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Development and Validation of an LC-MS/MS-ESI Method for Comparative Pharmacokinetic Study of Ciprofloxacin in Healthy Male Subjects

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ABSTRACT

A sensitive, specific and reproducible liquid chromatography coupled to tandem mass spectrometric method was developed and validated for the estimation of ciprofloxacin, an extensively used second-generation quinolone antibiotics, in human plasma. A liquid-liquid extraction of ciprofloxacin and the internal standard, ofloxacin, has been approached from the biological matrix using chloroform. Chromatographic separation was achieved in positive ion modes, isocratically on a 3.5 µm C18 analytical column (75 mm × 4.6 mm, i.d.) with 0.2 % formic acid solution in water: methanol (10:90, v/v) as mobile phase, at a flow rate of 0.5 mL.min^{-1}. The MS/MS ion transitions were monitored as 332.0→231.3 for ciprofloxacin and 362.2→261.0 for IS. The method showed good linearity in the range of 0.01–5.00 µg.mL^{-1} (r^2 > 0.99) with a good precision (3.37–12.60 %) and accuracy (87.25–114 %). At the same time, ciprofloxacin was found to be stable during stability studies viz. bench-top, auto-sampler, freeze-thaw cycle and long-term. The developed and validated method was successfully applied to measure plasma ciprofloxacin concentrations in a single dose bioequivalence study.

Introduction

Fluoroquinolones are one of the most promising and intensively studied drugs of day-to-day anti-infective therapy. Ciprofloxacin is the most widely used second-generation quinolone among them [1, 2]. It exhibits broad spectrum antimicrobial activity against both, gram-positive and gram-negative bacteria [3, 4] and it can be delivered through all the possible routes of drug administration routes, such as parental, topical and oral [5]. Ciprofloxacin mainly used for the treatment of respiratory tract infection, urinary tract infection, endocarditis, gastroenteritis, infection of bones and joints, prostatitis, anthrax, chancroid etc. [6].

Ciprofloxacin has been approved by the USFDA as an antimicrobial agent in the year 1987. Literature survey revealed that a number of methods are available for quantitative estimation of ciprofloxacin in plasma by using techniques like spectrophotometer [7, 8], high performance liquid chromatography (HPLC) [6, 9–22], capillary zone electrophoresis [23], microliquid chromatography mass spectrometry [24].

However, most of these published techniques have some limitations in their application. Previous researchers used protein precipitation technique in order to extract ciprofloxacin with a number of cleanup procedures [6, 13, 14, 17, 18, 25]. Major drawback of this cleanup procedure is its processing time and loss of analyte

sensitivity. Ciprofloxacin has also been estimated by using fluorescence technique using HPLC [10, 12] where the analyte was extracted by using protein precipitation. Though protein precipitation is a simple method but it can affect the life of the column which is great concern to the analyst. Sometimes protein precipitation also can be resulted in matrix effect.

Although, there is only one bioanalytical method using modern sensitive instrument like LC-MS/MS is available [26], we tried to make the method more sensitive and rapid compared to the published literature. In the proposed method by Kim et al. [26], the lower limit of quantification (LLOQ) was established as 0.05 µg.mL^{-1} with the retention time of ciprofloxacin at 1.79 min. Therefore, in this innovative bioanalytical technique we developed and validated more sensitive (LLOQ 0.01 µg.mL^{-1}) and faster elution of ciprofloxacin and the IS using ESI LC-MS-MS instrument to determine the pharmacokinetic parameters of the drug in human plasma. Electrospray Ionisation Source (ESI) is a robust ion source capable of interfacing to LC and demonstrated its application to a number of important classes of biological molecules [27]. The MS-MS combination provides sensitivity to detect more specific parent-daughter ion pair. Further, due to the amphoteric and polar nature of ciprofloxacin, extraction of the analyte has become difficult using the common and economic liquid-liquid extraction and/or solid-phase extraction procedures [12]. Consequently, in this present methodology we finally developed and validated a more sensitive LC-MS/MS technique for a quick and high-throughput screening of ciprofloxacin. Therefore, increased sensitivity could further be advantageous in determination of lower concentrations of ciprofloxacin, which will additionally support to obtain clearer pharmacokinetic profile during quantitative estimation of the drug in plasma, as evidenced by our findings on outcome of the bioequivalence study performed on 24 healthy adult male human volunteers.

Material and Methods

Materials

Working standard of ciprofloxacin (> 99 %) was obtained from Incepta Pharmaceuticals Ltd., Bangladesh and the internal standard, ofloxacin (> 99 %), was obtained from Rolax Pharma, India. Ofloxacin, an antimicrobial agent of the same group, is used as an internal standard (IS) in this experiment because it played an important role to minimize human error without interfering chemically with ciprofloxacin.

