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DEVELOPMENT AND VALIDATION OF A HPLC METHOD WITH UV DETECTOR FOR QUANTIFICATION OF GEMIFLOXACIN IN HUMAN PLASMA: APPLICATION TO BIOEQUIVALENCE STUDY

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ABSTRACT

A simple, sensitive, high throughput and specific HPLC method with UV detection was developed and validated to according to the FDA guideline for quantification of gemifloxacin in human plasma. The bioanalytical method consists of liquid-liquid extraction and quantification by UV detection. The analyte was separated isocratically on a Cyano column (250 X 4.6 mm, 5µ particle size) with a mobile phase consist of 1% v/v formic acid, acetonitrile and methanol in the ratio of 75:20:5 (v/v). The response to gemifloxacin was linear over range 0.025 to 2.000 µg/mL. The validation results demonstrate that the method had satisfactory precession and accuracy across the calibration range. Intra-and inter-day precession range from 0.676 to 1.222 and from 0.834 to 1.645% (RSD), respectively. The accuracy determine at three quality control samples was within 94.232 to 101.132%. There was no evidence of instability of the analyte in human plasma following the stability study. The method proved highly reproducible and sensitive and was successfully applied in bioequivalence study after oral dosing of gemifloxacin tablet in human volunteer. This method may be applicable for gemifloxacin single and multi dose pharmacokinetic study, tissue distribution study toxicokinetic study and tissue distribution modeling study in lower mammals.

INTRODUCTION: Gemifloxacin [(R, S) - 7- (3- aminomethyl- 4- syn- methoxyimino- 1- pyrrolidinyl) - 1 cyclopropyl- 6- fluoro -1, 4-dihydro- 4- oxo- 1, 8- naphthyridine - 3 - carboxylic acid methanesulfonate] (SB-265805; LB-20304a) (CAS number 175463-14-6), is a recently developed antibacterial compound with a broad spectrum of activity (Figure 1) [1-3].

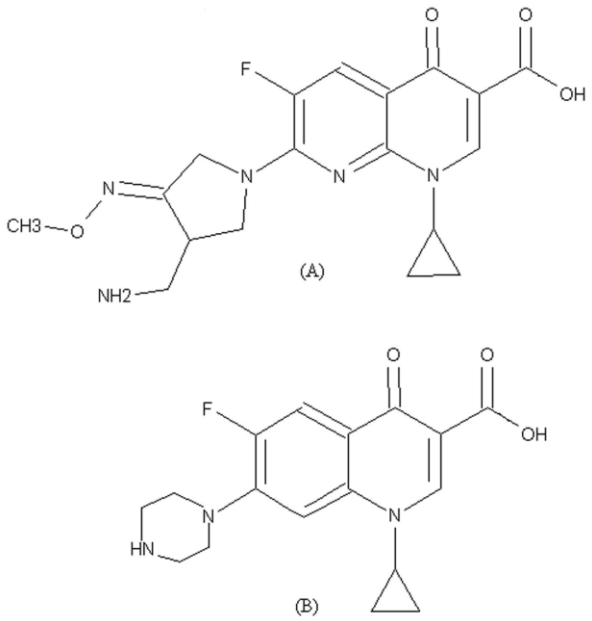

FIG. 1: STRUCTURAL REPRESENTATION OF; (A) GEMIFLOXACIN, (B) CIPROFLOXACIN (IS).

Gemifloxacin belongs to Flouroquinolone, which are used for management of various bacterial infections. It kills a variety of bacteria, and prevents their reproduction and is often used for the treatment of infections all over the body. It has shown potent antibacterial activity against clinical isolates and reference strains in both *in vitro* studies and experimental models of infection in animals [4-6]. It has particularly enhanced activity against gram-positive organisms, and displays fourfold higher activity than that of moxifloxacin against *Streptococcus pneumoniae* (MIC at which 90% of isolates are inhibited [MIC 90], 0.060 mg/ml) *in vitro* [5]. Gemifloxacin has also shown potent activity against other major pathogens involved in respiratory tract infections, including *Haemophilus influenzae* and *Moraxella catarrhalis* and the atypical organisms, *Legionella pneumophila*, *Chlamydia* spp., and *Mycoplasma* spp. . Furthermore, the compound has shown potent activity against many organisms that cause urinary tract infections. The adverse reaction profile is similar to that of older members of this class [8].

