INTERNATIONAL JOURNAL OF DRUG DISCOVERY AND HERBAL RESEARCH (IJDDHR)
10(4): Oct.-Dec.: (2020), 914-924
ISSN: 2231-6078

Research Article

BIOANALYTICAL, METHOD DEVELOPMENT AND VALIDATION OF SOME NOVEL DRUG IN HUMAN PLASMA

Heena Aday[1], Amit Gupta[1], Vivek Chourasia[1]

RKDF College of Pharmacy, SRK University, Bhopal, MP, India

Abstract

Aim of the study: Current research was aimed to develop a simple, rapid and sensitive HPLC method for the determination of gemiloxacin, and levofloxacin by UV detection in human plasma.

Materials and Methods: A robust, selective and sensitive HPLC Method with UV detection was developed to quantify Gemifloxacin in human plasma. It involved evaluation and optimization of the various parameters like sample preparation, chromatographic separation, detection and quantification. In this study a stock solution containing 1 mg/ml of GMF was prepared by dissolving the drug in methanol. This stock solution was further diluted to 10µg/ml with methanol. Based on the trial and error method ratio of the buffer and organic modifier (methanol and acetonitrile) was optimized. After selection of proper mobile phase, HPLC column was selected again by trial and error method. One of the most important parts of analysis in bioanalytical method is internal standard (IS). As thumb rule, a compound with structural similarity with the analyte or with significant absorbance at the detection wavelength is selected as IS in bioanalytical HPLC method. After selection of the internal standard, final tuning with mobile phase composition and buffer concentration was done based on the retention time of the Gemifloxacin and IS . The final mobile phase was selected so that it could elute the Gemifloxacin and IS with reasonable peak separation.

Results: The HPLC method described 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. All the validation data was in the range of USFDA guideline. 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.

Keywords: Gemifloxacin, HPLC, Bioanalytical Method Validation, UV.

*** Corresponding Author**
E.mail: vickyc.chourasia@gmail.com

Introduction

The initial drivers to measure the presence of drugs in biological fluids were to determine possible overdosing as part of the new science of forensic medicine/toxicology. The need to measure drug levels in biological fluids was further driven by the development of pharmacokinetics as a science in the 1930s.

In the early days of drug development, many of the assays for drugs in biological fluids were nonspecific and did not discriminate between the drug and its metabolites; for example, aspirin (circa 1900) and sulfonamides (developed in the 1930s) were quantified by the use of colorimetric assays, while antibiotics could be quantified by their ability to inhibit bacterial growth. The 1930s also saw the rise of pharmacokinetics – a driver for more specific assays.

The lack of specificity became a real issue as the identification of metabolites gathered speed for drugs in biological fluids. Their development as drugs in their own right made identification a commercial issue, as metabolites could be patented as 'new' drug entities. An understanding of the impact of the body's metabolism of drugs became essential for identifying the therapeutically active moiety, as well as possible 'toxic' metabolites.

The development of chromatographic techniques such as paper chromatography took place in the 1940s and allowed separation of the drug from its metabolites. Later in the 1950s, thin-layer chromatography was developed and used to quantify drugs in biological fluids, although its main application was in the separation of radiolabeled metabolites. Unfortunately, the sensitivity of these technologies was not sufficient to measure the new drugs of the 1950s, such as 'tricyclics' that had levels of ng/ml. There are several chromatography instrument can be used for the bioanalysis were HPLC and LC-MS, LCMS-MS and GC-MS in biological matrixes. Each of the instruments has its own merits and demerits. HPLC coupled with UV, PDA or fluorescence detector can be used for estimation of many compounds but it does not give the high sensitivity as required by some of the potent, low dose drugs and lacks selectivity. The main advantages of LCMS-MS include low detection limits, the ability to generate structural information, the requirement of minimal sample treatment and the

possibility to cover a wide range of analytes differing in their polarities. Depending on the sensitivity, selectivity and cost effectiveness of the method, a choice needs to be made between HPLC and LCMS-MS or GC-MS. But in normal laboratory offers a cost effective bioanalytical method, it is not possible to bear costly instrument like LCMS-MS or GC-MS. So we need to switch on the more economic analysis like HPLC. HPLC coupled with UV, PDA or fluorescence detector can be used for estimation of many compounds.

Chromatography

Chromatography is an analytical technique used for the separation of complex chemical mixtures into individual components.Chromatography (from Greek χρώμα : chroma, color and γραφειν : graphein to write) is the collective term for a set of laboratory techniques for the separation of mixtures. It involves passing a mixture dissolved in a "mobile phase" through a stationary phase, which separates the analyte to be measured from other molecules in the mixture based on differential partitioning between the mobile and stationary phases. Subtle differences in compounds partition coefficient results in differential retention on the stationary phase and thus separation. Chromatography may be preparative or analytical. The purpose of preparative chromatography is to separate the components of a mixture for further use (and is thus a form of purification). Analytical chromatography is done normally with smaller amounts of material and is for measuring the relative proportions of analytes in a mixture. The two are not mutually exclusive.

