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High Performance Liquid Chromatographic Assay of Ciprofloxacin in Human Plasma Using Fluorescence Detection

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ABSTRACT

An accurate, sensitive and reproducible High Performance Liquid Chromatographic (HPLC) method for the quantitation of Ciprofloxacin in plasma using Acebutolol as the internal standard has been developed and validated. The procedure involves protein precipitation with 7% perchloric acid. The drug and the internal standard were eluted from a 4-µm stainless steel Novapak C_{18} column (3.9 x 150 mm) with an average particle size of 4 µm at room temperature with isocratic mobile phase consisting of 10.5% acetonitrile in sodium dihydrogen phosphate buffer (pH 3.9), at a flow rate of 2 ml/min., and monitored in a fluorescence detector set at excitation wavelength 280 nm and emission wavelength 455 nm. Each analysis required no longer than 14 min. Quantitation was achieved by measurement of the peak height ratio of the drug to the internal standard, and the limit of detection for ciprofloxacin was 25 ng/ml.The intraday coefficient of variation (CV) ranged from 5.5% to 10.6% and interday CV ranged from 4.7 to 5.9% at four different concentrations.The mean relative recovery was 101.6%. Stability test shows that ciprofloxacin is stable in plasma for at least four weeks when stored at -20ºC. This method was applied for the determination of the pharmacokinetic parameters of ciprofloxacin after oral administration of 500 mg / tablet of two commercially available formulations to 6 human volunteers.

Key words: Ciprofloxacin ; High Performance Liquid Chromatography; Bioanalysis ;Stability ;Pharmacokinetic analysis.

1. INTRODUCTION

Ciprofloxacin hydrochloride is a synthetic broad spectrum antimicrobial agent which is clinically used in dosage ranged from 250mg to 1000mg depending on the type of infection and route of adminstration[1-3]. Ciprofloxacin hydrochloride, USP, a fluoroquinolone, is the monohydrochloride monohydrate salt of 1-cyclopropyl-6-fluoro-1, 4-dihydro-4-oxo-7-(1-piperazinyl)-3-uinolinecarboxylic acid (Figure 1).

Ciprofloxacin is available as a film-coated tablets (CIPRO) containing in 250 mg, 500 mg and 750 mg (ciprofloxacin equivalent) . The inactive ingredients are cornstarch, microcrystalline cellulose, silicon dioxide, crospovidone, magnesium stearate, hypromellose, titanium dioxide, and polyethylene glycol.

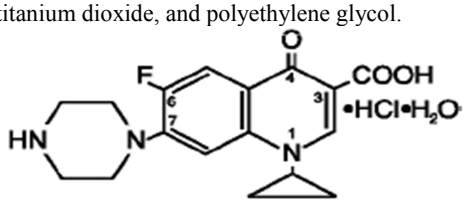

Figure 1. Ciprofloxacin is 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid.

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Several HPLC methods have been reported for the analysis of ciprofloxacin in biological fluids [4-12]. Some of these methods use ultraviolet (UV) detection [4-7], whereas others use expensive fluorescence detection [8-11]., a method that is not commonly available in every laboratory. Most of these methods do not include an internal standard (IS), which is crucial because the sample preparation methods involve more than one extraction step [4,5,7,9].

This manuscript describes a rapid, accurate, sensitive and specific and fully validated method that combines a simple procedure of sample preparation, an isocratic eluent of simple composition, and fluorescence detection. The method has been applied in pharmacokinetic studies in healthy volunteers.

2. EXPERIMENTAL

2.1. Apparatus

The following apparatus from Shimadzu Kyoto, Japan was used: a model (LC-10AD) solvent delivery pump, a system controller (SCL-10A), an automatic sample (SIL-10A) with a model 470 scanning fluorescence detector. Chromatographic separations were performed using a Novapak C_{18} stainless steel column (3.9 x 150 mm), with an average particle size of 4µm. A guard column of the same material was used (Waters Associates, USA).