Formic acid was purchased from Sisco Research Laboratories Pvt. Ltd (Mumbai, India) and methanol, chloroform (HPLC grade) were purchased from Merck Pvt. Ltd (Mumbai, India). HPLC grade water (resistivity of 18.2 MW cm) generated from Milli Q water purification system (Elix, Milli Q A10 Academic, Molsheim, France) was used throughout the analysis. The blank human plasma with potassium EDTA anticoagulant was collected from Clinical Pharmacological Unit (CPU) of Bioequivalence Study Centre, Jadavpur University, Kolkata, India. All other reagents used were of analytical grade (Merck Pvt. Ltd, Mumbai, India).

Apparatus and chromatographic conditions

The liquid chromatographic separation was performed using a Shimadzu scientific instrument (Shimadzu Corporation, Kyoto, Japan) comprising of LC-20AD pump, an on-line DGU-20A3 prominence solvent degasser, SIL-20AC auto-sampler, CTO-10ASVP column oven (Shimadzu, Kyoto, Japan) and controller (CBM20A Lite).

Different types of columns, such as C18, C8, cyano and different buffers [10 mM ammonium acetate (pH-5.0), 10 mM ammonium formate (pH-5.0) and 0.1–0.2 % of formic acid (without pH adjustment)] with different organic solvent (acetonitrile, methanol) at altered flow rates (0.30–0.50 mL.min^{-1}) were tested to achieve optimized chromatographic conditions (data were not relevant to the manuscript, as it does not generate any discussion).

Chromatographic separation was achieved by injecting 5 µL aliquots of the processed samples through C18 column (75 mm, 4.6 mm, 3.5 µm particles; Agilent, USA), maintained at room temperature with an isocratic mobile phase (methanol: 0.2 % formic acid, 90:10) delivering at flow rate of 0.5 mL.min^{-1} for a total run time of 3.0 min. The auto-sampler temperature was maintained at 10 ± 1 °C. Quantization of analyte and IS was achieved in multiple reaction-monitoring (MRM) mode by monitoring the ion transitions from m/z 332.0→231.3 (ciprofloxacin) and m/z 362.2→261.0 (IS) using a triple quadrupole mass spectrometer (API 2000) of AB Sciex Instruments (Toronto, Canada), equipped with an electrospray ionization source operating in positive polarity and with a Turboionspray™ interface at 400 °C. Unit-unit resolution was adopted in due course of the bioanalysis.

Mass spectrometric conditions were maintained as ions spray (IS) voltage 5 500 V, source temperature 400 °C, nebulizer gas 50 psi, auxiliary gas 55 psi and curtain gas 20 psi on an arbitrary scale. Compound dependent parameters are depicted in the ▶ **Table 1**. The instrument was controlled and the data integration was performed with analyst 1.5 software version (AB Sciex Instruments, Foster, C A, USA).

Preparation of stock solution

Two primary stock solutions of ciprofloxacin were separately prepared by dissolving the accurately weighed compounds in methanol to get a final concentration of 1.0 mg.mL^{-1} for calibration curve and quality control samples, respectively. They were successively diluted with methanol to prepare working solutions (ranging from 0.10–50.00 µg.mL^{-1}) and the calibration standards (ranging from 0.01–5.00 µg.mL^{-1}). Quality control (QC) samples at 3 concentrations (0.03, 1.50 and 4.00 µg.mL^{-1}) were prepared from the working stock solutions of 0.30, 15.0, 40.0 µg.mL^{-1}. The IS stock solution (1.00 mg.mL^{-1}) and a working IS solution (5.00 µg.mL^{-1}) were also prepared in methanol. All stock solutions and working standard solutions were stored in polypropylene vials at − 20 °C in a freezer.

▶ **Table 1** Compound dependent parameters for analysis of ciprofloxacin and IS (ofloxacin) using LC-MS/MS.

	For ciprofloxacin	For IS
Parent ion	332.0	362.2
Product ion	231.3	261.0
Declustering potential (DP)	25 V	22 V
Collision energy (CE)	52 eV	36 eV
Entrance potential (EP)	10 V	8.0 V
Collision cell exit potential (CXP)	6 V	5.0 V

Preparation of calibration and quality control samples

Seven-point calibration curve was prepared by spiking 30 µL of working solution into 270 µL blank human plasma in polypropylene tubes to obtain final concentrations of 0.01, 0.05, 0.10, 0.50, 1.00, 2.00 and 5.00 µg.mL^{-1} for the analyte and 30 µL of IS stock (5.00 µg.mL^{-1}) was added to each tube. Extraction recovery, accuracy and precision were assessed at lower limit of quantification (LLOQ – 0.01 µg.mL^{-1}), low quality control (LQC – 0.03 µg.mL^{-1}), medium quality control (MQC – 1.50 µg.mL^{-1}) and high quality control (HQC – 4.00 µg.mL^{-1}) concentrations levels. The stability studies were performed with LQC and HQC samples. The different quality control samples were prepared by spiking 30 µL of working solution into 270 µL blank human plasma into different tubes and depending on the nature of the experiment, the tubes were stored at different conditions until analysis [28].

