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The Development and Validation of HPLC-UV method for Analysis of Ciprofloxacin in serum and aqueous Humour

Abbas khan[1], Zafar Iqbal[1] , Jamshaid Ali Khan[1], Muhammad Imran Khan[1], Ghulam Sarwar khan[2] Muhammad Bilal[3], Tahir Mehmood Khan[4]

[1] Department of Pharmacy, University of Peshawar. Peshawar-25120. Pakistan
[2] Department of Community Medicine Khyber Medical College, Peshawar NWFP Pakistan
[3] Registrar Plastic surgery and burn Unit Khyber Teaching Hospital (KTH) Peshawar
[4] College of Clinical Pharmacy, King Faisal University, Saudi Arabia

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Abstract

A simple bioanalytical reversed phase HPLC method for the determination of the ciprofloxacin in serum, aqueous humour and ophthalmic drops was developed and validated. HPLC equipped with Hypersil C^{18} Column was operated in isocratic mode using acetonitrile and 0.25 M H_3PO_4 (60:40 v/v) as mobile phase that was pumped at rate of 1ml.min^{-1} and eluents were monitored using UV-Visible detector at 275 nm. The Ciprofloxacin was analysed in serum, aqueous humour and ophthalmic drops using acetaminophen as an internal standard (IS). The instrumental response was linear in the concentration range of 5 ng.ml^{-1} to 75 ng.ml^{-1} in aqueous humour and 10 ng.ml^{-1} to 70 ng.ml^{-1} in serum. The LOD and LOQ, precision and reproducibility of the method were good for pharmacokinetics studies.

Key words

Antibiotic ; Ciprofloxacin; HPLC; Human Blood serum; Aqueous Humour

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Corresponding Author

Prof. Dr. Zafar Iqbal. Department of Pharmacy, University of Peshawar. Peshawar-25120. Pakistan

Email: Zafardr61@yahoo.com

Introduction

Ciprofloxacin, 1-cyclopropyl-6 fluoro-1, 4-dihydro-4-oxo-7- (1 piperazinyl)-3 quinolone carboxylic acid, Showed better antimicrobial activity than the parents compounds nalidixic acid. Second-generation ciprofloxacin show greater potency, lower toxicity and broader antibacterial spectrum. The main difference between ciprofloxacin and other antibiotics is that it can be administered parentally, topically and orally. It is well absorbed and widely distributed into various body tissues and fluids. It is used in the urinary tract, respiratory tract and gastrointestinal tract, as well as skin, ocular disease and soft tissue infections [1, 2, 3, 4, 5 and 6].

Several HPLC methods have been reported for the analysis of ciprofloxacin in biological fluids [7, 8, 9, 10, 11, 12, 13, and 14] based on ultraviolet (UV) detection [7, 8, and 9] and fluorescence detection [11, 12, 13, and 14], because the sample preparation methods involve more than one extraction step [7, 8, 10 and 12]. The aim of the present study work to develop the method based on Hplc for the analysis of ciprofloxacin in aqueous humour , serum and pharmaceutical preparations.

Material and Methods

Chemicals and reagents. Ciprofloxacin hydrochloride, acetaminophen (Internal standard) provided by Ferozsons, Chemicals were analytical-reagent grade. HPLC-grade Acetonitrile, phosphoric acid were Purchased from Sigma Aldrich Chemie Gmbh Germany, Glass distilled water was used throughout analysis. All other chemicals used were of Hplc grade.

Instrumentation and chromatography.

Chromatography was performed using Perkin Elmer Series 200, HPLC equipped with UV-visible detector series 200. The analyte were separated using Hypersil C^{18} Column. (250 x 4.6 mm, 4.5 µ-particle sizes). Acetonitrile and 0.25 M H_3PO_4 (60:40 v/v) were used as mobile phase and pumped at a rate of 1ml.min^{-1}. The UV-visible detector adjusted at 275 nm. The column was protected with a pre-column (Guard-Pak(TM)) filled with a µBondapak (TM) C_{18} cartridge (Merck kGaA).

