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Modification and validation of liquid chromatographic method for the quantification of ciprofloxacin in human plasma and its application to a bioavailability study

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Abstract: A new simple, accurate, precise and sensitive liquid chromatographic method for the analysis of Ciprofloxacin in human plasma, suitable for quantification of drug was developed and validated using HPLC-UV method. The analyte was chromatographically separated from endogenous plasma components on a C-18 reversed phase column (5µm, 25cm × 0.46cm) and detected at 278nm. The sample pretreatment was carried out with acetonitrile on 200 µl of plasma. The Lower limit of quantification (LLOQ) was 0.04 µg/ml with linearity in the range 0.04-4 µg/ml and coefficient of correlation value (R^2)>0.995. The method was successfully validated as per current FDA guidance for necessary parameters and applied to a pilot bioavailability study conducted on six healthy volunteers with marketed Ciprofloxacin 250mg immediate release tablets. The plasma concentrations were subjected to non-compartmental analysis for calculation of pharmacokinetic parameters like C_{max}, T_{max}, AUC $_{0-t}$, AUC $_{0-\infty}$ and $t_{½}$ etc. The mean values of C_{max} and T_{max} were found to be 1.35±0.09µg/ml and 1.25±0.27h respectively while for other pharmacokinetic parameters including AUC $_{0-t}$, AUC $_{0-\infty}$ were found to be 5.98±0.96 µg/ml×h and 6.34±1.07µg/ml×h. The drug exhibited half-life ($t_{½}$) of 3.94±0.33h. The obtained results proved the suitability of the method for routine pharmacokinetic studies of Ciprofloxacin.

Keywords: Ciprofloxacin, plasma, HPLC, bioavailability.

INTRODUCTION

Ciprofloxacin, a well-known fluoroquinolone antibiotic frequently prescribed to patients with suspected Gram-negative infections while it displays broad-spectrum efficacy against both Gram-positive and negative bacteria. Among fluoroquinolones, it is the drug of choice for urinary tract infections (Van Bambeke, Michot *et al*., 2005).

The bactericidal activity depends on the ratio of area under the concentration–time curve from zero to 24 h to minimum inhibitory concentration. This ratio remains the vital predictor of clinical cure (Lode, Borner *et al*., 1998). Like any other drug, quantification of Ciprofloxacin in plasma is crucial for establishing its pharmacokinetic parameters.

Therefore, various bio-analytical methods for the measurement of ciprofloxacin concentrations in biological fluids have been published. These include high-performance liquid chromatography (HPLC-UV) methods (Khan and Khan, 2008, Krol, Beck *et al*., 1995, Mack, 1992, Pellegrino, Segoloni *et al*., 2008, Sowinski and Kays, 2004, Wu, Chein *et al*., 2008, Zhai, Korrapati *et al*., 1995), fluorescence detection (Sowinski and Kays, 2004, Watabe, Yokoyama *et al*., 2010) and both UV and fluorescence detection(Grondin, Zhao *et al*., 2011). Lately, HPLC coupled with tandem mass spectrometry (LC-MS/MS) for quantitative analysis in human plasma has also been published (Bannefeld, Stass *et al*., 1997). Capillary electrophoresis has also been reported (Matta, Chockalingam *et al*., 2018). These techniques possess various limitations. LC-MS/MS offers exceptional sensitivity and selectivity with short run time than HPLC-UV technique, but requires very expensive instrument and skilled expertise. This restrains its use in resource-limited laboratories. Consequently, an inexpensive, sensitive and selective HPLC method remains a method of choice, as separation of complicated mixtures based on polarities, and acid-base properties, can be done.

On the other hand, some of the published HPLC methods report lengthy, complicated, and laborious sample pretreatment and pretreatment procedures including solid phase extraction (SPE) (Zotou and Miltiadou, 2002), and longer run times (≥10 min)(De Smet, Boussery *et al*., 2009, Idowu and Peggins, 2004, Imre, Dogaru *et al*., 2003, van Geijlswijk, van Zanten *et al*., 2006). Additionally, few reported methods require volumes ≥1000µL of plasma for drug analysis (Idowu and Peggins, 2004) rendering them inappropriate for repeat sampling in

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pediatrics, geriatrics, and anemic patients where minimum volume of blood sample should be taken.