High performance liquid chromatography (HPLC)

High performance liquid chromatography is now one of the most powerful tools in analytical chemistry. It has the ability to separate, identify, and quantitate the compounds that are present in any sample that can be dissolved in a liquid. Today, compounds in trace concentrations may easily be identified. HPLC can be, and has been, applied to just about any sample, such as pharmaceuticals, bio analytical, forensic, food, cosmetics, environmental matrices, and industrial chemicals.

Method validation

Bioanalytical methods can be developed in the laboratory conducting the validation or obtained from another laboratory or literature. The results from a method validation can be no better than the quality of the method that was developed. Method validation is a process that demonstrates that the method will successfully meet or exceed the minimum standards recommended in the Food and Drug Administration (FDA) guidance for accuracy, precision, selectivity, sensitivity, reproducibility, and stability. The validation is performed using a control matrix spiked with the compounds to be quantified. While obtaining these parameters, other parameters are also determined during validation (eg, extraction efficiency, calibration range and response function positional differences within an analytical run, and dilution integrity for analyzing above limit of quantitation samples. For quantitative bioanalytical procedures, there is a general agreement, that at least the following validation parameters should be evaluated:

Gemifloxacin

The pharmacokinetics profile of Gemifloxacin is characterized by high concentration in plasma, tissues and body fluid and a long half-life permitting extended dosing intervals to be applied. If the free drug AUC/MIC ratio is >33.7, the probability of a favorable clinical outcome is quite high (>100%) for patients infected with gram-positive organisms.

Material and Methods

GEMIFLOXACIN

Instruments used for gemifloxacin analysis

HPLC analysis was performed using an Knauer 1000 Series pump combined with a Knauer 2500 series UV detector (Germany), and EZChrom (version 3.1.6) software. Three different types of columns used for the analysis were, C8 (Thermo, USA), HiberC18 (Merck, Germany),Cyano (Phenomenex, USA), 250X4.6mm, 5µ particle size. UV spectra were obtained o n a Jassco (V630) UV-Visible Spectroscopy system (Japan). Analyte weighing for preparation of calibration standards and quality control samples was done on a microbalance, Satorious SC103 (Germany).

Preparation of gemifloxacin stock solution

The stock solution of gemifloxacin (GMF) used during HPLC method development stage was prepared by dissolving the accurately weighted standard compound in methanol. Concentration of gemifloxacin stock solution was 1 mg/ml. Appropriate dilutions with mobile phase were made from the stock solution to prepare the working standard solutions for method development, calibration curve and quality control (QC) samples. The solution and working standard solutions were stored in polypropylene vials in a -20 °C freezer.

HPLC method development of gemifloxacin

A robust, selective and sensitive HPLC Method with UV detection was developed to quantify GMF in human plasma. It involved evaluation and optimization of the various parameters like sample preparation, chromatographic separation, detection and quantification. Steps involved in method developments are mentioned below in the order as they were followed.

Validation of developed bioanalytical HPLC method for GMF

The results of the pre-validation indicated that the final method validation could be performed. The HPLC method for GMF was validated to meet the acceptance

criteria of industrial guidance for the bio-analytical method validation (Food and Drug Administration of the United States, 2001).

Sample extraction procedure

0.450ml of blank plasma
↓
Add 0. 05ml of GMF (100µg/ml, 10µg/ml)
↓
Add 0.05ml of I.S (100µg /ml, 10µg/ml)
↓
Vortex for 2mins
↓
4.5ml of extracting solvent was added
↓
Extract the by hand mixing for 15mins
↓
Centrifuge for 20min at 5000rpm
↓
3.5 ml supernatantant was seperated
↓
Evaporate the supernatant under nitrogen stream at 40^0 C temperature
↓
Reconstitute the dried sample with 200µl of mobile phase
↓
Vortex for 2mins
↓
Centrifuge for 5 min at 5000rpm
↓
Inject the sample on the HPLC

Statistical analysis: The data were represented as Mean± SEM. The data on antiulcer activity of formulations were analyzed by one way Analysis of Variance (ANOVA), 'P' value less than 0.05 was considered as statistically significant.

Results and Discussion

Development of HPLC method for gemifloxacin

Determination of λmax for gemifloxacin

Maximum absorbance (λmax) of GMF (10 µl / ml) was found to be at 292 nm. It indicates that detection at 292 nm would be the most sensitive wavelength for HPLC work. This λmax was selected for HPLC method development of gemifloxacin.

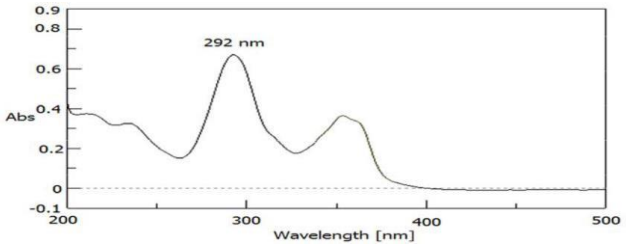

Figure.1 UV spectrophotometer scan for determination of λmax of gemifloxacin.