Calibration curve. Solution (500 µl.ml^{-1}) of the ciprofloxacin and acetaminophen, used as internal standard, were prepared in mobile phase and the diluted accordingly with the same solvent system. Calibration curve was constructed in the range of 5ng to 50 ng.ml^{-1}.

The calibration curve was also constructed in spiked

human serum and spiked bovine aqueous humour samples using acetaminophen as an internal standard.

Sample preparation

Serum. The human blood serum (50 µl) was transferred to glass vial with screw cap and the spiked serum were prepared by adding the required amount of Ciprofloxacin and acetaminophen, used as internal standard (IS) and then added and vortexed for 30 seconds. The 50 µl of *o*-phosphoric acid (0.25M) was added and vortexed for further 30 seconds and kept for 5 minutes at room temperature, to deprotenise the samples. Then acetonitrile (100 µl) was added and centrifuged for 10 min at 10,000 *g*. The clear supernatant was collected and evaporated at 45 ^{0}C under flux of nitrogen. The residue was then reconstituted in mobile phase (100 µL) and 20-µL sample was injected into HPLC.

Aqueous humour. The bovine aqueous humour (50 µl) was used in the method development. The sample was dprotenised and processed with the same procedure as described for the analysis of the serum.

Opthalmic solution (eye drops). Ophthalmic solution of ciprofloxacin (Ciloxan®) containing ciprofloxacin hydrochoride (0.3 %) was diluted with mobile phase to obtain the concentration 1.0, 5.0 and 10.0 ng. ml^{-1} of ciprofloxacin. The samples were prepared as described for serum with exclusion of the deprotenization step.

Limit of Detection and Limits of Quantifications. The method was validated for recovery, accuracy, precision, limit of quantification (*LOQ*), limit of detection (*LOD*), linearity, and selectivity. The % recovery in serum and aqueous humour sample was measured by comparing peak area of ciprofloxacin in spiked sample with the peak area in spiked sample from direct injection of an aqueous solution containing the same amount of drug (n = 6). *LOD* was expressed as a concentration for which the signal-to- noise ratio (S/N) was equal to 3.

LOQ of the assay was evaluated as the concentration for which S/N was equal to 10. The selectivity of the method was described as the resolution of the ciprofloxacin peak from other peaks in the matrics.

Statistical Studies. The mean ± SD concentration of the drug and other statistical analysis of the data was carried out using Minitab® a statistical software.

Stabilities Studies. Interday and intraday precision and stability of the method was studied by analyzing the spiked serum samples 500 ng.ml^{-1} and 100 ng.ml^{-1} and aqueous humour samples spiked with ciprofloxacin with 50 ng.ml^{-1} and 10 ng.ml^{-1}.

The specificity, precision and accuracy of the method was measured in serum and aqueous samples using standard protocols.

Results and discussion

Initially the levofloxacin, sparfloxacin was used as the internal standard but the resolution of the peaks was not very good then it was decided to use the acetaminophen as the internal standard. The retention times for ciprofloxacin and acetaminophen were 5 and 3 min, respectively (Fig 1). The blank serum and aqueous humour was used to establish the base line and then these serum samples were spiked with the ciprofloxacin to study any interference peaks from the biological matrices. Similarly the interference peaks were also studied for acetaminophen. The results showed no any interference from endogenous components.

Validation of the method

Linearity, precision, and accuracy. The linearity of the calibration curves was verified from 5 ng to 50 ng.ml^{-1} for ciprofloxacin in mobile phase and 10 ng.ml^{-1} to 75 ng.ml^{-1} in spiked human serum while for 5 ng.ml^{-1} to 75 ng.ml^{-1} for ciprofloxacin in spiked bovine aqueous humour. The linear curve was observed in the concentration range of the respective samples. And the r^2 value calibration curve in mobile phase, serum and aqueous humour samples was 0.999, 0.997 and 0.998, respectively. The regression equations shows good intercept and slopes values (shown in Fig.).