The current study was aimed at development and validation of a simple, inexpensive, selective and sensitive HPLC method having ultraviolent detection for the quantitative analysis of ciprofloxacin using small plasma volumes (200µL). The current method was successfully used for the determination of pharmacokinetics of ciprofloxacin (250mg) tablet administered in healthy human volunteers.

MATERIALS AND METHODS

Chemicals

Ciprofloxacin HCl was received as gift sample from Pharmagen Private Limited, Pakistan. The solvents used for analysis were of HPLC grade and procured from Merck, Darmstadt Germany.

Instruments

The HPLC system comprised of isocratic pump (LC-20 AD, Shimadzu, Kyoto, Japan), UV-visible detector (SPD-20A, Shimadzu, Kyoto, Japan) auto-sampler (SIL-20 AC, Shimadzu, Kyoto, Japan), a column oven (CTO-20A), and a degasser (DGU-20A5R). (Shimadzu LC-20A). The column used was C-18 (Teknokroma, Mediterrania Sea 18, 5µm, 250 x 4.6 mm i.d.).

Preparation of mobile phase and standard solutions

The mobile phase preparation was carried out by first preparing phosphate buffer (0.02M Sodium di-hydrogen phosphate) adjusted to pH 2.7 with dilute ortho-phosphoric acid followed by filtration through a 0.45µm filter in a filtration assembly. Acetonitrile was added in the buffer with ratio (77:23) buffer:ACN, filtered and further degassed through sonication for 10 min. Stock solution of Ciprofloxacin was prepared with concentration of 1 mg/ml in acetonitrile and subsequently diluted to 4µg/ml in plasma. This working solution was used for preparation of calibration standards with nominal concentrations of 0.04, 0.1, 0.5, 1, 2, 2.5, 3.0, and 4.0µg/ml. Three different concentrations were assigned as quality control samples with nominal concentrations of 0.12, 1.5 and 3.5µg/ml as QCL, QCM and QCH respectively.

Pretreatment of plasma samples

Plasma sample pre-treatment consisted of adding 0.2ml of acetonitrile to 0.2ml of plasma and thoroughly mixed on vortex mixer for one minute. The sample was then centrifuged at 10,000 rpm for 10 min. The upper layer (0.3ml) was separated by a micropipette and filtered through 0.22µ nylon syringe filter prior to transfer in auto-sampler vials for injection.

Chromatographic conditions

The separation of Ciprofloxacin from endogenous plasma peaks was carried out on HPLC (LC-20A, Shimadzu, Kyoto, Japan) comprising of auto-sampler (SIL-20A), isocratic pump (LC-20AD), column oven (CTO-20A), UV-Visible detector (SPD-20A) and column (Mediterranea Sea 18, 5 µm, 25 × 0.46) fitted with guard column (Merck, Lichrosorb®, RP 18, 5 µm). Lab Solutions software was used for data acquisition and analysis (version 5.65, Shimadzu Corporation). The pump was set at 1 mL/min flow rate while column oven was maintained at 40°C. The absorbance was observed at 278nm. The injection volume was 50 µl.

Method validation

Bio-analytical method validation was carried out as per recent FDA guidance document. (FDA Guidance, accessed: 29.05.2018).

Selectivity

For the investigation of selectivity, 6 samples of blank plasma, each from different source was pretreated with the prescribed method and chromatographed for detection of any interfering peak at analyte's retention time.

Linearity

The analytical range of the method was established between 0.04-4µg/ml with eight calibration point standard curve including 0.04, 0.1, 0.5, 1, 2, 2.5, 3.0, and 4.0µg/ml nominal concentrations.