Sample preparation

Plasma samples stored at − 20 ± 2 °C were thawed at room temperature on the day of extraction followed by mixing to ensure its homogeneity. Samples were prepared by taking 300.0 µL of human plasma with the addition of IS solution (30 µL of 5.00 µg.mL^{-1}) and then mixed for 1 min. Samples were extracted with 4.0 mL of chloroform by hand mixing for 12 min. After centrifugation for 10 min at 5 000 rpm, 3.2 mL supernatant was separated and dried at 40 °C under nitrogen atmosphere. Residue was reconstituted with 400 µL of the mobile phase and 5 µL of the reconstituted samples were injected into the LC-MS/MS system for quantification.

System suitability and carry over

System suitability test was described at the first American Association of Pharmaceutical Scientists (AAPS)/Food and Drug Administration (FDA) Bioanalytical Workshop held in 1990. The system suitability test was performed by 5 consecutive injections of system suitability sample (aqueous mixture of the 1.50 µg.mL^{-1} of analyte and 0.50 µg.mL^{-1} of IS) first in the run sequence [29]. Carry over from previous injections can affect the accuracy and precision of the following runs. Carry over was assessed by using our previously described method [29].

Method validation

Method validation was fully performed to meet the acceptance criteria of standard industrial guideline for bio-analytical method validation set by the Food and Drug Administration of the United States (2001) [30] for the assay of the analyte in human plasma. This bio-analytical procedure was validated for evaluating the method in terms of specificity, sensitivity, linearity, accuracy, precision, extraction recovery, matrix effect, and a battery of stability studies of the analyte like bench top, freeze thaw, long term stability studies etc.

Specificity and selectivity

The specificity of the method towards endogenous plasma matrix components, metabolites and concomitant medications was assessed by analyzing the blank sample from 6 different batches of pooled human plasma. The specificity of the method was performed to detect for any chromatographic interference at the retention time of the analyte and IS from endogenous plasma components. To accept the specificity test at least 4 out of 6 should have response less than 5 times of the LLOQ response.

Extraction recovery

The extraction recovery for analyte was determined by comparing the response of ciprofloxacin extracted from 6 replicates of LLOQ, LQC, MQC and HQC samples and the recovery of IS was determined at single concentration of 0.50 µg.mL^{-1}. Extracted samples were compared with the post extracted standards (blank matrix extracted and analyte spiked into the supernatant) for extraction recovery assess.

Matrix effect

Matrix effect can be defined as any change in the ionization process of an analyte due to a co-eluting compound, either plasma components, or metabolite or any co-administered drug which can either result in ion suppression or an enhancement. So, this may change the sensitivity, precision and accuracy of the analytical procedure [29]. Matrix effect was calculated by comparing the post extracted plasma QC samples (6 replicates) with the response of analytes from neat samples at equivalent concentrations [29, 31] whereas the matrix effect for IS was calculated at 0.50 µg.mL^{-1}.

Linearity

The 7-point calibration curve (0.01, 0.05, 0.10, 0.50, 1.00, 2.00 and 5.00 µg.mL^{-1}) was obtained by plotting the peak area ratio of the ciprofloxacin and IS against the nominal concentration of calibration standards in control human plasma. The results were fitted to linear regression analysis with the use of $1/x^2$ (x = concentration) weighing factor. The acceptance criterion for each back-calculated standard concentration was within ± 15 % deviation (SD) from the nominal value, except at LLOQ, which was set at ± 20 % [32]. To accept an analytical run, at least 75 % of the calibration standards had to meet the stated acceptance criteria.

Accuracy and precision

Intraday precision and accuracy were estimated by analyzing 6 replicates of LLOQ and quality control (LQC, MQC and HQC) samples during a single analytical run [33]. Whereas inter day precision and accuracy of the method (reproducibility or day-to-day variation) were evaluated by using 6 sets of LLOQ and quality control samples on 4 different runs. Precision was determined as % RSD (relative standard deviation), i. e., the ratios of standard deviation (SD) to the mean expressed as percentage. Accuracies expressed as a percentage of deviation from the respective nominal value. The criteria for acceptability of the accuracy up to 85–115 % from the nominal values and a precision of within ± 15 % RSD, except for the LLOQ, where it was found to be within 80–120 % for accuracy and less than 20 % of RSD.

Stability experiments

The working solutions and stock solutions of the analyte and IS were evaluated for their stability at room temperature for 8.0 h and freezer stability at 4–8 °C for one month when compared to the response with the freshly prepared stock. The stability of ciprofloxacin in plasma was determined by analyzing 6 replicates of LQC and

HQC samples to ensure the stability of the actual samples at different storage conditions. A battery of stability studies include stock solution stability, autosampler stability, bench top stability, freeze thaw stability and long term stability were performed during the method validation.

Autosampler stability: The autosampler stability of ciprofloxacin was determined by injecting replicate preparations of processed samples for up to 48 h (in the autosampler at 10 °C) after the initial injection.

Bench-top stability: It was assessed by analyzing the QC samples exposed at room temperature for 8 h, which safeguard long processing time.