Selection of the mobile phase

A number of mobile phases were tried for elution of GMF, out of which following three chromatograms are shown. They are: A. 10 mM Phosphate buffer: Acetonitrile:: 50:50 v/v, B. 10 mM Ammonium acetate: Acetonitrile:: 50:50 v/v, C. 10 mM ammonium formate: Acetonitrile:: 50:50 v/v.

Selection of the Stationary phase

By using C18, C8 column with different mobile phase composition such as 1%formic acid: Acetonitrile:: 50:50,55:45, 60:35, 65:35, 70:30and 75:25 v/v GMF peak was coming before 5 mints. GMF is the polar in nature, so it elutes very quickly in case of C8 or C18 column.

Selection of the Internal Standard (IS)

Based on the selected chromatographic condition (Mobile phase: 1%formic acid: Acetonitrile:: 50:50 v/v, Column Cyano), the different Internal Standard (IS) was experimented with the concentration of 10µg/ml. The internal standards (rofecoxib, valdecoxib & carbamazepine) were from different pharmacological classes with different structures than GMF apart from compound (ciprofloxacin, gatifloxacin & levofloxacin) with structural similarity.

Optimisation of the final mobile phase

After selection of column, and IS, the buffer composition in the mobile phase was tuned for improved chromatographic results. Different buffer composition mentioned in Table 5.1 were tried and change in RT for GMF and IS were observed.

Table.1 Effect of buffer composition of mobile phase on the RT of GMF and IS.

Buffer composition (%)	Retention time (RT)	
	Gemifloxacin (GMF)	Internal Standard (IS)
50	8.38	4.47
55	8.71	4.59
60	8.82	4.77
65	8.98	4.88
70	9.71	5.17
75	10.97	5.32

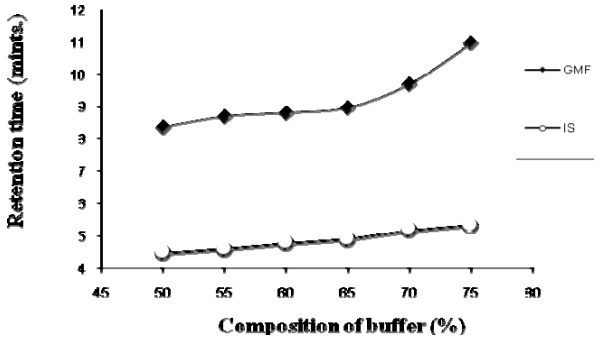

Figure:4 Effect of buffer composition on GMF and IS retention time

Table.2 Variation of peak resolution by adding the methanol in the Mobile phase.

Methanol Composition(%)	Resolution
0	4.31
5	4.95
10	6.17
15	6.94

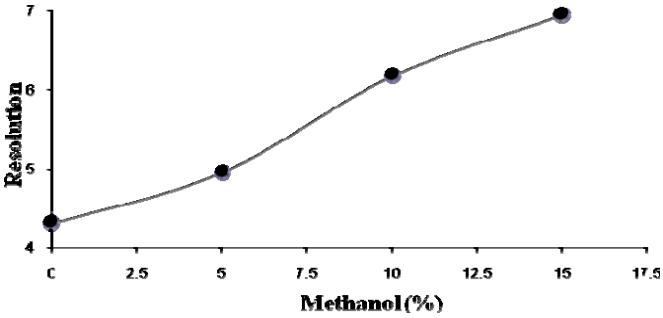

Figure. 5 Effect of methanol composition on GMF and IS peak resolution.

Table.3 Effect of buffer concentration (% of Formic acid) on the RT of GMF and IS.

Buffer concentration (% of Formic acid)	Asymmetry	
	Gemifloxacin GMF	Internal Standard (IS)
0.50	1.437	1.341
1.00	1.153	1.211
1.50	1.152	1.202
2.00	1.155	1.203