Limit of detection (LOD) and Limit of Quantification (LOQ). The limit of quantitation of the assay was evaluated as the concentration equal to ten times the value of the single-to-noise ratio. This was taken as the lowest concentration in the calibration range. The limit of detection (LOD) for aqueous humour and serum is 0.5 ng.ml^{-1} and 1 ng.ml-1 while limit of quantitation (LOQ) for Aqeous humour and human serum was 3.33 ng.ml^{-1} and for aqueous humour was 1.67 ng.ml^{-1}.

Specificity, selectivity, and stability. The interference from endogenous compound was investigated by the analysis of six samples of ophthalmic drops, human serum and bovine aqueous humour. No interference from endogenous compounds was observed, indicating the high selectivity of the method. The stability of ciprofloxacin solutions was verified by storing sample solutions refrigerated for six months. Their concentrations were measured periodically (after one, two, three and four weeks). Problems of stability are usually encountered mainly affecting serum and aqueous humour concentration at room temperature. From blood sampling to analysis, storage in the freezer eliminates decomposition.

Interday and Intraday Analysis. The intradays analysis of Bovine aqueous humour mean ± SD 0.05 µg.ml^{-1} and 0.01 µg.ml^{-1} at 9.am, 6 pm, and 9 pm were 0.047±0.001, and with 0087± 0.0001 with % RSD 2.12. and 1.31. while human serum 0.5 µg.ml^{-1} and 0.1 µg.ml^{-1} at 9.am, 1.pm and 6.pm mean ± SD were 0.48±0.0205 and 0.086±0.036 with % RSD 4.27 and 4.06. The interdays analysis (Three consecutive days) of bovine aqueous humour and spiked with ciprofloxacin is 0.047±0.001 and 0.0086±0.00025 with % RSD is 2.08 and 2.91 while for human serum is 0.48 ± 0.01 and 0.093 ± 0.004 with % RSD is 2.08 and 4.92. (Table 1)

Analysis of Ophthalmic drops . The ophthalmic drops were diluted to 1.0, 5.0 and 10.0 ng.ml-1 and analysed for the for ciprofloxacin contents. The concentration of the ciprofloxacin in 1.0, 5.0 and 10.0 ng.ml-1 was 99.2 %, 99.7%, 99.6%, respectively. The analysis of the samples of ophthalmic drops showed the results are in good agreement with the claimed contents.

Analysis of Pharmaceutical tablets. The Commercially available tablets Ciproxacin® contain 250mg, 500 and 750 mg Strength which were analyzed and results were in

range of 99.60 %, 99.20%, 99.7% with % RSD 0.20, 0.4 and 0.4 and are shown in table…

The present study describes a highly sensitive, accurate, and reproducible HPLC method for the determination of ciprofloxacin in ophthalmic preparations, Tablets, serum and aqueous humour. This method has several advantages over the previously reported methods [Jehl F et.al., 1985. Weber et. al., 1985. Vallee et. al., 1986. Groeneveld et. al., 1986. Nix et. al., 1985. Joos et.al., 1985. Borner et. al., 1986. Lovdahl et. al.,1993]. Sample preparation is simpler, and not time consuming. The analytes are analyzed using commonly available UV-Visible detector that gave very Good LOQ values. The method has been used successfully for the pharmacokinetics studies of ciprofloxacin. On the other hand, UV detectors give more reproducible and stable responses than fluorometric detectors [Gerson et. al.1980].