Quite methods have been published for the quantification of gemifloxacin in human plasma. A method was developed for the determination of gemifloxacin in human plasma using liquid chromatography-tandem mass spectrometry.[9] Another investigator has quantified concentrations of gemifloxacin in serum and urine by a reversed-phase liquid chromatography (LC) method with fluorescence detection [10]. HPLC with UV detection along with protein precipitation technique for gemifloxacin quantification in human plasma also has been reported [11]. Previous studies have shown that gemifloxacin displays a favorable plasma pharmacokinetic profile allowing a once-daily dosing regimen in human [10, 12].

Protein precipitation technique for sample preparation involves fewer steps, but during sample analysis column is blocked very often and provide high back pressure to the column and the sample filtration of protein precipitation is very difficult and expensive. Also in precipitation process, a guard column is required to protect and avoid damage to the column which is expensive. But liquid-liquid extraction process sample was very clean and no column damage was occurring as well as cost effective.

But no such method has been published for quantification or to observed pharmacokinetic profile of gemifloxacin by HPLC with UV detection in human plasma. More over many of those above mention methods has been developed in very

costly instrument like LC-MS/MS or HPLC with fluorescence detector, not available in many bioanalytical labs. From this ground it is clear that our method is very cost effective, simple, easier and may be applicable those bioanalytical labs witch have not LC-MS/MS or HPLC with fluorescence detector facility.

The main advantage of this method is the use of liquid-liquid extraction procedure from plasma. Which is easy and faster than the reported methods, which were utilized the extraction and fluorescence detection and protein precipitation method respectively [10, 11]. Our HPLC method was developed and validated with UV detection, which is less costly than the LC-MS/MS method [9]. So, this method may be used as a reliable and simpler assay method for the pharmacokinetics as well as bioavailability/bioequivalence study of gemifloxacin.

MATERIALS AND METHOD:

Materials and Reagents: Gemifloxacin mesylate (purity ≥ 99%) bulk drug was obtained from Acme formulation Pvt. Ltd., (Himachal Pradesh, India). Ciprofloxacin bulk drug (purity ≥99%) used as internal standard (IS), was supplied by Nobel Healthcare (Haryana, India). Formic acid (98%) (Analytical- reagent grade) and methanol (HPLC-grade) were purchased from Merck Pvt. Ltd. (Mumbai, India). HPLC grade water (resistivity of 18 M 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 EDTA-2K anticoagulant war collected from Clinical Pharmacological unit (CPU) of Bioequivalance Study Centre, Jadavpur University, Kolkata, India. All other reagents used were of analytical grade (Merck Pvt. Ltd. Mumbai, India).

Apparatus and Chromatographic Conditions: The HPLC apparatus (Knauer, Germany) adjusted with HPLC pump (Knauer 1000), Rheodyne injector (D-14163 Berlin), UV detector (Knauer 2500) and EZChrom (version 3.1.6) software. Reverse phase HPLC analysis was performed isocratically at room temperature using a Cyano, 250X4.6mm, 5µ particle size stainless steel column. A mixture of water containing 1% v/v formic acid, acetonitrile and methanol in the ratio of 75:20:5 (v/v) was used as mobile phase. The mobile phase was filtered through 0.45µm membrane filter. The eluent was monitored with a UV detector set at 292nm at a flow rate of 1mL/min and a sample size of 50µL was injected through the rheodyne injector.

Preparation of Stock and Standard Solution: The stock solutions of 1 mg/mL of the analyte (GMF) and IS were prepared in methanol, respectively. These solutions were further diluted suitably with the mobile phase to obtain a stock solution to prepare the working standard solution for calibration curve and quality control (QC) samples. An 8-point calibration curve was prepared by spiking appropriate amounts of working solution into the blank plasma to obtain final concentrations of 0.025, 0.050, 0.100, 0.250, 0.500, 1.000, 1.500 and 2.000µg/mL for the analyte.

The QC samples were also prepared in the same way to obtain the final concentrations of 0.075 (low QC), 1.250 (mid QC) and 1.800 (high QC) µg/mL. A working IS solution of 1.500µg/mL was prepared in mobile phase. All stock solutions and working standard solutions were stored in polypropylene vials in a -20°C freezer.

