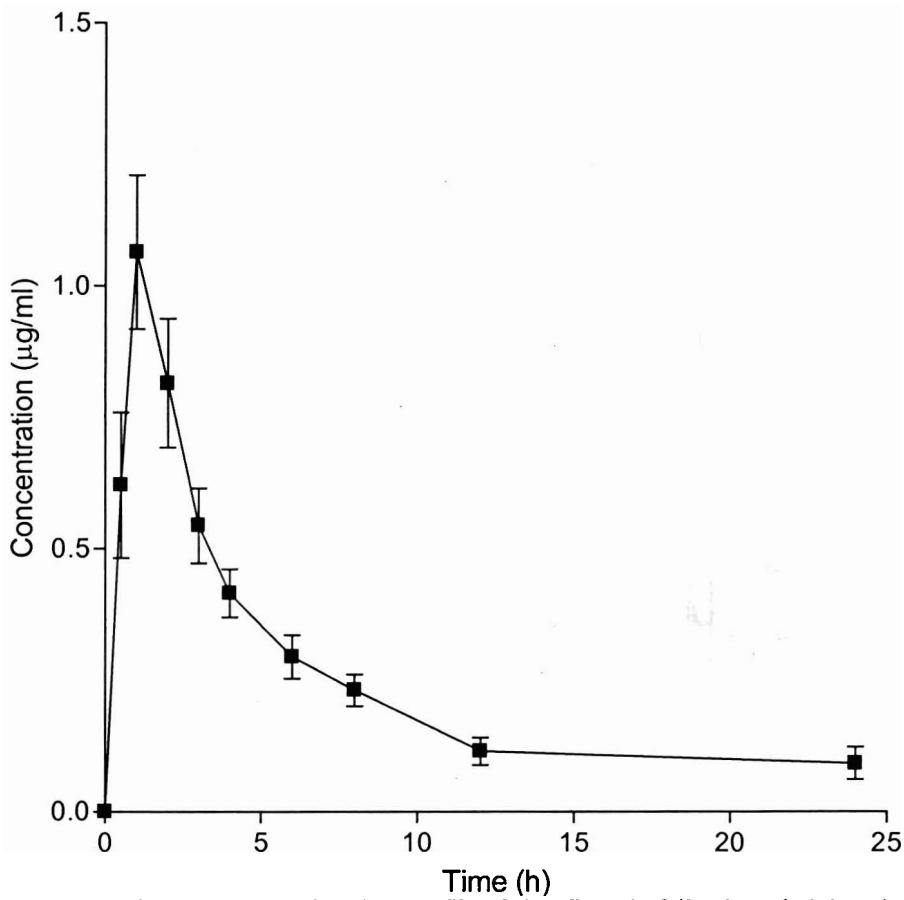

Figure 3: Mean (± SEM) plasma concentration-time profile of ciprofloxacin following administration of a single 500 mg oral dose to twelve healthy volunteers.

The mean recoveries of ciprofloxacin in plasma were 99.84 ± 0.60%, 99.16 ± 1.59 and 98.18 ± 3.95% at concentrations of 0.10, 1.0 and 2.50 µg/ml respectively while the percentage recoveries in urine, at concentrations of 0.5, 1.50 and 4.0 µg/ml, were 99.01 ± 1.49, 98.85 ± 1.95 and 99.97 ± 0.15% respectively (n = 5). The mean recovery of the internal standard was found to be 98.2 ± 2.1% in plasma and 97.8 ± 3.2% in urine (n = 15). Also, plasma and urine spiked with ciprofloxacin showed no significant loss of analyte during the period of storage.

The assay was precise and accurate. The results of the intra- and inter-assay precision and accuracy of the analytical method, for both plasma and urine, are shown in Tables 2 and 3 respectively. The overall precision of the assay is reflected by the mean variability of the quality control samples, which did not exceed 6% while the accuracy of the assay is reflected by an overall mean recovery that was greater than 98%. The limits of quantitation were the lowest quantifiable concentrations of 10 ng/ml in plasma and 50 ng/ml in urine with CV <10%.

The applicability of this method for the evaluation of ciprofloxacin in human pharmacokinetic studies was demonstrated successfully. The drug was well tolerated at the administered dose and no adverse effects were reported. The mean concentration-time profile for ciprofloxacin after administration of a single 500 mg oral dose to twelve healthy volunteers is shown in Figure 3. All the calculated pharmacokinetic parameters

derived from the plasma concentration-time profiles (Table 4) were in good agreement with previously reported values [Vance-Bryan *et al.*, 1990; Chukwuani *et al.*, 1998; Shah *et al.*, 1999; Lubasch *et al.*, 2000).

Conclusion

In summary, the analytical method described here proved to be simple, fast, reproducible, and selective with a good accuracy and precision, and allowed for numerous samples to be processed within a short period of time. Since practicality and low instrument cost are essential features of routine laboratory test, this method is well within the capabilities of the average analytical laboratory. Furthermore, the high sensitivity of the method makes it applicable to pharmacokinetic studies and routine monitoring of ciprofloxacin levels in humans.

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