Freeze-thaw stability: This stability study was conducted by analyzing the samples which were undergone 3 freeze-thaw cycles. The samples frozen for at least 12 h and their unassisted thawing at room temperature represent one cycle. The cycle was repeated for 3 times and then analyzed.

Long term stability: Samples at LQC and HQC level were stored at around − 20 °C for 30 days and analyzed to ensure the long term stability of ciprofloxacin in human plasma.

All stability studies were performed against freshly spiked calibration standards and compared with the freshly prepared LQC and HQC samples. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (i. e., 85–115 % of nominal value) and precision (i. e., ± 15 RSD %) as compared with freshly spiked samples [29, 34].

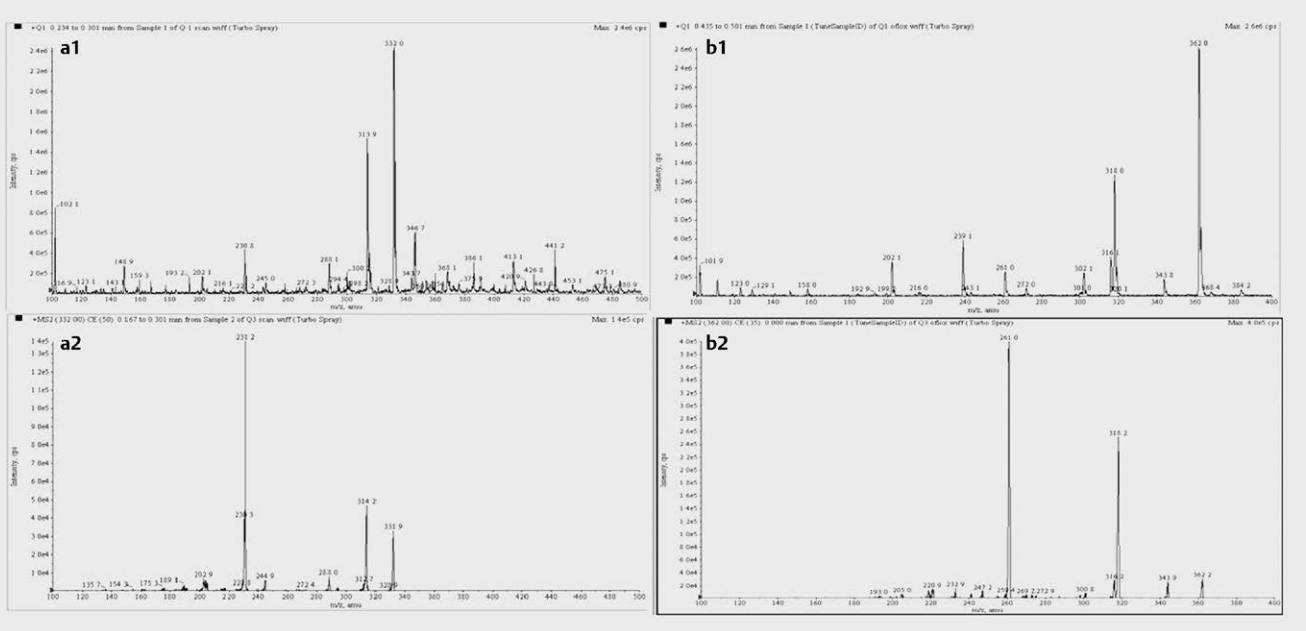
Dilution integrity

Dilution integrity was executed to make sure that the samples (which cross upper limit of quantification) could be diluted with blank matrix without affecting the final concentration. Plasma sample at higher concentration (20 µg.mL^{-1}) of ciprofloxacin was prepared and diluted with blank plasma to bring the concentration within the calibration range. Accordingly, 6 replicates of samples with 10 times dilution were analyzed with the calibration curve. To establish dilution integrity, diluted samples should comply with both the precision (i. e., ± 15 % RSD) and accuracy (i. e., ± 15 %).