Preparation of Calibration and Quality Control Samples: Liquid- liquid extraction procedure was used for the extraction of the GMF from the plasma. For calibration standards and quality control samples, an aliquot quantity of 0.45ml

plasma sample was taken in a 10mL stopper test tube and 0.05mL internal standard was added and mixed. (50µL of 30µg/mL) equivalent to 1.500µg and vortex mixing for 30 sec. Then 100µL of 10mM tris buffer [Tris (hydroxymethyl) amino- methane] was added and vortex mixing for 30 sec.

To it, 4.5ml of mixed solvent (Chlorofoform-Isopropyl alcohol = 90:10, v/v) was added and mixed for 15 min followed by centrifugation at 5000 rpm for 20 min. The organic layer was separated, transferred to a separated test tube and evaporated to dryness under the stream of N_2 at 40°C. The residue was reconstituted in 200µl of mobile phase, filter through 0.22µm membrane filter and 50µl was injected into the HPLC system.

For calibration standards, an aliquot of 50µL of working stock solution of GMF was spiked into 500µL of blank plasma in polypropylene tube and 25µL of IS was added. Samples for the determination of recovery, precision and accuracy were prepared by spiking the blank plasma with the analytes at lower limit of quantitation (LLOQ), low quality control (LQC), mid quality control (MQC) and high quality control (HQC) concentrations into different tubes. The samples for the stability studies were also prepared in the same way at appropriate concentrations (LQC, MQC and HQC) into different tubes and, depending on the nature of the experiment; the samples were stored at -20°C until analysis.

Method Validation: The method was validated to meet the acceptance criteria of industrial guidance for the bioanalytical method validation [13].

Linearity and LLOQ: To establish linearity, a series of calibration standards were prepared by adding a known concentration GMF and IS to drug free human plasma and analysed. The lowest concentration on the standard curve with detector response five times greater than the drug free (blank) human plasma was considered as the LLOQ. The analyte peak in LLOQ sample should be identifiable, discrete and reproducible with a precision of 20% and an accuracy of 80 to120%.

Specificity and Selectivity: This test was performed by analyzing the blank plasma samples from six different sources to detect for any chromatographic interference at the retention times of the analyte and IS.

Precision and Accuracy: Three validation batches were processed on three separate days. Each batch included two set of blank (zero concentration) samples and two samples containing only IS, two set of calibrators and six replicates of each LLOQ, low-, medium-, and high-concentrations of QC samples. The results from QC samples in six runs were used to evaluate the accuracy and precision of the method. Accuracy was determined by the ratio of determined concentration and actual concentration multiplied by 100%, and precision was evaluated by the percentage relative standard deviations (RSD %). The criteria for acceptability of the data included accuracy within ±15% deviation (SD) from the nominal values and a precision of within ±15% relative standard deviation (RSD), except for LLOQ, where it should not exceed ±20% of SD.[13]

Extraction recovery: The extraction recovery of analyte was determined by measuring the peak areas of the drug from the prepared plasma quality control samples. 0.075, 1.250 and 1.800µg/mL were taken as LQC (low quality control), MQC (medium quality control) and HQC (high quality control) samples for drug and 400ng/mL for IS, respectively. The peak areas of extracted LQC, MQC and HQC were compared to the absolute peak area of the un-extracted samples containing the same concentration of the drug as 100%.

Stability study: The stability of the analytes after three freeze and thaw cycles was determined at low, middle, high QC samples. The samples were stored at -20°C for 24 h and thawed unassisted at room temperature. After completely thawing, the samples were refrozen for 12-24 h. After three freeze- thaw cycles, the concentration of the samples were analyzed.

For ambient and bench top stability study the QC samples were kept at room temperature for 8 h and 24 h respectively, then extracted and analyzed. Stability was concluded when the % RSD was within ±15% of the actual value.

Six aliquots of each low, middle and high QC samples were kept in deep freezer at -20±5°C for one month and three months. The samples were processed and analyzed and the concentrations obtained were compared with the actual value of QC samples to determine the short term stability and long term of analyte in human plasma. Samples were concluded stable if the %RSD of the stability samples was within ±15% of the actual value.

