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RAPID DETERMINATION OF CIPROFLOXACIN CONCENTRATION IN HUMAN PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A simple, precise, and rapid high performance liquid chromatography (HPLC) method for the determination of ciprofloxacin level in human plasma using gatifloxacin as an internal standard (IS) was developed and validated. 0.5 ml Plasma samples containing ciprofloxacin were mixed with 200 µl of IS (50 µg/ ml in buffer) and filtered through a centrifugal filter device by centrifuging at 4000 rpm for 30 minutes. A 100 µl of ultra-filtrate clear solution was injected in the HPLC system. The compounds of interest were efficiently separated on Xterra RP-18 (4.6 x 150 mm, 5-µm) steel column (25ºC) proceeded by a universal sentry guard column (symmetry C_{18}, 5-µm insert) and detected with a photodiode array detector set at 276 nm. The mobile phase consisted of a mixture of 0.025 M of sodium phosphate monobasic (pH = 3.0, adjusted with phosphoric acid) and acetonitrile (85:15, v:v). The mobile phase was delivered at a flow rate of 1 ml/min and run for 10 min. No interference in blank plasma or by commonly used drugs was observed, and the detection limit of ciprofloxacin was 0.05 µg/ml. The relationship between ciprofloxacin concentration in plasma and peak area ratio of ciprofloxacin /IS was linear ($R^2 \geq 0.9997$) in the range of 0.1 – 12.0 µg/ml. Intra- and inter-day coefficient of variation and bias were ≤ 7.0% and ≤ 11.1%, respectively. Mean extraction recovery of ciprofloxacin and the IS from the plasma samples were ≥ 90%, and 85%, respectively. Using the method, ciprofloxacin in found to be stable in human plasma for 16 weeks at -20 ºC. Further, the method was successfully employed to measure ciprofloxacin level in plasma samples from a healthy volunteer.

KEYWORDS: Ciprofloxacin, Gatifloxacin, Human plasma, HPLC.

INTRODUCTION

Ciprofloxacin (CAS: 85721-33-1), 1-cyclopropyl-6-fluoro-1, 4-dihydro-4-oxo-7-[1-piperazinyl]-3-quinoline carboxylic acid, is a synthetic broad-spectrum antimicrobial fluoroquinolone that has activity against both gram-negative and gram-positive bacteria. It is used in the treatment of a wide range of infections.[1-2] Its absolute bioavailability is about 70% with a mean peak plasma concentration of 1.5 to 2.9 µg/ml at 1-2 hours after the ingestion of a 500 mg therapeutic dosage.[3-4]

Various analytical methods have been reported in the literature for the determination of ciprofloxacin in pharmaceutical preparations and in biological matrixes. The methods include high-performance liquid chromatography (HPLC),[5-16] atomic absorption spectrometric,[16] and LCMS/MS.[17] Most of the HPLC assays for ciprofloxacin in human plasma required a relatively large sample volume.

We describe a simple and reliable analytical method for the quantitative determination of therapeutic levels of ciprofloxacin in human plasma. The method was used to determine long-term stability of ciprofloxacin in human plasma and for pharmacokinetic study conducted on healthy volunteers.

MATERIAL AND METHODS

Apparatus

Chromatography was performed on a Waters 2998 photodiode (Waters Associates Inc., Milford, MA, USA) consisting of a quaternary pump, autosampler, column thermostat, photodiode array detector, and Xterra RP-18 (4.6 x 150 mm, 5-µm) steel column at 25°C, preceded by a universal sentry guard column (Symmetry C_{18}, 5-µm insert). Data were collected with a Pentium IV computer using Empower Chromatography Manager Software.

Chemical and reagents

All reagents were of analytical grade unless stated otherwise. Ciprofloxacin standard was purchased from Bayer Leverkusen, Germany. Gatifloxacin was provided by Jamjoom pharma, Jeddah, Kingdom of Saudi Arabia. Acetonitrile, phosphoric acid, and sodium phosphate monobasic (all HPLC grade) were purchased from Fisher Scientific, Fairlawn, NJ, USA. HPLC grade water was prepared by reverse osmosis and was further purified by

passing through a Synergy Water Purification System (Millipore, Bedford, MA, USA). Drug-free human plasma was obtained from the blood bank of King Faisal Specialist Hospital & Research Centre (KFSHRC), Riyadh, Saudi Arabia. Samples from healthy volunteers were collected after obtaining approval from the Research Ethical Committee of KFSHRC.

Chromatographic conditions

The mobile phase consisted of a mixture of 0.025 M of sodium phosphate monobasic (pH = 3.0, adjusted with phosphoric acid), and acetonitrile (85:15, v: v). Before delivering into the system, the mobile phase was filtered through 0.45 µm polyetersulfone membrane and sonicated under vacuum for 5 minutes. The analysis was carried out under isocratic conditions using a flow rate of 1 ml/min at 25°C and a run time of 10 minutes. A photodiode array detector set at 276 nm was used.