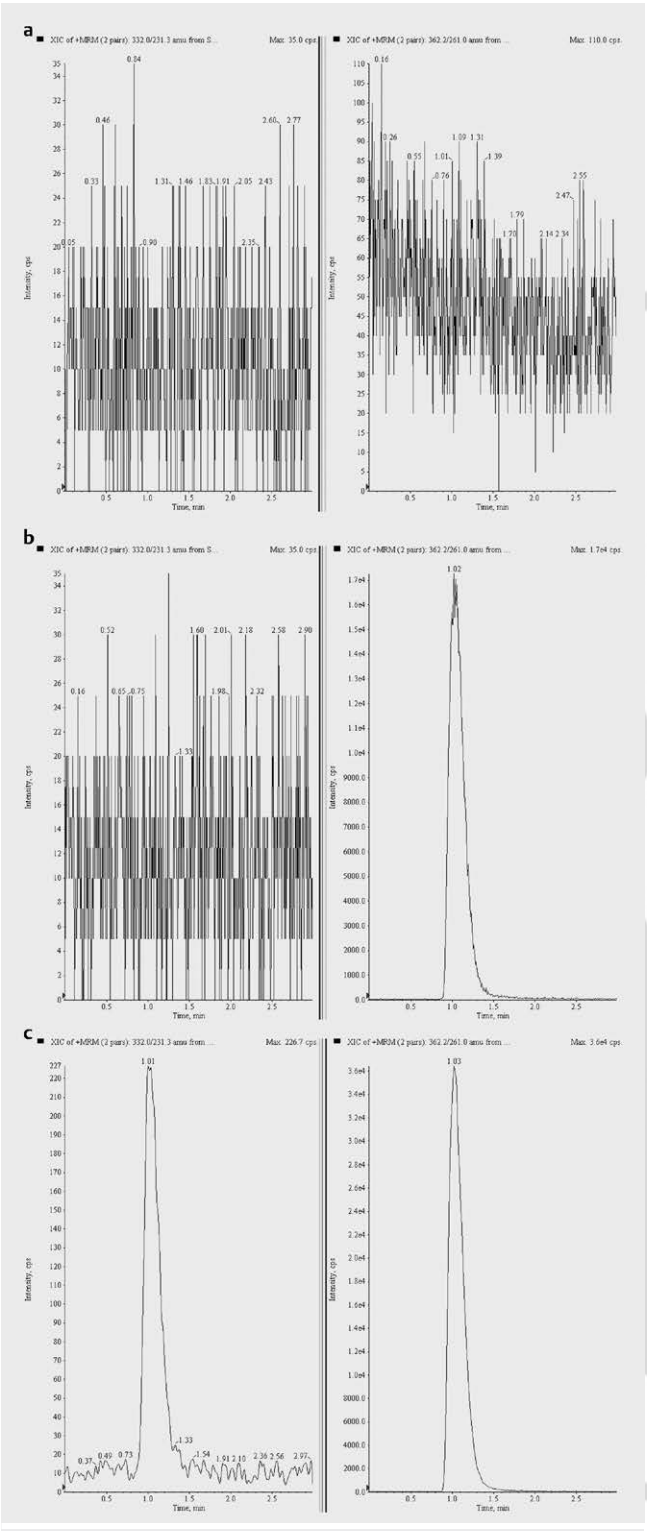
Application to bioequivalence study in healthy volunteers

The developed and validated method was applied to estimate the plasma concentration of the drug obtained from a randomized, 2-treatment, 2-period, open level, crossover bioequivalence study of ciprofloxacin tablet formulation (500 mg) manufactured by Incepta Pharmaceuticals Ltd., Bangladesh, in comparison with Ciflox tablet (containing ciprofloxacin 500 mg), manufactured by Bayer, Germany in 24 healthy male Indian human volunteers under fasting condition. This biostudy was performed according to the Declaration of Helsinki (2013) [35] and with a prior approval from the ethics committee of Jadavpur University, Kolkata, India. Before admission to the clinical study, each healthy volunteer signed a written informed consent document. The volunteers, included in the study, were housed in a closed facility and provided standard diet as per the diet chart in the study protocol. Following a 10 h fasting, they were dosed with the medication as per the randomization schedule. A total of 13 blood samples were collected at 0.00 h (predose) and 0.50, 1.00, 1.50, 2.00, 2.50, 3.00, 4.00, 6.00, 8.00, 10.00, 12.00 and 24.00 h post dose. Blood samples were collected in 5 mL vacutainers containing potassium EDTA at each time point through an indwelling catheter placed in one of the forearm vein. Collected blood samples were immediately centrifuged at 3 000 rpm for 10 min at 4 °C, plasma was separated and stored in polypropelene tubes with appropriate labeling at − 20 °C biofreezer [34, 36, 37].

Volunteer plasma samples were processed and analyzed by the bioanalytical method as described above. Pharmacokinetic parameters like area under the plasma-concentration-time curve from zero to the last measurable plasma sample time point and to infin-



▶ **Fig. 1** Parent ion scanning spectra of ciprofloxacin **a1** and IS **b1** Product ion scanning spectra of ciprofloxacin **a2** and IS **b2**.



▶ **Fig. 2** Typical MRM chromatograms of ciprofloxacin and IS in **a** blank human plasma; **b** blank human plasma spiked with IS; **c** blank human plasma spiked with ciprofloxacin at LLOQ (0.010 µg/mL) and IS.

ity (i. e., AUC_{0-t} and $AUC_{0-\infty}$), maximum plasma concentration (C_{max}), time to reach maximum plasma concentration (t_{max}), elimination rate constant (K_{el}) and elimination half-life ($t_{1/2}$) for the period of 0–24.0 h by non-compartmental method were evaluated by using WinNonlin™ Enterprise, Version: 5.3.