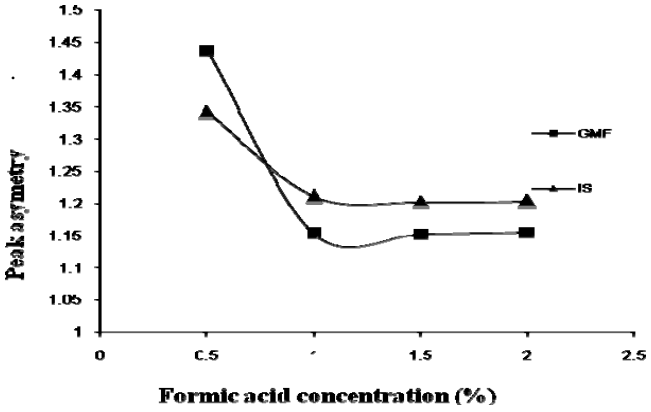

Figure. 6 Effect of buffer concentration on GMF and IS peak asymmetry

Optimization of the flow rate

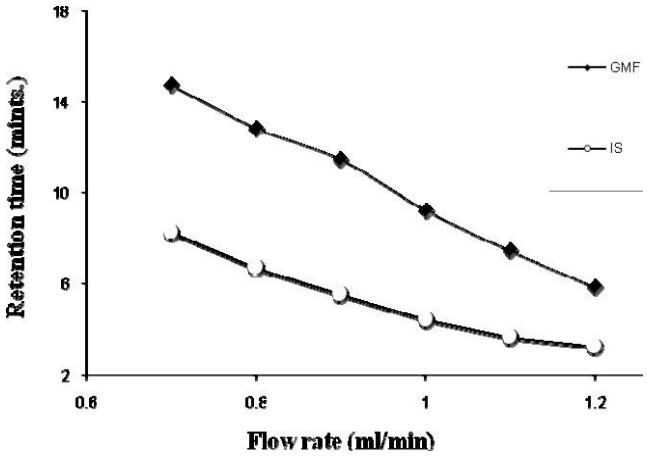

Figure. 8 Effect of the flow rate on resolution of GMF and IS

Final chromatographic conditions of bioanalytical method of GMF

Estimating LOD and LLOQ (Signal- to-noise method) of GMF

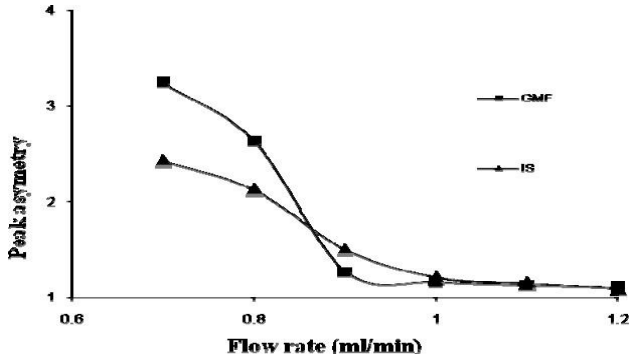

Figure. 9 Asymmetry of GMF and IS change on variation of the flow rate

The LOD was therefore found to be about 8 ng/ml where signal to noise ratio was 3 and LLOQ was 25 ng/ml where signal to noise ratio was more than 5.

Method validation for bioanalytical studies of gemifloxacin

Specificity and Selectivity

Stability of GMF and IS stock solution

The stock solution of both GMF and IS was kept in three different storage conditions and peak area was calculated of each condition. It can be seen from the Fig. 5.17 and 5.18, GMF and IS shows significant decrease in peak area with time, under the condition of room temperature and normal deep freezer. The stock

solution was stable at - 20 °C for 30 days and stable at normal deep freezer about a week.

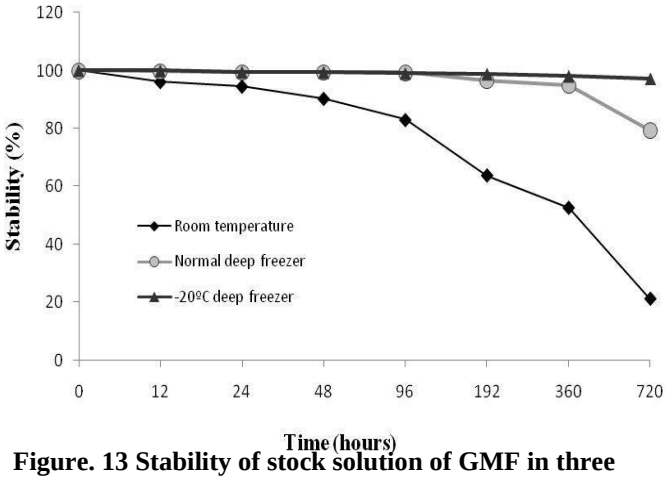

Figure. 13 Stability of stock solution of GMF in three different conditions.

Table:4 Linear regression data for the determination of GMF 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 Correlation coefficient (r^2)	f = 1.004 C + 0.009 0.999

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

Precision and Accuracy for plasma analysis of GMF

Table.5 Accuracy and precision of data of the analysis human plasma (n= 6).

Quality control sample	Spiked concentration (µg/ml)	Mean (µg/ml)	SD	Accu racy(%)	RSD(%)
Intra-Day					
LLOQ	0.025	0.024	0.0003	94.232	1.194
LQC	0.075	0.075	0.0005	98.066	0.676
MQC	1.250	1.264	0.0088	101.133	0.699
HQC	1.800	1.788	0.0219	99.349	1.223
Inter-Day					
LLOQ	0.025	0.024	0.0004	95.064	1.645
LQC	0.075	0.073	0.007	97.702	1.014
MQC	1.250	1.261	0.0105	100.902	0.834
HQC	1.800	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.