2.2. Reagents

All solvents used were of HPLC grade. While other chemicals and reagents were of analytical grade. Ciprofloxacin and acebutolol hydrochloride were obtained from Sigma Chemical (St Louis, MO, USA)

2.3. Standard Solutions

Ciprofloxacin (10 mg) was dissolved in 10 ml of deionized water (Milli-Quarter). This stock solution was diluted 10-fold and 100 fold in sodium dihydrogen phosphate buffer to give working standard solutions of 100 µg/ml and 10 µg/ml respectively. The internal standard, acebutolol (100 mg) was dissolved in 100 ml of deionized water (Milli-Quarter). This stock solution was diluted 10-fold in 7% per chloric acid to give a working standard solution of 100 µg/ml. These solutions were stable at -20□C for at least one month

2.4. Chromatographic Conditions

The mobile phase consisted of 10.5% acetonitrile in 0.1 M sodium dihydrogen phosphate buffer solution (pH3.9). The mobile phase was degassed daily by passing it through a 0.22-µm membrane filter (Millipore, Bradford, MA, USA). The mobile phase was pumped isocratically at a flow rate of 2 ml/min., at ambient temperature. The chart speed was 0.3 cm/min., and the effluent was monitored at wavelength of 280 and 455 nm respectively and at attenuation 7.

3. PROCEDURE

Ciprofloxacin working standard (100 µg/ml) was added to a 2-ml micro-centrifuge tubes in volumes of 0, 5, 10, 20, 40 and 80 µl of (10 ug/ml) working standard and 16, 24, 40, 80 and 100 ul of (100 ug/ml) working standard. Drug-free plasma was added to complete volume to 2 ml, vortex-mixed for 30 s, to provide calibration standards of 0.0 (no Ciprofloxacin added), 25, 50, 100, 200, 400, 800, 1200, 2000, 4000 and 5000ng/ml. In addition, different aliquots of ciprofloxacin stock solution were used to spike blank plasma to prepare quality control (QC) samples at 150, 500, and 3000 ng/ml. Each of these standard solutions were distributed in disposable polypropylene microcentrifugre tubes (1.5 ml; Eppendorf) in volumes of 300 µl and stored at -20°C pending analysis. For preparation of samples for injection onto HPLC system, a 300-µl aliquot of the internal standard (100 µg/ml) was added to 100 µl plasma sample, and shaken on a vortex-mixer for 30 s. and centrifuged at 10,000 r.p.m. for 10 min. Aliquots prepared samples were loaded in the autosampler tray and volumes of 50 µl were injected. Calibration samples and QC samples were processed identically as described.

4. VALIDATION

4.1. Linearity, accuracy, precision and sensitivity

For the determination of linearity, accuracy, precision and sensitivity, twelve standard calibration curves of 10 points (non-zero standards) and 12 sets of three spiked quality control samples were prepared and analyzed on day one. The validation on day two and three included the analysis of plasma sample representing two complete standard calibration curves plus six sets of three quality control samples. The calibration curves were evaluated individually by linear regression.

4.2. Validation Results

The validation procedures and results are presented below. All samples used for the validation tests were prepared by spiking interference-free pools of heparinized plasma with prepared standards to give the specified final concentrations.

A. Linearity:

Plasma standards were at ten non-zero concentrations over the range of 25 to 5000 ng/ml. Standards were analyzed in replicates of twelve. The average peak height ratios were plotted against the concentration. The slope, intercept, and the correlation coefficient (r) were determined by the method of weighted least squares (linear model) and the data are collected.

B. Precision and Accuracy

Replicate samples (n = 12) spiked at three different concentrations (150, 500, and 3000 ng/ml) were used to

assess intraday and interday precisions of ciprofloxacin assay in plasma. Selection of concentrations for analysis was made to allow for definition of precision at low, medium and high concentrations of the linear range. Precision is expressed as the percent coefficient of variation (% C.V.), and accuracy is expressed as a percent

C. Stability

Stability in heparinized plasma through 6 freeze-thaw cycles (-20 ± 5ºC to room temperature) has been confirmed (Table 5). Samples, after thawing, were allowed to stand on the bench top, under room lighting until 2 hours had elapsed since their removal from the freezer.