Stability

The stability studies were conducted by exposure of spiked plasma samples to different environmental and sample exposure conditions likely to be observed during a routine PK study including stock solution, freeze-thaw, auto-sampler and long-term stability. For stock solution stability, a nominal concentration of 2µg/ml prepared in mobile phase was used. Long term, freeze-thaw, auto-sampler stabilities were investigated on QC samples prepared in plasma. Stock solution and long-term stability was investigated for four weeks in order to cover the expected duration of study.

Analytical recovery

Analytical recovery of Ciprofloxacin from plasma matrix was obtained using three replicates of quality control samples prepared in plasma as well as mobile phase and the peak areas thus obtained were compared and the exercise was performed on three consecutive days.

Accuracy and precision

The accuracy and precision (inter and intra-day) were determined by five replicates of each Ciprofloxacin plasma calibration standard sample in the concentration range 0.04-4µg/ml along with quality control samples for three consecutive days. Calibration curves were constructed each day and concentrations of calibrators were obtained from standard curve whereas mean, SD and % CV were determined for all concentrations. The statistical analysis for mean, SD and % CV was carried out through MS Excel software version 2010.

Sensitivity and limit of detection

Determination of Lower Limit of quantification (LLOQ) and detection (LOD) was carried out on five different calibration standards in plasma i.e. 0.01, 0.02, 0.03, 0.04, and 0.05 µg/ml.

Application of validated method to bioavailability study

The developed bio-analytical method was applied to a bioavailability study of Ciprofloxacin, conducted on six healthy male, adult human subjects after acquiring ethical approval from Ethical review board of the University of Karachi (Approval no. IBCPH-10). Each subject was counseled regarding the study followed by acquisition of written informed consent prior to administration of Ciprofloxacin marketed product (Ciproxin® 250mg). The drug administration was carried out at fasted state and the blood samples were collected at 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12 and 16 h in vacutainers through IV cannula. Plasma was obtained by centrifugation of blood sample at 5000 rpm for 5 minutes in refrigerated centrifuge. The samples were analyzed as per the proposed method followed by determination of PK parameters through PK Solver (Excel add-in).

RESULT

The bio-analytical method was validated as per recent FDA guidance for bio-analytical method validation. (FDAGuidelines, accessed: 27.05.2018). During the experiment for establishing selectivity of the method, it was found devoid of any interfering peak at 8.5 min which is the retention time of analyte as shown in fig 1 (a) and the endogenous plasma peaks were well resolved as depicted in fig 1 (b). The linearity of the analytical method was observed from 0.04-4.0µg/ml with R^2 value 0.9975 as depicted in figure 2. The accuracy denoted with reference to the (%) of the nominal concentration was found between 97.38-104.48% (table 1) with precision in terms of %CV was within 2.15-3.93%. The lowest concentration with acceptable accuracy i.e. limit of quantification (LLOQ) was found to be 0.04 µg/ml and the lowest detected concentration (LOD) was 0.01 µg/ml as shown in fig 1 (c) and fig 1 (d). Analytical recovery for the quality control samples tested for three consecutive days was found consistent and reproducible with 98.82, 96.53 and 98.06% for Low, medium and high QC samples respectively (table 2). The analyte was found stable in stock solution, freeze/thaw, long-term and auto-sampler stability studies, the results of which have been depicted in table 3. The plasma samples were analyzed through the reported method while the average plasma concentration-time profile has been shown in fig 3. The plasma concentrations thus obtained were analyzed for PK parameters including C_{max}, T_{max}, AUC $_{o-t}$, AUC $_{0-\infty,}$ AUMC, MRT and kel the values of which have been presented in table 4