Evaluation of pharmacokinetic parameters

Reference & Test preparation are used

Reference preparation:

Tablet GEMBAX (containing Gemifloxacin Msylate 320 mg)

Mfg.by:M/S Ranbaxy Laboratories Ltd., plot no.90, Sector –32, Gurgaon – 122001, India

Batch No.: R21208

Mfg. Date: January, 2020

Exp. Date: December, 2021

Test preparation:

Tablet Gemifloxacin Mesylate 320 mg (containing Gemifloxacin Mesylate 320 mg)

Mfg. by: M/S Acme Formulation Pvt. Ltd., Ropar Road, Nalagarh, District Solan, HimachaL Pradesh – 174101

Batch No.: Trial Batch 1

Mfg. Date: June, 2020

Exp. Date: February, 2021

Table.6 Demographic Data of 12 Volunteers

Vol. No.	Sex	Age	Height (cm)	Weight (kg.)	BMI (kg/m^2)
1	M	29	172.72	75	25.14
2	M	21	170.18	65	22.44
3	M	26	165.10	71	26.05
4	M	30	172.72	75	25.14
5	M	27	170.18	62	21.41
6	M	*32*	157.48	69	27.82
7	M	*34*	162.56	57	21.57
8	M	*29*	157.48	66	26.61
9	M	*30*	172.72	70	23.46
10	M	*25*	175.26	73	23.77
11	M	*34*	167.64	67	23.84
12	M	*28*	165.10	61	22.38
Mean		**28.75**	**167.43**	**67.58**	**24.14**
S.D.		**5**	**5.98**	**5.68**	**2.05**

Pharmacokinetics & statistical analysis

The plasma samples were analyzed by the proposed analytical method (as described in section 5.1.8 & 5.1.9) and the plasma concentrations and mean plasma concentrations were calculated and shown in Table 5.15 - 5.16. And the mean plasma concentration-time profile curves were shown in Figure. 5.20 for both Test and Reference. The reference tablet as a single dose in the fasting state produced the maximum plasma concentration of 1.313± 0.052 µg/ml (Cmax) at the time of 1.500± 0.369h (Tmax) whereas the test product in the fasting state produced the maximum plasma concentration of 1.318± 0.059 µg/ml (Cmax) at the time of 1.417± 0.469h (Tmax).

Conclusion

The HPLC method described for analysis of fluroquinolones antibiotic - gemifloxacin, are very specific and sensitive. The methods developed in our laboratory are very simple utilizing liquid-liquid extraction procedure, which make the method high throughput for analysis. All the validation data were met the of range acceptance criteria of USFDA guideline. Moreover this bioanalytical method has been applied successfully to the bioequivalence studies of respective drugs in plasma of human volunteers. The values of the main pharmacokinetic parameters Cmax, tmax, AUC0– t, AUC0–∞, t½ and Kel were comparable between the reference and test products. The proposed method to analyze the analytes 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 helpful for carrying out pharmacokinetic studies of respective drugs in laboratories that lack sophisticated analytical instrument of LC-MS/MS.

Reference

1. Ahuja. S. (2007). Rasmussen. H. HPLC Method Development for Pharmaceuticals, Chapter 11:HPLC method development for drug discovery LC-MS assay in rapid PK 1st ed; Elsevier Inc.: London.; P.191-219.

2. Breda .C.A., Breda. M., Frigerio E. (2004).Bioanalytical method validation: a risk-based approach

3. Chakrabarty.U.S., Das. A., Bhaumik.U., Chatterjee.B., Ghosh. A., Bose .A, Sengupta. P,Nandi. U. and. Pal. T. K. (2009). Rapid and Sensitive LC Method for the Analysis of Gemifloxacin in Human Plasma.Chromatographia.9-10(69), 853-858.

4.Dadgar. D. & Burnett, P.E.(1995). Issues in Evaluation of Bioanalytical Method Selectivity and Drug Stability. Journal of Pharmaceutical and Biomedical analysis. 14, 23 -31.

5. Development and validation of RP-HPLC assay for levofloxacin in rat plasma and saliva: Application to pharmacokinetic studies. African Journal of Pharmacy and Pharmacology. 5(13),1612-1618,

6. Liu. R. H., Foster .G, Cone. E. J., and Kumar S. D., (1995) Selecting an appropriate Isotopic Internal Standard for Gas Chromatography/Mass Spectrometry Analysis of Drugs of Abuse Pentobarbital Example. Journal of Forensic Sciences. (4)6, 983-989.

7. Pharmacokinetics and safety of NM441, a new quinolone, in healthy male volunteers. Journal of Clinical Pharmacology. 34, 930-937.

8. Saxena. C.H., Srtvastava J.R., Goswami. P.,(1971).Bacterial infection of the newborn. Pediatric Clinics of India.6,63.

9. Analytical Method Validation: Bioavailability, Bioequivalence and Pharmacokinetics Studies. Journal of Pharmaceutical Sciences. 81, 309-312.

10. Sarkar. A.K., Ghosh. D., Das .A., Selvan P.S., Gowda. K.V., Manda .U, BoseA.D., Selvan ..S. Agarwal, Bhaumik .U, Pal T.K.(2008). Simultaneous determination of metoprolol succinate and amlodipine besylate in human plasma by liquid chromatography–tandem mass spectrometry method and its application in bioequivalence study. Journal of Chromatography B. 873, 77–85.

