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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CIPROFLOXACIN IN HUMAN SERUM

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A simple high performance liquid chromatographic method is described for the determination of ciprofloxacin in serum. Extraction of the drug from serum was achieved by protein precipitation using trichloroacetic acid. Analysis was performed with a mobile phase comprising of 9% acetonitrile in 2.5% tetrabutyl ammonium hydroxide buffer (pH was adjusted to 2.5 using orthophosphoric acid). The mobile phase was pumped through a nucleosil C-18 column at a flow rate of 1.0 ml.min^{-1} at 40°C. The analyses were detected spectrofluorometrically The excitation and emission wavelengths were 277 nm and 456 nm respectively. The detection limit was 0.0075 μg.ml^{-1} for ciprofloxacin. The method is applicable for the determination of ciprofloxacin in human serum for clinical studies.

Keywords : *Ciprofloxacin, HPLC, Serum sample*

Introduction

Ciprofloxacin belongs to the new quinolone group of antibiotics with an extended antibacterial spectrum. It is also described as a gyrase inhibitor because of its mode of action and has rapid bactericidal activity not only in the multiple phases but also at the resting phase of bacteria. It is rapidly and well absorbed after oral administration and distributed in the many tissues and body fluids. It is largely excreted as unchanged substance and elimination is predominantly *via* the kidneys (1). Several HPLC methods concerning determination of Ciprofloxacin have been described (2-7). The present paper describes a simple and rapid HPLC method for the determination of ciprofloxacin in serum.

Materials and Methods

Ciprofloxacin, enrofloxacin and metabolite-I (M-I) were obtained from Bayer AG, FRG. Double distilled deionised water was used. Acetonitrile was Lichrosolve grade (Merck, Darmstadt, Germany) and used without further purification. Orthophosphoric acid and tetrabutyl ammonium hydroxide were of analytical grade (Merck). The buffer consisted of 10 ml 25% methanolic solution of tetrabutyl ammonium hydroxide in one litre of water and pH was adjusted to 2.5 with orthophosphoric acid. The validation samples were prepared in pooled human serum.

Instrumentation: The HPLC system used consisted of the following components: a Merck-Hitachi Lachrome pump-7100, Shimadzu Rf-10 Spectrofluorometric detector, equipped with PE-Nelson 1022 computer Integrator and rheodyne injector holding a 50μl loop.

Chromatographic conditions: Isocratic separation was achieved using a Nucleosil ODS column (250 mm x 4.0 mm I.D., 10 μm). The mobile phase was acetonitrile and buffer (9:91) (buffer consisted of 10 ml of 25% methanolic solution of tetrabutyl ammonium hydroxide in one litre of water and pH was adjusted to 2.5 with orthophosphoric acid). The flow rate was 1.0 ml.min^{-1}. The excitation and emission wavelengths were 277 and 456 nm respectively for the detection of the analysis. The injected volume was 10 μl. Chromatography was performed at 40±1°C.

Standard and stock solutions: A concentrated stock solution of ciprofloxacin (1 mg.ml^{-1}) was prepared in methanol and used for up to 30 days. Enrofloxacin was used as the internal standard

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(IS). A concentrated solution (1mg.ml^{-1}) of IS was prepared in 1% orthophosphoric acid solution and used for up to 7 days. A solution of metabolite-1 (of Ciprofloxacin) was also prepared using methanol. The concentration was 0.425 μg.ml^{-1}.

Spiked serum validation samples were prepared by spiking known concentration of standard solution of ciprofloxacin (5%) to the blank pooled serum (95%). These samples were aliquoted and stored at -70°C until analysis.

Sample extraction: Extraction of the drugs from serum was performed as follows. An aliquot (100 μl) of the internal standard solution (0.250 μg.ml^{-1}) was added to 850 μl of serum and vortexed. To the above serum sample a 200 μl of 20% trichloroacetic acid solution was added for deproteinization while vortexing and centrifuged at 3500 rpm (4°C) for 20 min. 10 μl of supernatant was injected on to the column.

Standard curve: Calibration standards were prepared using 0.0075, 0.0150, 0.0300, 0.0751, 0.1502, 0.4506, 1.1265 and 2.2530 μg.ml^{-1} concentration of ciprofloxacin in human serum and in water. These samples were treated as indicated above.

The validation of this procedure was performed in order to evaluate the method for recovery, linearity, accuracy, precision, sensitivity and stability on five separate days. The validation set consisted of 1 calibration curve (8 different concentration of ciprofloxacin, blank and blank with IS and metabolite-I) and 4 replicate of quality control samples (0.0250, 0.3754, 0.7508 and 1.5016 μg.ml^{-1}) respectively.