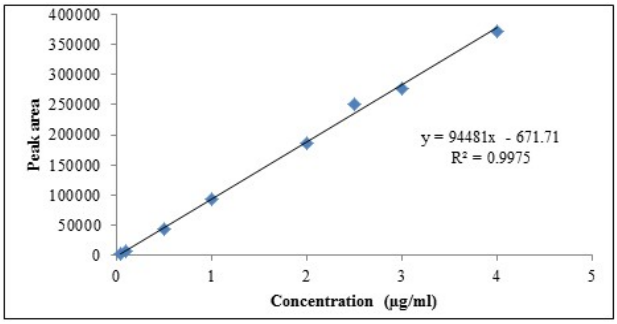

Fig. 2: Calibration curve showing linearity of bio-analytical method

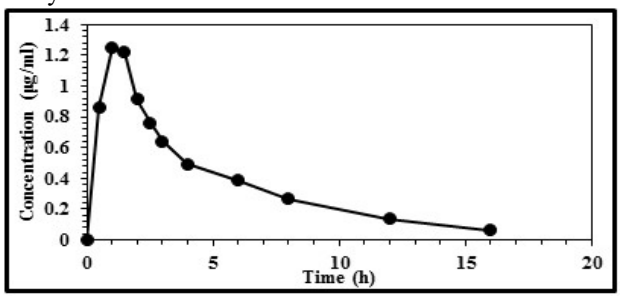

Fig. 3: Mean concentrations vs. time plot after administration of Ciprofloxacin tablet 250mg

DISCUSSION

For the quantitation of Ciprofloxacin in human plasma, a sensitive and specific HPLC-UV method was developed and validated as per FDA guidance for selectivity, sensitivity, precision & accuracy, analytical recovery and stability. The aim was to establish a suitable analytical method that can be applied to low volumes of plasma sample which is an essential requirement in case of pediatric and geriatric patients. The currently reported method employed a reversed phase column Mediterrania Sea 18, Teknokroma (250 mm × 4.6 mm, 5µm). The reported method by Janis *et al*; involved use of mobile phase with 0.02M phosphate buffer (pH 2.7) and ACN in the ratio 77:23 for the separation of analyte peak from plasma on an equivalent C-18 reverse phase ACE® 5 column (250mm × 4.6 mm, 5µm). The sample preparation method in the current method was different with reference to protein precipitation and direct injection after filtration of the supernatant, unlike Janis *et al* who dried the collected supernatant after protein precipitation. The latter technique significantly prolongs the sample preparation time because an aqueous ACN mixture takes much longer to evaporate than pure organic solvent. Secondly, the LLOQ they reported was 0.05µg/mL which was higher than the currently developed method i.e. 0.04µg/mL. Another method reported by Khan *et al*. employed a complex mobile phase using N,N- Dimethyl formamide, Sodium Dihydrogen Phosphate Dihydrate (15:6:79) and pH 3.0 adjusted with phosphoric Acid (85%).

Table 1: Accuracy and precision of quality control and calibration standards in plasma

Spiked concentration (µg/mL)	Intra-day Assay (n = 5)			Inter-day Assay (n = 15)		
	Mean ± SD	Precision (% CV)	Accuracy (%)	Mean ± SD	Precision (% CV)	Accuracy (%)
0.04	0.041 ± 0.02	3.61	99.49	0.041 ± 0.001	2.75	100.98
0.1	0.101 ± 0.01	2.73	100.25	0.099 ± 0.002	2.20	98.59
0.5	0.52 ± 0.04	2.32	99.43	0.499 ± 0.004	3.05	99.78
1	1.02 ± 0.08	3.05	101.60	1.008 ± 0.007	2.63	100.83
2	1.97±0.05	2.41	98.42	2.011 ± 0.038	2.37	100.55
2.5	2.61±0.06	2.45	104.48	2.550 ±0.066	2.18	102.02
3	2.92 ±0.08	2.62	97.38	2.995 ± 0.064	2.93	99.84
4	4.01±0.14	3.49	100.26	3.976 ± 0.040	3.02	99.41
3.5 (QCH)	3.55±0.097	2.74	101.52	3.56 ± 0.06	2.37	101.64
1.5 (QCM)	1.51 ± 0.055	3.66	100.52	1.50 ± 0.02	3.93	100.29
0.12 (QCL)	0.121 ± 0.003	2.15	100.44	0.121 ± 0.003	2.49	100.52