11. Roy. B., Choudhury. H., Das. A., Das. K. A., Pal. T.K. (2011). Development and validation of a liquid chromatography tandem mass spectrometry method to determine ulifloxacin, the active metabolite of prulifloxacin in rat and rabbit plasma: application to toxicokinetic study. Biomedical Chromatography. 25(8), 890–901.

12. Naxing. X.R. , Leimin.F., Matthew. R. J., Tawakol. A. (2007). El-Shourbagy,Recent advances in high-throughput quantitative bioanalysis by LC–MS/MS. Journal of Pharmaceutical and Biomedical Analysis .44, 342–355.

.

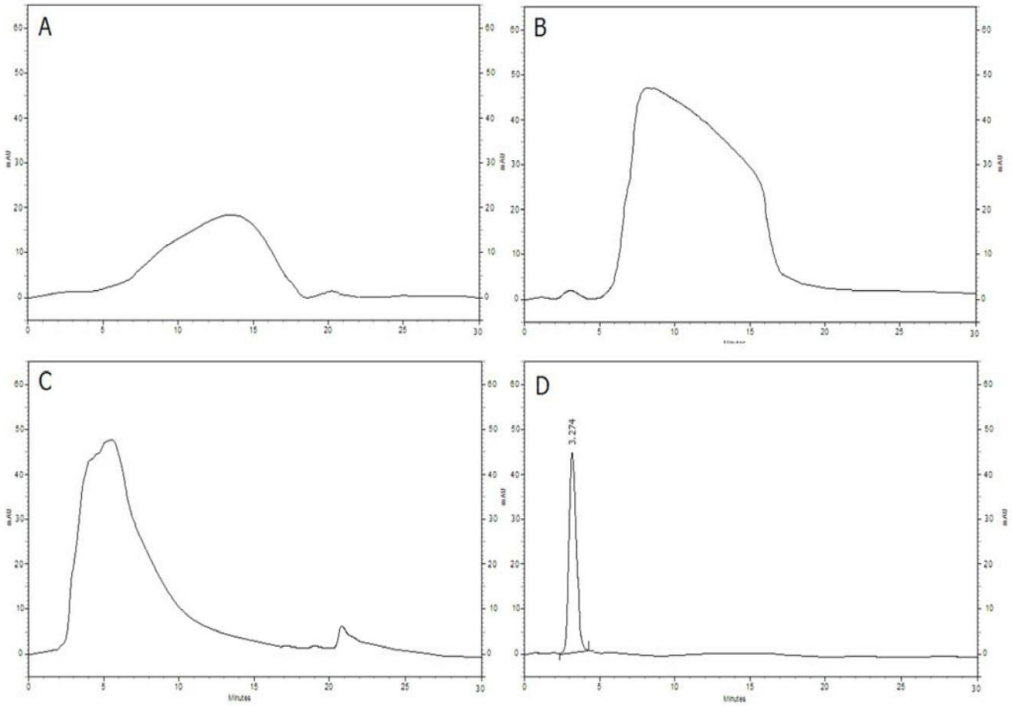

Figure.2 Chromatograms showing GMF peak with mobile phase composition of A. 10 Mm Phosphate buffer : Acetonitrile:: 50:50 v/v, B. 10 mM Ammonium acetate: Acetonitrile:: 50:50 v/v,C. 10 mM Ammonium formate:Acetonitrile:: 50:50 v/v. D. 1%formic acid: Acetonitrile:: 50:50 v/

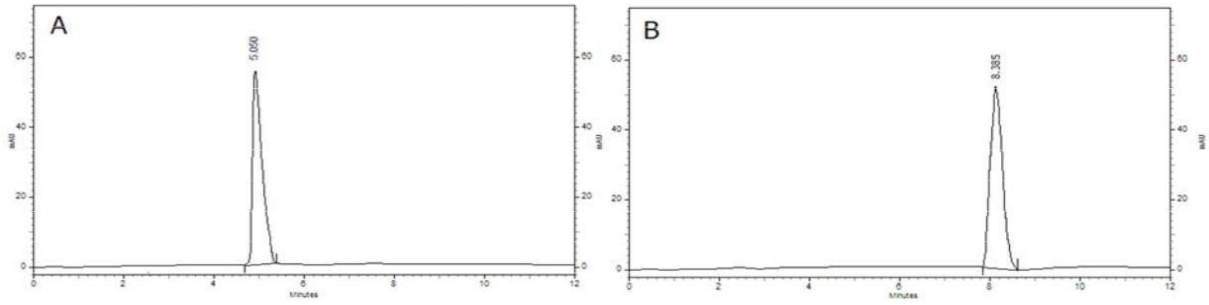

Figure.3 Chromatograms showing GMF peak using C8 (A) and Cyano column (B).