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Table 1 Intraday and Interday Precisions and Accuracy Study Determination of Ciprofloxacin in Serum and Aqueous Humour of Human Volunteer

Human Serum										
Added ug.ml^{-1}	**Intraday**					**Interdays**				
Serum	**9A.M**	**1.PM**	**6.PM**	**Mean**	**% RSD**	**Day1**	**Day 2**	**Day 3**	**Mean**	**% RSD**
0.5ug.ml-1	0.47± 0.043	0.51± 0.065	0.48±0.052	0.48±0.0205	4.27	0.49±0.046	0.47±0.035	0.48±0.024	0.48±0.01	2.08
0.1 ug.ml^{-1}	0.09±0.005	0.084±0.0075	0.086±0.0053	0.086±0.036	4.06	0.098±0.002	0.092±0.021	0.089±0.004	0.093±0.004	4.92
Aqueous humour										
0.05ug.ml-1	0.048± 0.0051	0.046 ± .0042	0.047 ± 0.071	0.047±0.001	2.12	0.047±0.0041	0.049±0.0071	0.048±0.0041	0.047±0.001	2.08
0.01 ug.ml^{-1}	0.0087±0.00074	0.0089±0.0009	0.0087± 0.00072	0087±0.0001	1.31	0.089±0.0009	0.0086±0.0005	0.0084±0.0006	0.0086±0.00025	2.91

Table 2 Pharmaceutical analysis of Ciprofloxacin Tablets

Tab (mg)	Mean	% age	S.D	% RSD
250	498	99.6	1	0.200
500	248	99.2	1	0.403226
750	748	99.7	3	0.40107

Table 3 Accuracy Reproducibility and Recovery of Ciprofloxacin from Serum and human aqueous humour

Parameter	Added amount	Water	Serum	Aqueous humour
Reproducibility	5 $ng.ml^{-1}$	4.95 ± 0.006	4.86 ± 0.002	4.874 ± 0.092
Precision	5 $ng.ml^{-1}$	4.89 ± 0.008	4.82 ± 0.006	4.84 ± 0.012
% Recovery	20 $ng.ml^{-1}$	97.3 ± 2.3	92.0 ± 4.1	93.5 ± 3.9
Injection Repeatability	20 $ng.ml^{-1}$	19.8 ± 0.23	19.6 ± 0.33	19.9 ± 0.13