Results and Discussion

Optimization of liquid chromatographic and mass spectrometric conditions

Parent and product ion scan for ciprofloxacin were performed both in positive and negative ion mode and optimized mass spectrometric conditions with high sensitivity were achieved for the analyte and IS in positive ion mode where the m/z parent-product transition pair of ciprofloxacin was 332.0→231.3, respectively and m/z parent-product transition pair of IS was 362.2→261.0, respectively (▶ **Fig. 1**).

Compound dependent parameters DP, EP, FP and ion-source parameters such as ion spray voltage, curtain gas were optimized to polish the mass spectrometric method. CE, CXP, CAD gases were optimized to achieve intense product ion for analyte and IS. Detailed optimization of mass spectrometry conditions were provided in the methodology section. Following a developed MS/MS method, liquid chromatographic conditions were optimized in order to achieve a good chromatographic separation and improved peak shape by optimizing mobile phase composition and column.

First of all mobile phase with different buffers in combination with various organic solvent in varied composition were tried to achieve optimal chromatographic separation, appropriate retention time and good peak shape for analyte and IS to improve the sensitivity and throughput of the analysis. As per the procedure described earlier, improved peak shape with high throughput analysis and higher sensitivity were achieved by delivering 0.50 mL. min^{-1} of mobile phase consisting of 0.2 % formic acid and methanol (premixed) in ratio of 10:90 through C18 column of 75 mm length. The total chromatographic run time was 3 min with the retention time of 1.07 min for ciprofloxacin and 1.05 min for IS. Following optimization of LC-MS/MS parameters, m/z 332.0→231.3 for ciprofloxacin and m/z 362.2→261.0 for IS, the developed method was validated for quantification of analyte in bioequivalence study samples.

The retention time of the drug and IS in this current study was very less than that of the established methodology by Kim et al. [26], where it was found to be 1.79 and 1.51 min, respectively for ciprofloxacin and IS. Although, the run time of both the studies are seem to be same at this point, our methodology could easily be modified with lesser run time which can be helpful to obtain a rapid, economic, and high-throughput screening for ciprofloxacin.

Method validation

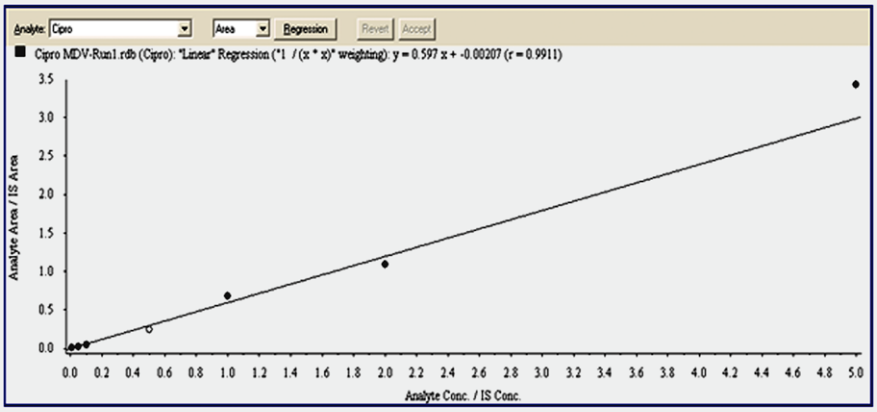
Specificity

No significant interfering peaks from 6 different lots of pulled human blank plasma were observed at the retention times of analyte and IS. A typical MRM chromatogram for the blank human plasma (free of analyte and IS), human plasma matrix spiked only with IS and plasma matrix spiked with analyte at LLOQ concentration and IS have been represented in ▶ **Fig. 2**.

▶ **Table 2** Extraction recovery of ciprofloxacin in human plasma[a] (n = 4).

Analyte	Spiked Concentration (µg/mL)				
	LLOQ (0.010)	LQC (0.030)	MQC (1.50)	HQC (4.00)	Mean
	Recovery (%)	Recovery (%)	Recovery (%)	Recovery (%)	Recovery (%)
Ciprofloxacin	89.12 ± 7.540	85.24 ± 5.704	87.98 ± 4.178	90.48 ± 6.103	88.21 ± 2.224
IS (at 0.5 µg/mL)					93.31 ± 3.380

[a] The data presented in this table as mean ± SD %



▶ **Fig. 3** Representative calibration curve for ciprofloxacin. (Color figure available online only).

▶ **Table 3** Linear regression data for the determination of ciprofloxacin added to human plasma [a] (n = 4).

Spiked Concentration (µg/mL)	Measured concentration (µg/mL)
0.01	0.01 ± 0.001
0.05	0.05 ± 0.006
0.10	0.10 ± 0.007
0.50	0.48 ± 0.038
1.00	1.05 ± 0.069
2.00	2.11 ± 0.114
5.00	5.13 ± 0.192
Equation	y = 0.597 × − 0.00207
r^2	0.991

[a] Mean peak area ratios of ciprofloxacin to IS presented by y. Correlation coefficient (r^2) is the linearity of the calibration curve used for this treatment

Extraction recovery and matrix effect

The mean recovery for LLOQ and 3 different QC samples was found to be 88.21 ± 2.224 %, whereas recovery of IS was reported to be 93.31 ± 3.380 %. The results obtained from the current experiment indicated that extraction of ciprofloxacin from human plasma by using liquid-liquid extraction with chloroform is adequate and reproducible throughout the level (▶ **Table 2**). Average matrix effect values were calculated as the % RSD of 6 replicates at LLOQ and 3 QC's level for analyte and 6 replicates at single concentration for IS. Findings were found to be less than 2 % for both the components, hence there is no enhance or suppression on ionization of analyte and IS.