Final chromatographic conditions of bioanalytical method of GMF
Estimating LOD and LLOQ (Signal- to-noise method) of GMF

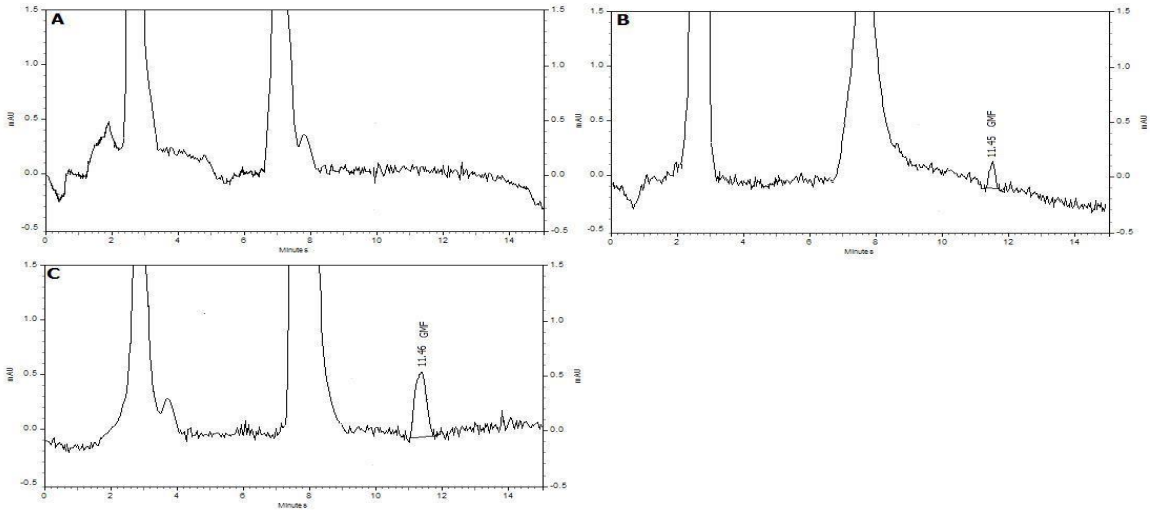

Figure.10 Chromatograms run to determine LOD and LLOQ of GMF. A. Blank plasma chromatogram B. Chromatogram of LOD of GMF C. Chromatogram of LLOQ of GMF.

Method validation for bioanalytical studies of gemifloxacin
Specificity and Selectivity

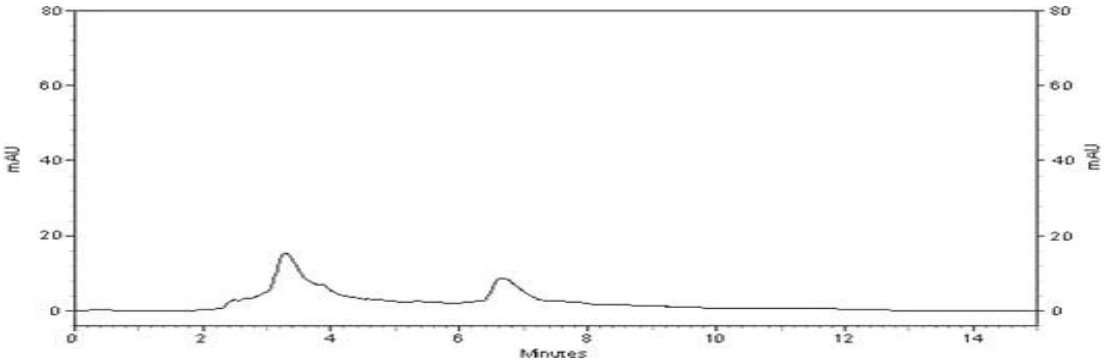

Figure.11 Blank human plasma (free of GMF and IS) [Double blank].

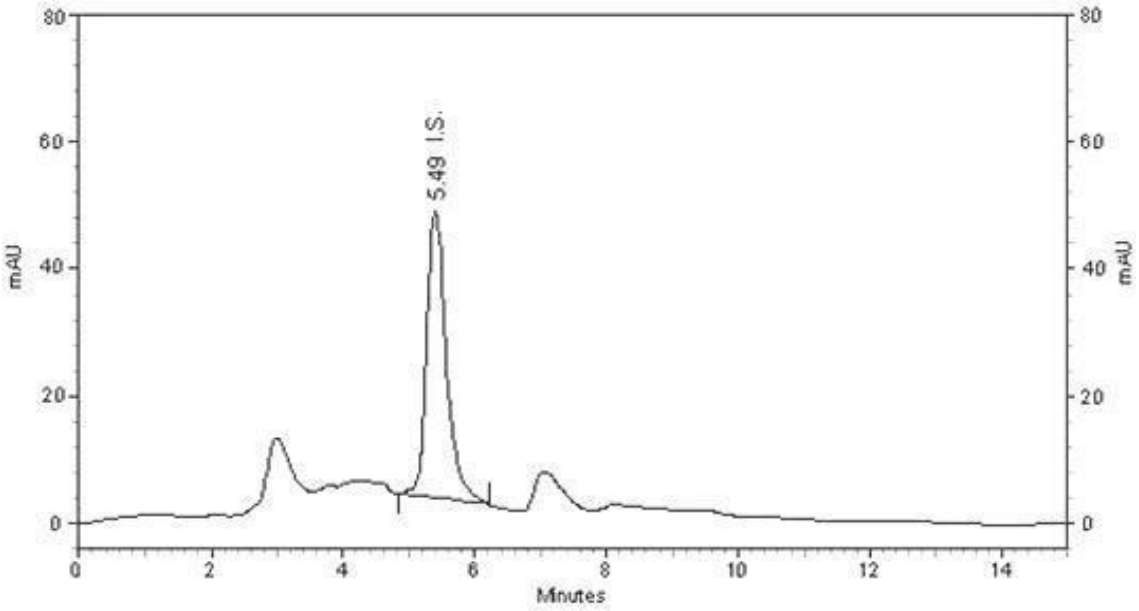

Figure.12 Blank human plasma spiked with IS [Single blank].