Table 2: Analytical recovery data for Ciprofloxacin in plasma

QC Level	Day 1 (%)	Day 2 (%)	Day 3 (%)	Mean (%)
QCL	98.50	99.12	98.84	98.82
QCM	96.43	97.45	95.72	96.53
QCH	96.30	99.08	98.81	98.06

Table 3: Stability data for Ciprofloxacin in quality control samples prepared in plasma

Stability studies of Ciprofloxacin in plasma			
Nominal concentration (µg/mL) Ciprofloxacin	QCL	QCM	QCH
	0.12	1.5	3.5
Auto-sampler stability (8 h)			
Mean ± SD	0.1204±0.0008	1.524±0.027	3.580±0.063
Mean % recovery	101.63	100.49	103.82
Bench-top stability (8 h)			
Mean ± SD	0.116±0.0007	1.473±0.09	3.429±0.057
Mean % recovery	96.67	98.20	97.97
Freeze-thaw stability (72 h)			
Mean ± SD	0.119±0.001	1.534±0.063	3.445±0.103
Mean % recovery	99.68	102.49	98.06
Long term stability (after four weeks)			
Mean ± SD	0.122±0.003	1.491±0.084	3.411±0.083
Mean % recovery	101.89	100.39	95.80
Stock solution stability	Week 1 98.19	Week 2 101.42	Week 4 102.45

Table 4: Pharmacokinetic parameters of Ciprofloxacin calculated from concentration vs. time data

Pharmacokinetic Parameter	V1	V2	V3	V4	V5	V6	Mean± SD
$t_{½}$ (h)	4.52	3.69	3.60	4.08	3.86	3.90	3.94±0.33
T_{max} *(h)*	1.50	1.00	1.50	1.00	1.50	1.00	1.25±0.27
C_{max} (µg/ml)	1.23	1.35	1.29	1.37	1.35	1.49	1.35±0.09
AUC 0-t (µg/ml×h)	6.35	6.10	6.53	7.18	5.14	4.58	5.98±0.96
AUC 0-∞ (µg/ml×h)	6.89	6.38	6.78	7.76	5.42	4.81	6.34±1.07
AUMC (µg/ml×h^2)	42.95	34.32	37.55	48.79	26.97	22.46	35.51±9.80
MRT (h)	6.23	5.38	5.54	6.29	4.97	4.67	5.51±0.65