Recovery: The percent recoveries were determined by measuring the peak areas of ciprofloxacin and the internal standard respectively from the prepared serum validation samples at concentration of 0.0250, 0.3754, 0.7508 and 1.5016 μg/ml. The peak areas of serum validation samples were compared to the absolute peak area of the unextracted aqueous standard containing the same concentration of ciprofloxacin and the internal standard.

Accuracy and limit of quantitation: Quantitation was based on the peak area measurement. The of quantitation was defined as the lowest ciprofloxacin concentration, which can be determined with confidence on a day to day basis. The linearity of the method was assessed from 0.0075 to 2.2530 μg/ml of ciprofloxacin concentration. Each concentration was measured five times.

Accuracy was evaluated by calculating the mean percent difference of theoretical values and measured values.

Precision: Both within day and between day reproducibility were tested. The quality control concentrations were 0.0250, 0.3754, 0.7508 and 1.5016 μg.ml^{-1} of ciprofloxacin. Five samples of each QC concentration were tested on the same day and the resulting coefficient of variation (CV) indicated the within-day reproducibility. Aliquots of the same QC sample were tested once a day during five days and the resulting CV indicated the between day reproducibility.

Specificity: To evaluate the specificity of the method, drug free serum sample was carried through the assay procedure and the retention times of the endogenous compounds in the serum were compared with those of ciprofloxacin, metabolite-I or enrofloxacin. Specificity of the method was assessed to test the matrix influence between different serum samples.

Stock solution stability: The working aqueous solution (0.7508μg/ml) of ciprofloxacin was repeatedly (n=3) injected into the chromatograph immediately after preparation (time 0) and at 3, 6 and 9 hours after bench top storage at room temperature and at 4°C. This injection protocol was repeated after a 1, 3, 6, 8, 15, 30 and 60-day storage of this solution at 4°C.

Bench-top stability: Quality control samples were analysed in triplicate immediately after preparation (time 0) and after bench-top storage at 20°C for 3, 6 and 9h. The peak areas of samples were compared with the peak areas of the samples at 0-time. In fact this test indicated the stability of the ciprofloxacin in the thawed serum at 20°C on the bench-top.

Freeze-thaw stability: The freeze-thaw stability of the QC samples was analysed after one, two or three freeze-thaw cycles. The peak areas of these samples were compared with the freshly prepared serum samples of the same concentration.

Long term stability: QC concentrations of 0.0250, 0.3754, 0.7508 and 1.5016μg/ml^{-1} of ciprofloxacin in serum were analysed in triplicate and injected into the chromatograph immediately after preparation (time and day 0). The injection protocol was repeated after 1, 8, 15, 30 and 60-day storage of these samples at -70°C to check possible degradation during storage.

Results and Discussion

A representative chromatogram of the blank serum, serum containing metabolite-I (M-I) and ciprofloxacin along with enrofloxacin (IS) is shown in Fig.1. The pH of the mobile phase was 2.5, which prevents the tailing of the drug. There was clear resolution of the metabolite-I, ciprofloxacin and enrofloxacin, which have retention times of 4.70, 6.10 and 7.25 min. respectively.

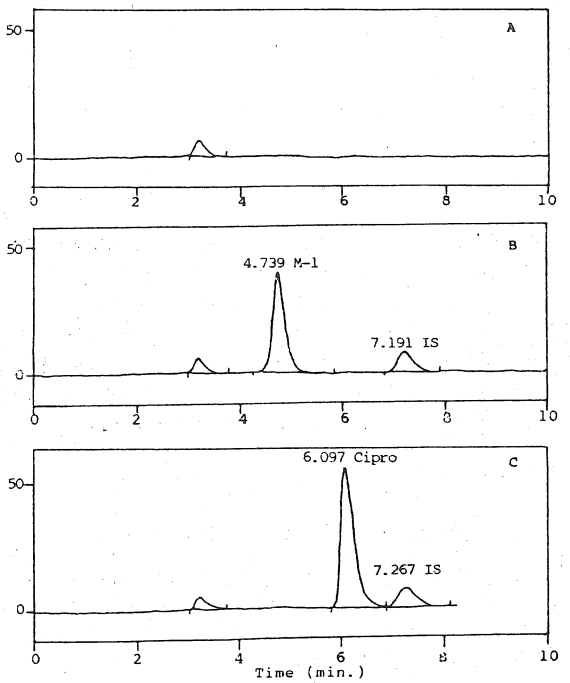

Fig.1: Chromatograms (A) blank serum; (B) serum spiked with 0.425 μg.ml^{-1} M-I; (C) serum spiked with 0.425 μg.ml^{-1} Ciprofloxacin.