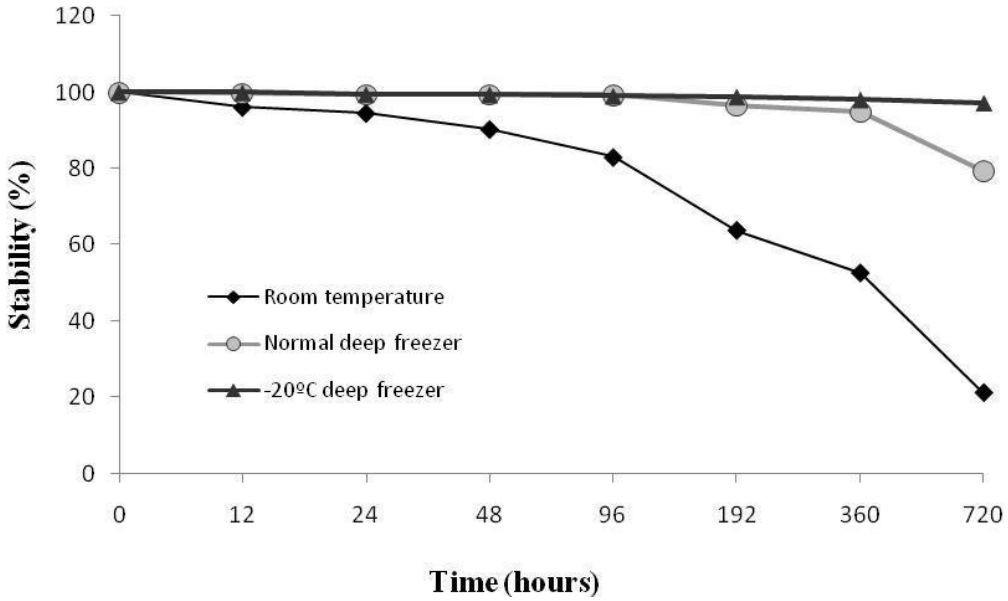

Figure. 13 Stability of stock solution of G MF in three different conditions.

Table.7 Pharmacokinetic Parameters in 12 volunteers with the Test and Reference Preparation

Pharmacokinetic parameters	**Reference Preparation (A)**		**Test Preparation (B)**	
Cmax (µg/ml.)	**Mean**	1.313	**Mean**	1.318
	±S.D.	0.052	**± S.D.**	0.059
tmax (hr.)	**Mean**	1.500	**Mean**	1.417
	±S.D.	0.369	**±S.D.**	0.469
AUC 0-t (µg. hr./ml.)	**Mean**	10.385	**Mean**	10.130
	± S.D.	0.669	**± S.D.**	0.625
AUC $_{0-\infty}$ (µg. hr./ml.)	**Mean**	10.974	**Mean**	10.746
	± S.D.	0.657	**± S.D.**	0.625
kel (hr.$^{-1}$)	**Mean**	0.123	**Mean**	0.122
	± S.D.	0.005	**± S.D.**	0.004
t 1/2 (hr.)	**Mean**	5.631	**Mean**	5.671
	± S.D.	0.247	**± S.D.**	0.005
Relative Bioavailability%	**100%**		**97.55%**	

Source	**D.F.**	**S.S.**	**M.S.S.**	**F.**	**P.**
Subjects	11	0.0146	0.0013	0.2512	N.S
Treatment	1	0.0001	0.0001	0.0282	N.S
Period	1	0.0003	0.0003	0.0551	N.S
Error	10	0.0529	0.0053	-	-
Total	23	0.0679	-	-	-

Table. ANOVA Summary for in Ln C_{max} Values Ln transformed data

Source	D.F.	S.S.	M.S.S.	F.	P.
Subjects	11	0.00827	0.00075	0.25040	N.S
Treatment	1	0.00008	0.00008	0.02591	N.S
Period	1	0.00015	0.00015	0.04855	N.S
Error	10	0.03003	0.00300	-	-
Total	23	0.03853	-	-	-

Table. ANOVA Summary for AUC 0-t Values untransformed data

Source	D.F.	S.S.	M.S.S.	F.	P.
Subjects	11	5.9817	0.5438	1.7127	N.S
Treatment	1	0.3891	0.3891	1.2255	N.S
Period	1	0.0604	0.0604	0.1902	N.S
Error	10	3.1751	0.3175	-	-
Total	23	9.6060	-	-	-