Preparation of standard and quality control samples

Stock solution (1 mg/ml) of ciprofloxacin and gatifloxacin (internal standard, IS) were prepared in mobile phase and water, respectively. They were diluted with blank human plasma or mobile phase, respectively, to produce working solutions of ciprofloxacin (100 µg/ml) and IS (5 µg/ml). Calibration curve standards (nine concentrations) in the range of 0.1 – 12 µg/ml were prepared in human plasma. Six quality control (QC) samples (0.1, 0.3, 6.0, 7.0, 10.8, and 11 µg/ml) were also prepared in human plasma. 0.5 ml aliquots were transferred into Teflon-lined, screw-capped, borosilicate glass culture tubes (13 x 100 mm) and stored at –20 ºC until used.

Sample preparation

Aliquots of 0.5 ml blank plasma, calibration curve, quality control, or volunteer samples were filtered using Centrifugal Filter Units (Millipore, Waters USA) after mixing with 200 µl of IS (gatifloxacin, 50 µg/ ml buffer), vortexing for 30 seconds, and centrifuging at 4000 rpm for 30 min. A 100 µl of ultra-filtrate clear solution of was injected in HPLC system with a run time of 10 min.

Stability studies

Three QC samples (0.3, 6.0 and 10.8 µg/ml) were used for ciprofloxacin stability studies: Five aliquots of each QC sample were extracted and immediately analyzed baseline and five aliquots were stored at -20ºC for 16 weeks before being processed and analyzed (long-term freezer storage stability).

Method validation

The method was validated according to standard procedures described in the US Food and Drug Administration (FDA) bioanalytical method validation guidance.[18] The validation parameter included: specificity, linearity, accuracy, precision, and recovery.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

Figure 1 depicts the chemical structures of ciprofloxacin and gatifloxacin. The optimal experimental conditions were a mobile phase composed of 0.025 M of sodium phosphate monobasic (pH = 3, adjusted with phosphoric acid) and acetonitrile (85:15, v: v), and a flow rate of 1 ml/min. Under these conditions ciprofloxacin, gatifloxacin, and components of plasma exhibited a well-defined separation within 10 minutes run. The retention times of ciprofloxacin and gatifloxacin, were around 5.3 and 8.9 minutes, respectively.

Specificity

Specificity is defined as the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. Potential interfering substances in plasma samples include endogenous components, metabolites, and decomposition products. We screened six batches of blank plasma and seven frequently used medications (ranitidine, acetaminophen, ibuprofen, nicotinic acid, ascorbic acid, caffeine, and asprin) for potential interference. No interference was found in plasma and none of the drugs co-eluted with ciprofloxacin or the IS. **Figure 2** depicts a representative chromatogram of drug free human plasma used in preparation of QC samples.

Limit of detection & quantification and linearity

The limit of quantification was defined as the lowest concentration on the calibration curve that can be determined with acceptable precision and accuracy (i.e., coefficient of variation and bias ≤20%). The limit of quantification of ciprofloxacin in human plasma was 0.1 μg/ml. Calibration curves were linear with an $R^2 \geq 0.9991$. **Figure 3** shows an overlay of chromatograms of a typical calibration curve. The mean (SD) of slope, intercept, and (coefficient of variation) of the ten curves were 0.0135 (0.0488), -0.020 (0.020), and 0.991 (0.006), respectively. The suitability of the calibration curves was confirmed by back-calculating the concentration of ciprofloxacin in human plasma from the calibration curves (**Table 1)**. All calculated concentrations were well within the acceptable limits.

Precision and bias (inaccuracy)

The intra-day and inter-day precision and bias of the method were evaluated by analyzing four QC (0.1, 0.3, 6.0, and 10.8 µg/ml). The intra-day precision and bias (n = 10) ranged from 3.0% to 7.0% and from –2.0% to 11.0%, respectively. The inter-day precision and bias were determined over three different days (n=20). The inter-day precision and bias ranged from 4.9% to 6.4% and from 0.7% to 9.2%, respectively. The results are summarized in **Table 2**.

Recovery

The absolute recovery of ciprofloxacin was assessed by direct comparison of the absolute peak areas from plasma and mobile phase samples, using five replicates for each of the 4 QC concentrations (0.1, 0.3, 7.0, and 11.0 µg/ml). The recovery of the IS was determined by comparing the peak areas of the IS in 5 aliquots of human plasma spiked with 200 µl of IS (50 µg/ml) with the peak areas of equivalent samples prepared in mobile phase. The results are presented in **Table 3**.