Pharmacokinetic and bioequivalence study: The above mentioned validated method was successfully used to investigate the concentration of gemifloxacin in human plasma for a bioequivalence study. The study was approved by the Bio-Ethical Committee of Jadavpur University, Kolkata, India. It was an open, randomized, crossover study to assay relative bioavailability of gemifloxacin in twelve healthy male volunteers with age between 21-28 years following single dose administration of gemifloxacin 320mg tablet.

Test preparation was gemifloxacin 320mg tablet manufactured by M/S Acme Formulation Pvt. Ltd., (Himachal Pradesh, India). Tablet Gembex (containing gemifloxacin 320mg) manufactured by M/S Ranbaxy Laboratories Ltd, (Gurgaon, India) was used as Reference preparation. Blood samples were collected predetermined time at 0 hr. (before drug administration), 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 4.0, 8.0, 12.0, 24.0 and 48.0 hrs (after drug administration) into the tubes containing EDTA-2K (Merck Pvt. Ltd., Mumbai, India) as an anti-coagulant. Plasma was separated by centrifuging the blood and stored frozen at -20°C until analysis. Plasma (500µL) samples were spiked with IS and processed.

Plasma concentration–time data of each analyte was analyzed by non-compartmental method using WinNonlin Version 5.1 (Pharsight Corporation, Mountain View, CA, USA) software. The pharmacokinetic parameters like area under the plasma-concentration-time curve from time zero to the last measurable Gemifloxacin sample time and to infinity (AUC_{0-t} and $AUC_{0-\infty}$), maximum concentration (C_{max}), time to maximum concentration (t_{max}), elimination rate constant (K_{el}) and elimination half-life ($t_{1/2}$) were determined for the period of 0 to 48 h.

RESULTS AND DISCUSSION:

Linearity and LLOQ: Initially, validation was performed to evaluate the calibration, accuracy and precision of GMF in human plasma. The calibration curve was constructed by plotting the peak area ratio (*f*) of GMF to internal standard versus the nominal concentration (C) of the analyte. The plasma calibration curves were linear (r^2>0.999) over the concentration range of 0.025-2.000µg/mL. The standard curve had a reliable reproducibility over the standard concentrations across the calibration range.

The linearity was determined by un-weighted linear regression analysis. The regression equation of the calibration curve was then used to calculate the concentration of GMF in human plasma. The back calculated values of the

concentrations were statistically evaluated for human plasma. The back calculated concentrations (mean±SD) from the representative calibration standards by HPLC determination for GMF and the corresponding regression equations are given in **Table 1**. The average regression (n = 6) for GMF in human plasma was found 0.9992. Additional evaluation of the lower limit of quantitation (LLOQ) confirmed that those concentrations fitted with the proposed criteria. It is clear that (from Table 1) our method exhibited good sensitivity.

TABLE 1: LINEAR REGRESSION DATA FOR THE DETERMINATION OF GEMIFLOXACIN ADDED IN HUMAN PLASMA (n= 6)*

Spiked Concentration (µg/mL)	Measured concentration (µg/mL) ±SD
0.025	0.024±0.0002
0.050	0.049±0.0001
0.100	0.099±0.0007
0.250	0.252±0.0019
0.500	0.502±0.0068
1.000	1.015±0.0076
1.500	1.512±0.0109
2.000	1.994±0.0421
Equation	f = 1.0045 C + 0.009
Correlation coefficient (r^2)	0.9992

* SD, standard deviation; n = number of sample; f = peak area ratio

Specificity: Representative chromatograms of blank plasma, blank plasma spiked with GMF and IS, and volunteer plasma sample after administration of an oral dose of 320 mg GMF are shown in **Figure 2**. The analyte separated well from IS under the described chromatographic conditions. Total run time of the chromatogram was 15 min and the retention time of drug and IS were about 5.5 and 11.5 min, respectively. No interfering peaks at these times were found in the chromatogram obtained from blank plasma.