Extraction and chromatographic analysis of the six different blank serum samples confirmed that there were no endogenous peaks that coeluted either with ciprofloxacin or its active metabolite-I (M-I).

The lower limit of quantitation was 0.0075 μg.ml^{-1}. The linearity study was carried out with concentration ranging from 0.0075 to 2.2530 μg.ml^{-1} serum. The regression analysis between the peak area ratio and concentration ratio revealed that the method was linear (r^2>O.999). The equation of the regression line is y=0.2672x-0.01186 where y = concentration ratio and x = area ratio. The extraction recovery from the serum spiked with ciprofloxacin was 52.08±1.41% (Table 1).

Table 1. Extraction recoveries of ciprofloxacin

Concentration (μg.ml^{-1})	*Recovery (percent)
0.0250	49.29 ± 1.29
0.3754	53.92 ± 1.56
0.7508	48.56 ± 1.15
1.5016	56.54 ± 1.36

*As compared to the unextracted aqueous samples (n=5)

For serum, the intra-assay precision was characterised by a coefficient of variation (C.V.) of 2.15% (0.0250), 3.29% (0.3754), 2.81% (0.7508) and 3.15% (1.5016 μg/ml) (Table 2).

Table 2. Precision of ciprofloxacin assay

Concentration (μg.ml^{-1})	Intra assay CV (%)	Inter assay CV (%)
0.025	2.15	5.86
0.3754	3.29	7.93
0.7508	2.81	4.69
1.5016	3.15	6.27

The values for inter-assay precision respectively were 5.86, 7.93, 4.69 and 6.27%. Accuracy for targeted values were below 15% and 10% for the low and high quality control levels respectively (Table 3).

Bench-top stability of the quality control samples was determined at 0, 3, 6 and 9 hours. Comparison was done against

the 0-hour sample. The bench-top stability of the samples ranged from 100.68 to 106.07% (Table 4).

Table 3. Accuracy of ciprofloxacin assay (n=5)

Theoretical value ($\mu g.ml^{-1}$)	Measured value ($\mu g.ml^{-1}$)	% Difference from theoretical value
0.0075	0.0084	12.00
0.0150	0.0161	7.33
0.0301	0.0281	6.64
0.0751	0.0734	2.26
0.1502	0.1515	0.86
0.4506	0.4352	3.41
1.1265	1.0918	3.08
2.2530	2.2618	0.39

Table 4. Bench top stability of ciprofloxacin in human serum (n=3)

Time (hr)	Concentration ($\mu g.ml^{-1}$)			
	0.0250	0.3754	0.7508	1.5016
0	100.00	100.00	100.00	100.00
3	105.84	101.15	102.29	100.68
6	106.07	102.34	101.87	103.16
9	104.84	105.21	103.48	104.96

When stored at -70°C for two months in serum, ciprofloxacin did not reveal any appreciable degradation with all samples retaining more than 95% of their original concentrations. The freeze-thaw stability of ciprofloxacin after a 1, 2 or 3 cycle was ranged from 93.83 to 102.15% (Table 5). The working solution of ciprofloxacin was stable both at room temperature and at 4°C. The serum samples of ciprofloxacin before and after extraction were stable at the various concentrations tested. The percentage of change compared to day and time 0 was -0.99% (0.025), +1.73% (0.3754), -1.56% (0.7508) and +2.61% (1.5016 $\mu g.ml^{-1}$) after 24 hours.

Table 5. Freeze-Thaw & Long term stability of ciprofloxacin in human serum

Freeze-thaw stability (cycle)	QÇ concentration ($\mu g.ml^{-1}$)			
	0.0250	0.3754	0.7508	1.5016
1	99.16	94.45	101.15	94.88
2	93.83	102.15	99.96	97.68
2	96.56	98.47	97.12	101.53
Long-term Stability(Day)				
1	99.01	101.73	98.44	102.61
8	97.79	100.84	99.20	99.15
15	98.25	101.23	98.45	98.89
30	96.23	102.67	97.76	99.75
60	97.77	101.97	98.38	98.26

The HPLC method for ciprofloxacin described here represents a rapid, sensitive, efficient and precise analytical approach for quantitating this drug in serum. The primary metabolite **(M-I)** was also separated by this method and can be validated separately. The analytical characteristics of the proposed method and the minimum sample handling as well as low injection volume were satisfactory for pharmacokinetic and clinical studies.

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