D. Recovery

The recoveries (relative and absolute) of Ciprofloxacin from plasma were quantitated using spiked plasma standards (150, 500, and 3000 ng/ml). The relative analytical recovery was measured in the following way: the drug and internal standard were added to drug-free plasma (twelve replicates for each standard) to achieve the concentrations shown in Table 6. The spiked plasma was then analyzed by the developed method. The relative recovery was calculated by comparing the concentrations obtained from the drug-supplemented plasma with actual added amounts

The absolute recoveries were obtained by comparing the observed concentrations obtained from the processed standard samples to direct injections of stock aqueous solutions prepared at concentrations which represented 100% recovery.

E. Clinical application and pharmacokinetic analysis

Two commercially available tablet formulations of ciprofloxacin were administered to 6 healthy male volunteers in a two-way crossover design with two-week washout period between each treatment. The approval of Human Ethics Committee approved the study and the consent of the volunteers were obtained. The volunteers fasted overnight before the drug administration (1x500 mg tablet) for at least 10 hours. The volunteers remained ambulatory and no smoking or strenuous activity was permitted during the study. The volunteers did not take any other medications for at least two weeks prior to and during the study. Multiple blood samples (10 ml) were collected in evacuated glass tubes (heparinized vacutainers, Beckton and Dickinson, Rutherford, NJ, USA) through an indwelling cannula placed in the forearm veins or directly from the vein before (0 hour) and at 0.25, 0.5, 0.75, 1.0,1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 8.0, 10.0, 12.0, 14.0, 16.0, and 24.0 hours post-dosing. The blood was then slightly shaken and centrifuged at 3000 rpm for 10 minutes and the plasma was stored frozen at -20 C pending analysis. The pharmacokinetic parameters for ciprofloxacin were determined from the plasma concentration-time data. The maximum plasma concentrations (C_{max}) and time to reach maximum plasma concentrations (T_{max}) were obtained directly by inspection of the individual drug plasma concentration-time profiles, and were used as measures of rate of absorption. The area under the plasma concentration-time curve up to the last time (t) showing a measurable concentration (C_t) of the analyte (AUC_{0-t}) was determined by using the linear trapezoidal rule. The apparent elimination rate constant (K_{el}) was calculated by the technique of least-squares regression from the data of the last 7-13 points (non-zero) of each plasma concentration-time curve. The $AUC_{0-\infty}$ values (express the magnitude of absorption) were determined by adding the quotient of $*C_t$ and the appropriate K_{el} to the corresponding AUC_{0-t}, that is :

$$AUC_{0-\infty} = AUC_{0-t} + *C_t / K_{el}$$

where $*C_t$ is the last measurable plasma oncentration.
The apparent elimination half-life ($t_{½}$) of iprofloxacin in plasma was calculated by using the following equation:

$$t_{½} = (\ln 2) / K_{el}$$

The rate of absorption was also evaluated by means of the ratio $C_{max}/AUC_{0-\infty}$ [10-11]. This ratio is held to be a good parameter for evaluation of the absorption rates in bioequivalence studies of immediate release formulation [11]. The ratio $AUC_{0-t}/ AUC_{0-\infty}$ was determined as an indicator for the adequacy of the sampling times.

T he absolute recoveries were obtained by comparing the observed concentrations obtained from the processed standard samples to direct injections of stock aqueous solutions prepared at concentrations which represented 100% recovery.

5. RESULTS AND DISCUSSION

The present study describes a highly sensitive, accurate, and reproducible HPLC method for the determination of ciprofloxacin in human plasma. This method has several advantages over the previously reported methods [4-17].Sample preparation is simpler, and the chromatographic column and IS used are commercially available. The procedure for sample preparation is rapid and inexpensive. Because the IS and samples containing unknown concentrations are handled simultaneously, errors of manipulation are taken into account. The low limit of quantification (LOQ) obtained indicates the high sensitivity of the described method compared with the previous methods of analysis of ciprofloxacin, which rendered this method particularly useful for pharmacokinetic studies.