Calibration curve

Calibration curve was constructed by plotting the peak area ratios (i. e., peak area of analyte/peak area of IS) vs. concentration (▶ **Fig. 3**). As the process was well developed, therefore, the plasma calibration curve containing ciprofloxacin was found to be linear over the concentration range of 0.01–5.00 µg.mL^{-1}. LLOQ was found to be 0.01 µg.mL^{-1}. Finally from the raw data of ▶ **Table 3**, it was revealed that the percentage accuracy obtained for the mean back-calculated concentrations of 4 calibration curves in the range of 87.25–114.00 % for ciprofloxacin, while the range of precision (%RSD) values were 3.37–12.60 %.

Based on the published literature on established LC-MS/MS methodology for the estimation of ciprofloxacin from human plasma, Kim et al. [26] reported 0.05 µg.mL^{-1} as the LLOQ for ciprofloxacin. Whereas, current method was found to be more sensitive as the LLOQ for the drug was established to be 0.01 µg.mL^{-1}. Therefore, from our research findings it can clearly be demonstrated to obtain clearer pharmacokinetic picture of ciprofloxacin in human plasma as traces of the drug can be estimated.

Accuracy and precision

A developed method with excellent interday and intraday precision and accuracy represents reproducibility and reliability of the method within the analytical range. From the results it was found that the intraday precision and accuracy for the analyte were ranging from 1.52–3.71 % and 89.78–95.96 %, respectively.

On the contrary, interday %RSD of ciprofloxacin was found to be in the range of 1.64–3.15 % while the accuracy was from 91.34 to 95.65 % (results are depicted in ▶ **Table 4**). The depicted findings satisfied the acceptable criteria of USFDA guideline for bioanalytical method; therefore, the developed method demonstrated the reproducibility of its results.

Stability

Results of the experimentation on different stability conditions for the accuracy and precision of LQC and HQC samples were within the acceptable limit of the recommended USFDA guideline. Ciprofloxacin was found to be stable for 3 freeze thaw cycles and also no detectable loss was observed in spiked plasma quality control samples stored at −20 ± 2 °C for duration of one month. Further, the experimental findings on autosampler stability (for duration of 48 h) and on bench top stability (for 8 h) were also found stable. From the overall findings of the stability study data of ciprofloxacin depicted the stability of the compound in human plasma (▶ **Table 5**). These findings are comparable to the reported stability results of ciprofloxacin in human plasma by various researchers [12, 16]. Further to mention that the working and stock solutions of the analyte and IS were found to be stable on storage at room temperature for a period of 8 h and for a period of 1 month at 4–8 °C.

Dilution integrity

Outcomes of the current experiment revealed that the accuracy and precision of the diluted samples were within the specified range (± 15 %). This certainly ensures that the concentrated plasma samples which are greater or equal to upper limit of quantification can be diluted and analyzed without interfering the final outcome of the concentration of actual samples.

▶ **Table 4** Accuracy and precision of the LC-MS/MS method for determination of ciprofloxacin in human plasma [a] (n = 4).

Nominal concentration (µg/mL)	Measured concentration (Mean ± SD) (µg/mL)	Precision RSD %	Accuracy (%)
Intraday			
0.01	0.01 ± 0.0003	3.10	89.78
0.03	0.03 ± 0.001	3.71	92.74
1.50	1.41 ± 0.026	1.84	93.88
4.00	3.84 ± 0.059	1.52	95.96
Interday			
0.01	0.01 ± 0.0003	3.15	91.34
0.03	0.03 ± 0.001	3.14	93.25
1.50	1.41 ± 0.034	2.39	93.63
4.00	3.83 ± 0.063	1.64	95.65

Accuracy (%) = [(measured concentration/spiked concentration) × 100]; CV (%), coefficient of variation = [(SD/mean) × 100]