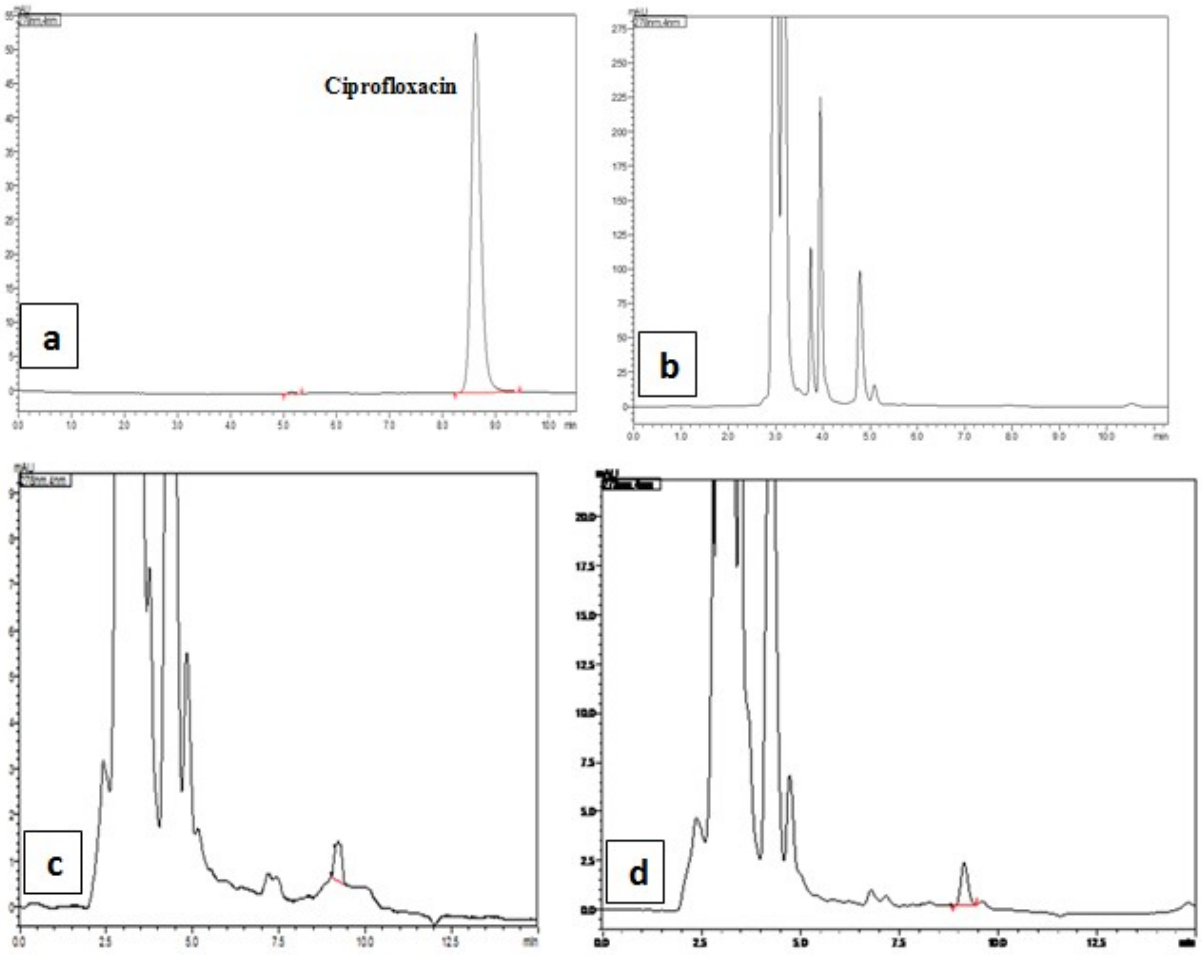

Fig. 1: Representative chromatograms showing Ciprofloxacin in mobile phase (a), Blank plasma (b), Lower limit of quantification (c) and Limit of detection (d).

Secondly the plasma standard samples were prepared by adding working solution prepared in organic solvent rather than plasma which is not recommended since it lacks similarity to natural plasma samples. Though HPLC with fluorescence detection (Watabe, Yokoyama *et al*., 2010) and LC-MS/MS (Bannefeld, Stass *et al*., 1997) have also been used but these techniques are much more costly than the conventional UV based methods. The current method satisfactorily quantified the analyte in plasma with reference to PK profiling of the drug. Upon investigation of the pharmacokinetic parameters calculated by non-compartmental analysis of concentration versus time data, the values for Cmax, Tmax, AUC 0-t and AUC 0-∞ were 1.35±0.33h, 1.25±0.27h, 5.98±0.96 (µg/ml×h) and 6.34±1.07 (µg/ml×h) respectively. Abul Kalam *et al*. conducted a bioequivalence study of Ciprofloxacin on 24 healthy male volunteers and reported C*max*1.49± 0.085µg/ml, T*max* 1.2±0.27 h, AUC_{0-t} 5.82±0.38 and 5.79±0.67mg/L×h for reference and test products respectively while the $AUC_{0-\infty}$ values for test and reference products were 6.86±0.93 and 6.92±0.92 mg/L×h (Kalam Azad, Ullah *et al*., 2007).

CONCLUSION

On the basis of the obtained results it can be concluded that a simple and sensitive bio-analytical method for determination of Ciprofloxacin in human plasma has been developed which is suitable for application in PK studies conducted on special population. On the other hand it can be used in the conventional bioequivalence studies.

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