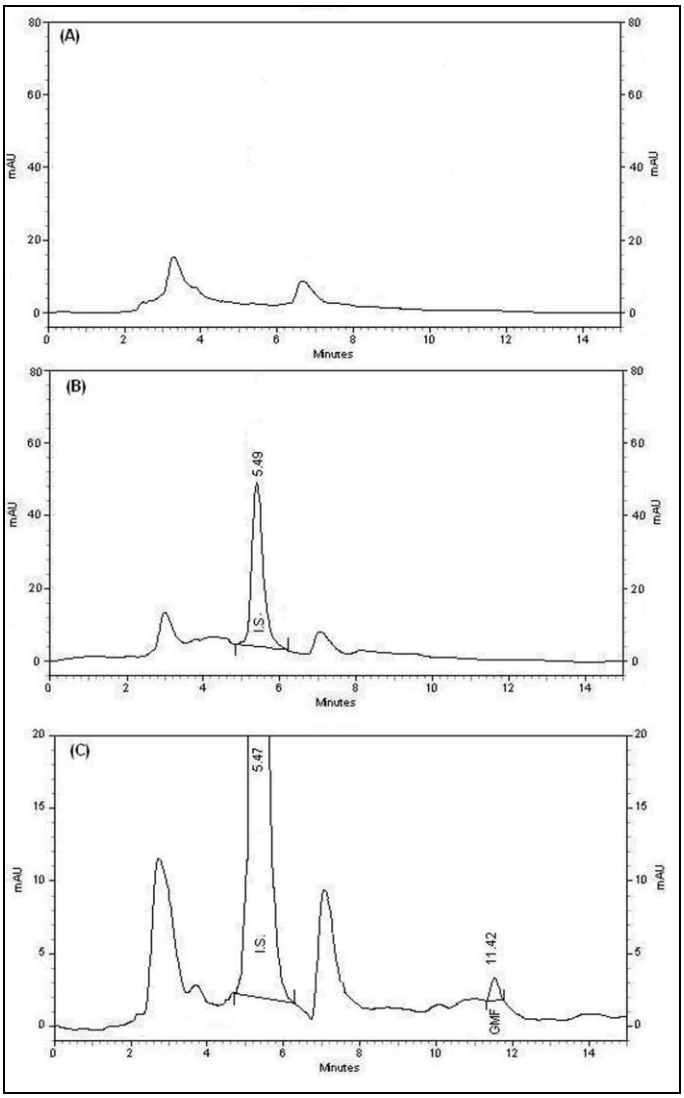

FIG. 2: REPRESENTATIVE CHROMATOGRAM OF;
(A) BLANK HUMAN PLASMA (FREE OF ANALYTE AND IS)
(B) HUMAN PLASMA SPIKED WITH ANALYTE IS (50µL OF 30.00µg/mL EQUIVALENT TO 1.50µg/mL) AND
(C) HUMAN PLASMA SPIKED WITH ANALYTE (GMF) AT LLOQ (0.025µG/ML) AND IS (50µL OF 30.00µg/ml EQUIVALENT TO 1.5µg/ml).

Accuracy and precision: The intra- and inter-day precision (%RSD.) values of GMF for various concentrations ranged from 0.676% to 1.194% and 0.834% to 1.645% respectively. At the same concentrations, the percentage of accuracy was in the range of 94.232%-101.133% and 95.064%-100.902% respectively. Both accuracy and precision were in the acceptable range for bioanalytical

purpose. The precision and accuracy data for both intra and inter day assays of three QC samples are presented in **Table 2**. The assay method demonstrated high degree of accuracy and precision.

TABLE 2: ACCURACY AND PRECISION OF DATA OF THE ANALYSIS HUMAN PLASMA (n= 6)*

	Spiked concentration (µg/mL)	Intra-day				Inter-day			
		Mean (µg/mL)	SD	Accuracy (%)	RSD (%)	Mean (µg/mL)	SD	Accuracy (%)	RSD (%)
LLOQ	0.025	0.024	0.0003	94.232	1.194	0.024	0.0004	95.064	1.645
LQC	0.075	0.075	0.0005	98.066	0.676	0.073	0.0007	97.702	1.014
MQC	1.250	1.264	0.0088	101.133	0.699	1.261	0.0105	100.902	0.834
HQC	1.800	1.788	0.0219	99.349	1.223	1.802	0.0247	100.090	1.369

*Accuracy (%), [(measured concentration/spiked concentration) x 100]; SD, Standard deviation; RSD (%), relative standard deviation [(S.D./mean) x 100]; LLOQ, limit of quantification; LQC, low quality control; MQC, middle quality control; HQC, high quality control ; n = number of sample

Extraction recovery: Recovery results presented that the maximum recovery was achieved at low-(0.075µg/ml), medium-(1.250µg/ml) and high-(1.800µg/ml) quality control samples was 94.521%-97.647%. The extraction recovery was found to be satisfactory as it was consistent, precise and reproducible. Thus single step liquid-liquid extraction procedure used in this method proved to be efficient and simple enough to extract GLF from human plasma.