Bioequivalence study

The bioequivalence study was successfully completed with 24 healthy adult human subjects in fasting state without any major deviation in the approved protocol. Developed bioanalytical methodology was successfully applied for the accurate determination of ciprofloxacin concentration in the plasma samples obtained from this bioequivalence study. Both the formulations were well tolerated by the participants and no untoward adverse event observed in the subjects during the entire duration of study. No clinical and abnormal laboratory parameters showed on completion of the study. No dropout or withdrawn was reported in the study. Mean pharmacokinetic parameters following administration of both, test and reference formulations, were presented in ▶ **Table 6**. The AUC_{0-t} value for ciprofloxacin test and reference formulations are 11.33 ± 3.338 µg.h.mL^{-1} and 12.51 ± 1.852 µg.h.mL^{-1}, respectively. Maximum plasma concentration (C_{max}) were found to be 2.98 ± 0.438 µg.mL^{-1} and 2.90 ± 0.390 µg.mL^{-1}at the time 1.75 ± 0.552 h and 1.96 ± 0.440 h for the test and reference formulations, respectively. The values of area under the plasma concentration-time curve from time zero to infinity ($AUC_{0-\infty}$) were found to be 11.54 ± 3.404 µg.h.mL^{-1} and 12.75 ± 1.893 µg.h.mL^{-1}, respectively for the test and reference formulations of ciprofloxacin. The mean ± SD plasma levels of ciprofloxacin after the oral administration of a single dose of test and reference formulations are shown in ▶ **Fig. 4**. Finally, the relative bioavailability of the formulation was found to be 90.57 % and therefore, it can be depicted from the acquired results that the test formulation of ciprofloxacin should be bioequivalent to the reference formulation.

Conclusion

In this article we have established development and validation of a simple, specific, sensitive and stable ESI-LC-MS/MS method for estimation of ciprofloxacin in human plasma. This advanced method was found to be highly precise and accurate which involves liquid-liquid extraction procedure of ciprofloxacin for the estimation of the analyte from plasma matrix. This approach will allow a simple and economical detection of ciprofloxacin in small volume of human plasma. This method has been successfully applied for the determination of ciprofloxacin in human plasma matrix of the volunteers in a bioequivalence study. This assay procedure would fur-

▶ **Table 5** Short-term and long-term stability data of ciprofloxacin in human plasma [a] (n = 6).

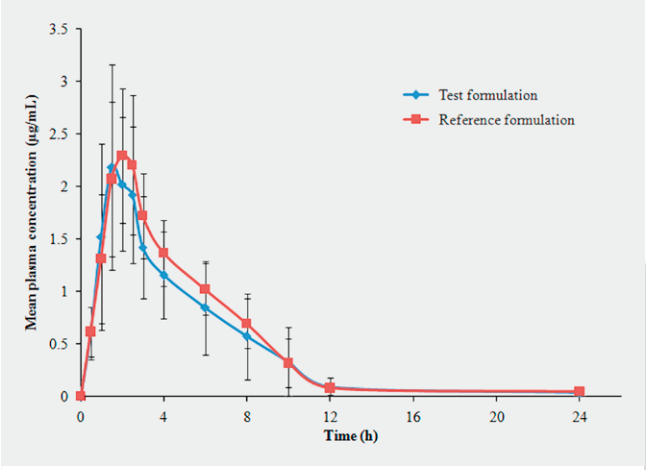
Storage condition	LQC (0.03 µg/mL)		HQC (4.00 µg/mL)	
	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)
Freeze-thaw cycle (3 cycle)	95.99	4.640	96.94	3.399
Autosampler (24.0 h)	93.70	4.163	95.37	3.624
8 h bench top	94.57	5.323	94.31	2.578
Long term (30 days)	93.03	4.468	104.44	3.491

[a] The data presented in this table is the percentage of measured value vs. theoretical value with CV % (n = 6)

▶ **Table 6** Pharmacokinetic parameters of ciprofloxacin in healthy human volunteer following single oral dose of ciprofloxacin 500 mg tablet of the test and reference formulations in healthy male human volunteers (n = 24, mean, mean ± SD).

Pharmacokinetic parameters	Ciprofloxacin	
	Reference (Mean ± SD)	Test (Mean ± SD)
AUC_{0-t} (μg.hr/mL)	12.51 ± 1.852	11.33 ± 3.338
$AUC_{0-\infty}$ (μg.hr/mL)	12.75 ± 1.893	11.54 ± 3.404
C_{max} (μg/mL)	2.90 ± 0.390	2.98 ± 0.438
T_{max} (h)	1.96 ± 0.440	1.75 ± 0.552
K_{el}	0.20 ± 0.020	0.20 ± 0.016
$t_{1/2}$ (h)	3.53 ± 0.381	3.49 ± 0.288
Relative bioavailability (%)	100	90.57

C_{max}, maximum plasma concentration; t_{max}, time require to achieve maximum concentration; AUC_{0-t}, area under the plasma concentration time curve from time zero to t h; $AUC_{0-\infty}$, plasma concentration–time curve from time zero to infinity; $t_{1/2}$ (h), elimination half life; K_{el}, elimination rate constant



▶ **Fig. 4** Graphical presentation of mean plasma concentration-time profile of ciprofloxacin after single oral dose administration of test and reference preparations in 24 healthy male human volunteers. (Color figure available online only).

ther be applicable in preclinical pharmacokinetic, regulatory toxicokinetic, clinical kinetic study and bioequivalence study.

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Conflict of Interest

The authors confirm that this article content has no conflict of interest.

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