Another advantage of this method is the use of an isocratic mobile phase of simple composition. The proposed method has been applied for the analysis of ciprofloxacin in the plasma of healthy volunteers in a single-dose pharmacokinetic study where the concentrations of the drug is expected to be much lower than those observed at steady state.

The results of twelve calibrations curves showed good linearity with correlation coefficient of 0.9996. The slope, intercept, and the correlation coefficient (r) were determined by the method of weighted least squares (linear model) and the data are presented in Table 1.

Table 2 : Intraday Precision of Ciprofloxacin Assay in Plasma.

	Added Conc. ng/ml		
	150	500	3000
Run	**Measured conc. ng/ml**		
1	155	533	2855
2	163	564	3420
3	154	533	3300
4	145	524	3279
5	137	527	3163
6	140	517	3406
7	142	535	2817
8	146	517	3232
9	158	482	2810
10	146	468	2820
11	163	509	2778
12	146	482	2854
Mean	149.6	515.8	2948
± S.D.	8.8	28.5	313.2
% C.V.	5.9	5.5	10.6
Accuracy %	99.7	100.3	98.3
Bias (%)	0.3	0.3	1.7

Table 3 : Interday Precision of Ciprofloxacin Assayin Plasma.

	Added conc. ng/ml		
	150	500	3000
Run	**Measured conc. ng/ml**		
1	145	530	3074
2	146	523	3295
3	167	513	3129
4	142	531	3098
5	158	475	3070
6	157	491	2818
7	147	515	2865
8	150	528	3169
9	161	550	3320
10	162	538	3243
11	142	539	2871
12	139	560	2815
Mean	152	524	3064
± S.D.	8.9	24.8	182.6
% C.V.	5.9	4.7	6.0
Accuracy%	101.3	104.8	102.1
Bias (%)	1.3	4.8	2.1

Table 4 : Freeze-thaw Stability in Plasma.

	Added conc. ng/ml		
	150	500	3000
Cycles	**Measured conc. ng/ml**		
1	156	514	3159
2	162	536	3178
3	149	508	3212
4	155	504	3074
5	150	498	3086
6	152	485	2904
Mean	154	508	3102
± S.D.	4.8	17.1	110.8
% C.V.	3.1	3.4	3.6
Accuracy	102.7	101.6	103.4
Bias (%)	2.7	1.6	3.4

The mean relative recovery of ciprofloxacin from plasma ranged from 99.7 to 103.2%. , The mean absolute recovery of ciprofloxacin from plasma ranged from 97.9 to 106.9%. .

The method reported above offers advantages over the previously described HPLC method using fluorescence detection such as a simple procedure of sample preparation and the use of an isocratic eluent of simple composition.

6. PHARMACOKINETIC STUDY

The mean plasma concentration profiles of ciprofloxacin after administration of 500 mg tablet from two commercially available formulations to 6 healthy male volunteers is calculated. The calculated pharmacokinetic parameters (AUC, C_{max}, T_{max}, Kel, $t_{1/2}$, and C_{max} / $AUC_{0\rightarrow\infty}$ of the two brands are shown in Table 5. The data obtained in this study are in agreement with the previously published results [18-19]

Table 5. Mean pharmacokinetic parameters of ciprofloxacin after administration of the two commercially available brands to 6 healthy male subjects.

Parameter	Brand A	Brand B
$AUC_{0\rightarrow t}$ (µg.hr/ml)	12.697 ± 2.993	12.534 ± 3.907
$AUC_{0\rightarrow\infty}$ (µg.hr/ml)	13.174 ± 3.086	13.009 ± 3.174
C_{max} (µg/ml)	2.913 ± 0.698	2.724 ± 0.593
T_{max} (hr)	1.354 ± 0.510	1.490 ± 0.407
K_{el} (hr^{-1})	0.169 ± 0.035	0.165 ± 0.033
$t_{1/2}$ (hr)	4.278 ± 0.876	4.340 ± 0.803
$C_{max}/AUC_{0\rightarrow\infty}$ (hr^{-1})	0.225 ± 0.046	0.213 ± 0.031

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