Stability study: Each stability test included six replicates of three levels of QC samples. All stability results, as well as the linear regression correlation coefficients of calibration curves generated from each stability test run for the analyte are presented in **Table 3**. In human plasma, GMF was stable for three freeze/thaw cycle to three month frozen condition at -20°C. No detectable loss of GMF in the stored samples was detected. The experimental data of the stability tests, i.e. 24 h ambient temperature, bench-top (8 h), three repeated freeze- thaw cycles and at -20°C for 30 days, 90 days respectively showed that predicted concentrations for analyte at LQC, MQC and HQC samples deviated within the assay variability limits (±15%) of the nominal concentrations.

TABLE 3: STABILITY DATA OF GEMIFLOXACIN IN HUMAN PLASMA (n= 6)*

Storage condition	LQC (0.075 µg/mL)	MQC (1.250 µg/mL)	HQC (1.80 µg/mL)	r^2
3 Freeze-thaw cycle	94.128±0.0016	95.684±0.0123	96.209±0.0121	0.9951
24 h ambient	93.438±1.4229	96.368±0.4349	94.923±1.3335	0.9997
8 h bench top	95.289±1.0677	95.539±1.3397	94.121±0.9049	0.9933
1 month frozen (-20 ^{o}C)	92.718±1.5865	93.299±0.7252	91.592±0.8695	0.9986
3 months frozen (-20 ^{o}C)	90.064±0.8247	91.275±1.7625	91.062±0.4601	0.9980

*LLOQ, limit of quantification; LQC, low quality control; MQC, middle quality control; HQC, high quality control ; n = number of sample. The data presented in this table is the percentage of measured value vs. theoretical value with S.D. in parentheses; r^2 (correlation coefficient), linearity of the calibration

Evaluation of Pharmacokinetic and bioequivalence study: The above mentioned bioanalytical method was used to determine the plasma concentration of gemifloxacin for a bioequivalence study. The mean (±SD) plasma level of gemifloxacin for test and reference preparation after the oral administration of a single dose 320mg gemifloxacin tablet in 12 healthy human volunteers are shown in **figure 4**. The representative chromatograms of the test and reference product of gemifloxacin 320mg tablet after oral administration in healthy human volunteer **figure 3**. All the pharmacokinetic parameters are presented in **Table 4**. The values of the main pharmacokinetic parameters like C_{max}, t_{max}, AUC_{0-t}, $AUC_{0-\infty}$, $t_{½}$ and K_{el} were similar between the reference and test products.