Robustness and ruggedness

Robustness of a method is a measure of its capacity to remain unaffected by small variations in method conditions. The robustness of the current assay was evaluated by altering the strength of the sodium phosphate buffer (±0.005 M), pH (±0.20), and volume of acetonitrile (±2.0%) in the mobile phase. No significant changes were observed. Ruggedness was tested by conducting split sample test. Two split samples (concentration 0.58 and 7.0 µg/ml) were analyzed by two blinded technologists on two different instruments. The accuracy of the reported concentrations was within the acceptable limits (bias ≤5.0%).

Stability

Stability of analytes in biological matrices is an important pre-analytical variable. It is necessary to perform stability studies of the analyte to determine the range of appropriate conditions and time of storage. The long term stability of ciprofloxacin was carried out using three QC (0.3, 6.0, and 10.8 µg/ml). Ciprofloxacin level was measured, after storing samples for 16 weeks at -20 ºC. The mean measured (SD) levels were 0.32 (0.018), 6.67 (0.355), and 11.21(0.309) µg/ml, respectively. These results indicate that ciprofloxacin is stable (101%, 104% and 104%) respectively, at -20 ºC for 16 weeks.

Application to a volunteer sample

Figure 4 depicts an overlay chromatogram of samples collected from a volunteer before and 3.0 hours after the ingestion of a single dose of 500 mg ciprofloxacin. Measured levels of ciprofloxacin were zero and 0.28 µg/ml, respectively.

Table 1: Back calculated ciprofloxacin concentrations from ten calibration curves

Nominal level (µg/ml)	Calculated level (µg/ml)		CV (%)	Bias (%)
	Mean	SD		
0.1	0.118	0.019	16.1	18.2
0.4	0.415	0.022	5.3	3.8
0.6	0.597	0.013	2.1	-1.6
0.8	0.801	0.014	1.8	0
1.0	0.997	0.015	1.5	0
4.0	4.036	0.026	0.6	0.7
6.0	5.999	0.068	1.1	0
8.0	7.850	0.087	1.1	-2.5
12.0	12.083	0.073	0.6	0.6

SD, standard deviation. CV, standard deviation divided by mean measured concentration x100

Bias = (mean measured concentration – nominal concentration divided by nominal concentration) × 100.

Table 2: Intra and inter-day precision and bias of ciprofloxacin assay

Nominal level (µg/ml)	Measured level (µg/ml)		CV (%)	Bias (%)
	Mean	SD		
Intra-day (n=10)				
0.1	0.1099	0.007	7.0	9.9
0.3	0.3101	0.015	5.0	3.4
6.0	6.6788	0.200	3.0	11.1
10.8	10.5820	0.459	4.3	-2.0
Inter-day (n= 20)				
0.1	0.1065	0.006	6.4	6.5
0.3	0.3044	0.015	4.9	1.5
6.0	6.5511	0.376	5.7	9.2
10.8	10.8827	0.572	5.3	0.7

SD, standard deviation. CV, standard deviation divided by mean measured concentration x100

Bias = (mean measured concentration – nominal concentration divided by nominal concentration) × 100.

Table 3: Recovery of ciprofloxacin and the internal standard from 0.5 ml of human plasma.

Concentration (µg/ml)	Human Plasma	SD	Mobile Phase	SD	Recovery (%)
Ciprofloxacin					
0.1	2929	35	2948	6	99
0.3	8108	107	8882	132	91
7	206618	790	226338	827	91
11	323605	943	357587	875	90
Internal standard					
0.2	75433	500	83826	1108	85

** Mean peak area (SD), n = 5.*

FIGURE CAPTIONS

Fig. 1 Chemical structures of ciprofloxacin and gatifloxacin (IS).

Fig. 2 Representative chromatogram of a drug-free human plasma. The arrows indicate the retention times of ciprofloxacin and the internal standard (gatifloxacin, IS).

Fig. 3 Overlay of chromatograms of extracts of 0.5 ml human plasma spiked with the internal standard (IS) and zero or one of nine concentrations of ciprofloxacin.

Fig. 4 An overlay of chromatograms of plasma samples obtained from a healthy volunteer before (A) and 3 hours after (B) a single oral 500 mg ciprofloxacin dose.