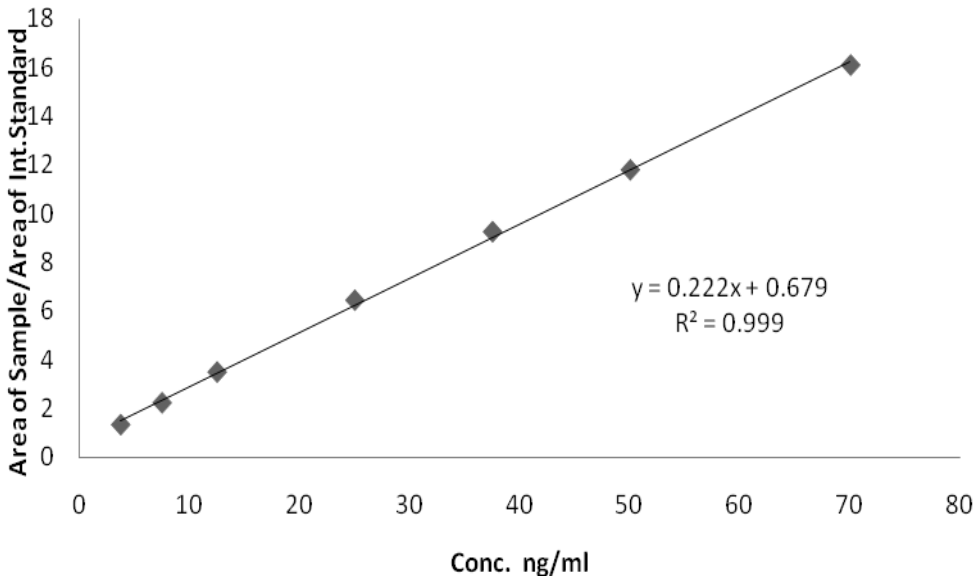

Figure 1 Calibration Curve of Ciprofloxacin in Mobile Phase

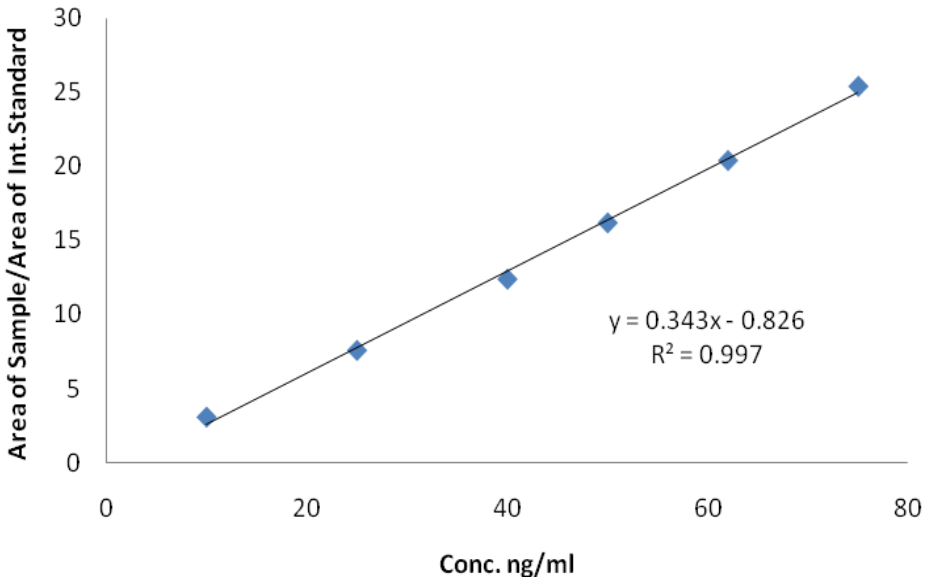

Figure 2 Calibration Curve of Ciprofloxacin in Serum

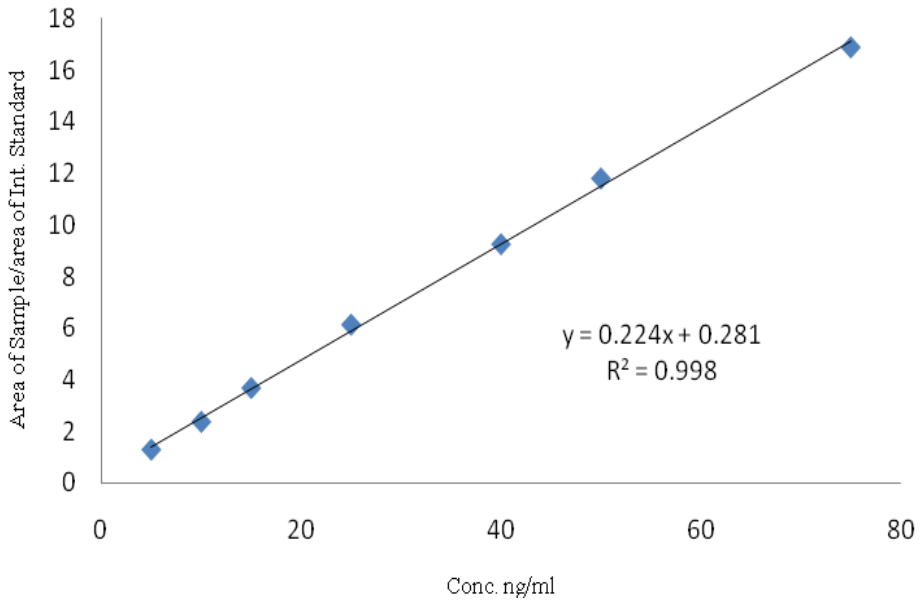

Figure 3 Calibration Curve of Ciprofloxacin in Aqueous humour