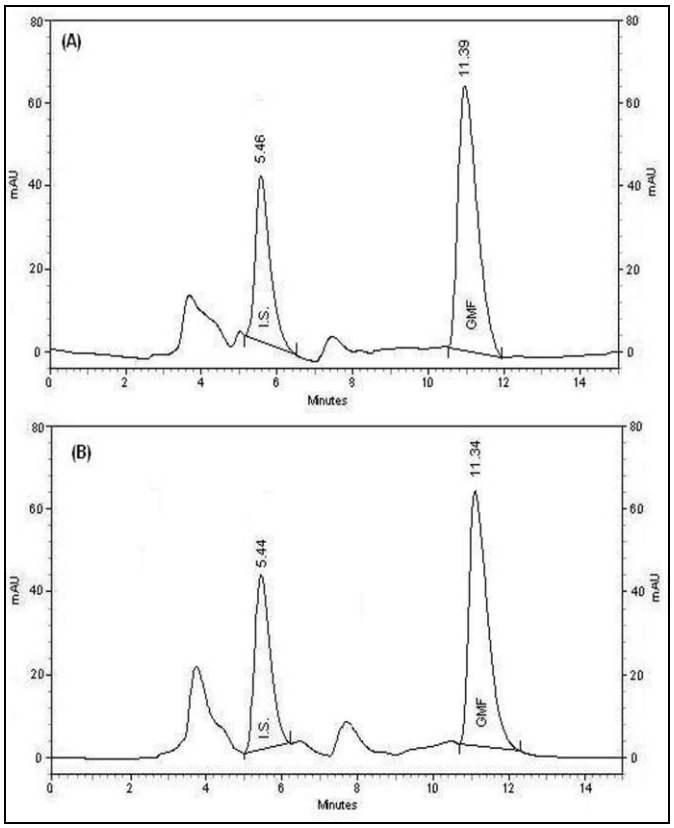

FIG. 3: (A) REPRESENTATIVE CHROMATOGRAM OF VOLUNTEER (NO. 7) PLASMA SPIKED WITH IS (50µL OF 30.00 µg/ml EQUIVALENT TO 1.50µg/ml) AT 1.5 H AFTER ADMINISTRATION OF REFERENCE TABLET (GEMBEX; RANBAXY LABORATORIES LTD, GURGAON, INDIA) CONTAINING 320mg GEMIFLOXACIN. (B) REPRESENTATIVE CHROMATOGRAM OF VOLUNTEER (NO. 7) PLASMA SPIKED WITH IS (50µL OF 30.00µg/ml EQUIVALENT TO1.50µg/ml) AT 1.5 H AFTER ADMINISTRATION OF TEST TABLET (ACME, HIMACHAL PRADESH, INDIA) CONTAINING 320mg GEMIFLOXACIN.

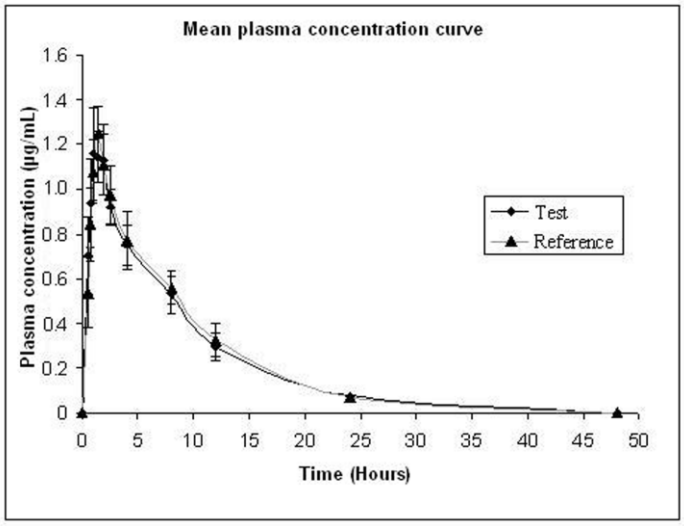

FIG. 4: PLASMA CONCENTRATION (MEAN±SD) VS TIME PROFILE OF GEMIFLOXACIN IN HUMAN PLASMA.

TABLE 4: PHARMACOINATIC PARAMETERS OF GEMIFLOXACIN IN HUMAN PLASMA FOLLOWING ORAL DOSING OF 320 mg (n= 12)*

Pharmacokinetic parameters	Reference	Test
C_{max} (µg/mL)	1.313±0.052	1.318±0.059
t_{max} (h)	1.500±0.369	1.417±0.469
$t_{1/2}$ (h)	5.631±0.247	5.671±0.005
AUC_{0-48} (µg/mL.h)	10.385±0.669	10.130±0.625
$AUC_{0-\infty}$ (µg/mL.h)	10.974±0.657	10.746±0.625
K_{el}	0.123±0.005	0.122±0.004

*C_{max}, maximum plasma concentration; t_{max}, time require to achieve maximum concentration; AUC_{0-48}, area under the plasma concentration time curve from time zero to 48 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; n= number of volunteer.

CONCLUSION: The HPLC method described here for analysis of gemifloxacin in plasma is very simple, specific and sensitive. The method developed in our laboratory is very simple utilizing liquid-liquid extraction procedure, which make the method high throughput for analysis. Moreover this bioanalytical method has been applied successfully for the bioequivalence study of gemifloxacin in human volunteer. The values of the main pharmacokinetic parameters like C_{max}, t_{max}, AUC_{0-t}, $AUC_{0-\infty}$, $t_{½}$ and K_{el} were similar between the reference and test products.

The proposed method to analyze gemifloxacin in plasma by HPLC with UV detection happens to be first of its kind described so far in the literature. This new method will be of immense helps for carrying out pharmacokinetic study of gemifloxacin in laboratories that lack sophisticated analytical instrument of LC-MS/MS.

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