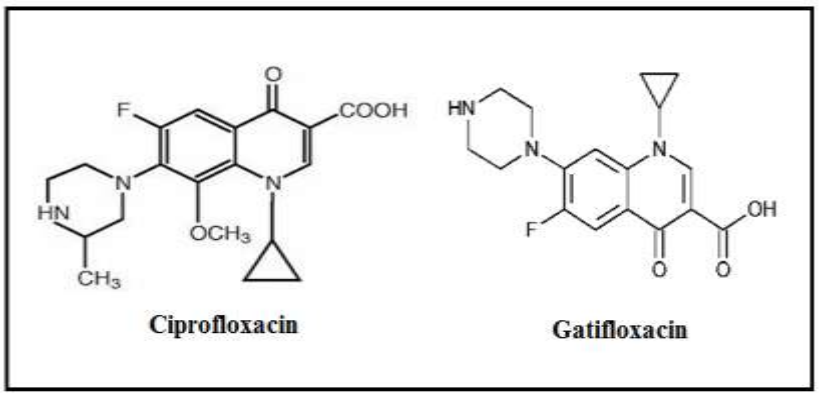

Figure 1

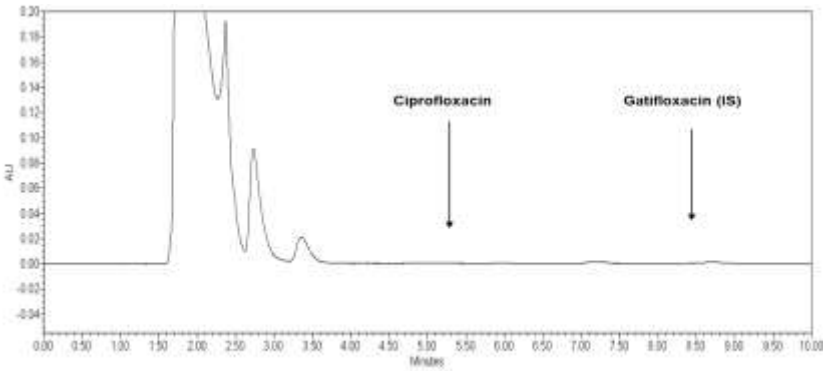

Figure 2

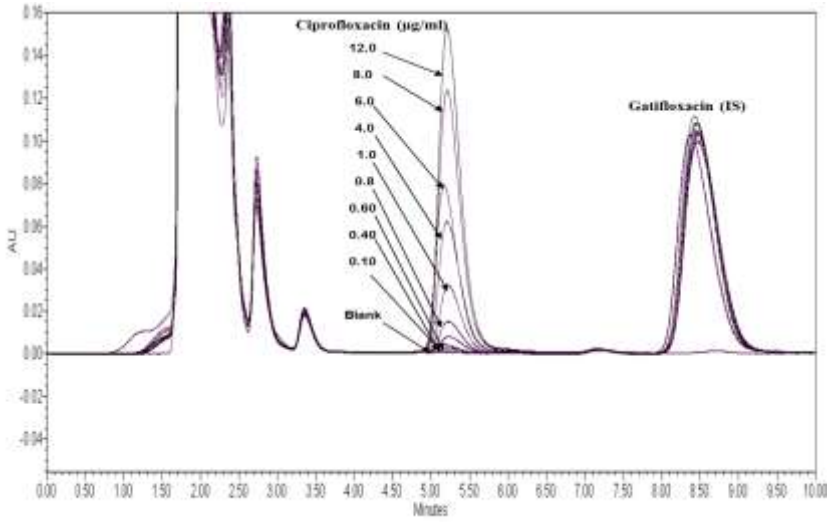

Figure 3

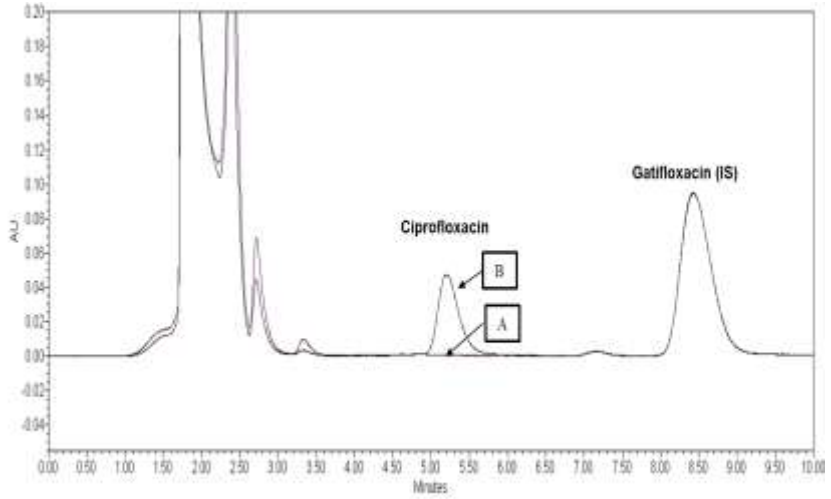

Figure 4

CONCLUSION

The described HPLC assay is precise, accurate, and rapid. It requires only 0.5 ml plasma and utilizes a simple and convenient method for sample preparation. The assay was successfully applied to monitor 16 weeks stability of ciprofloxacin at -20 ºC and to determine ciprofloxacin concentration in a healthy volunteer.

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