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Validated Stability Indicating Assay of Gemifloxacin by different Chromatographic and Spectrophotometric methods of analysis.

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ABSTRACT

Two chromatographic and three spectrophotometric methods have been developed for the determination of gemifloxacin (GF) in bulk powder and pharmaceutical preparations. The first method depends on RP-HPLC, separation of drug and its degradation products was successfully achieved on a Hypersil BDS C18 column using mobile phase consisted of citrate buffer (adjusted to 2.5 pH by citric acid):Acetonitrile (70:30, v/v) at 1 ml/min flow rate and 267 nm wavelength of detection. The second method which achieved successful separation of drug and its degradation products depends on TLC densitometry using a developing system consisted of chloroform : methanol : toluene : diethylamine : water (33.6:33.6:16.8:10.8:6,by volume) with 20 µl spotting volume and 260 nm wavelength of detection. Three UV methods have been developed for GF estimation in presence of its degradation products as stability indicating techniques. These methods are first derivative (D^1), first derivative of ratio spectra (DD^1) and second derivative of the difference absorption (ΔD^2) of acidic GF solutions in 0.1N HCl against its alkaline solutions in 0.1N NaOH as blanks. All the proposed methods were validated and successfully applied for determination of GF in pure form and in pharmaceutical preparations with good recovery. The results obtained by applying the proposed methods were statistically analyzed and compared with those obtained by the manufacturer method and no significant difference was found.

Keywords: Gemifloxacin, RP-HPLC, TLC, UV Spectrophotometry, Derivative Spectroscopy.

INTRODUCTION

GF is a new synthetic third generation fluorinated quinolone antibacterial used in the treatment of severe systematic infections as bronchitis pneumonia as it has a broad spectrum activity against many pathogenic gram –ve and +ve bacteria including many of the so called

atypical respiratory pathogens [1]. It overcomes the microbial resistance against common classes of antibiotics which is increasingly important global problem [2] as it is a significant phenomenon in terms of its clinical and economic impact. Patients who were infected with resistant organisms had longer hospitalizations than those infected with susceptible bacteria. In addition, increased costs were associated with infection caused by resistant species and increased mortality, despite the fact that patients received appropriate antimicrobial therapy [3].

GF is 7 [3-(aminomethyl)-4-(methoxyimino)-1-pyrrolidinyl]-1-cyclopropyl-6-fluoro-1, 4-dihydro-4oxo-1, 8-naphthyridine-3-carboxylic acid was prepared in 1995 and 1997 [4]. The great bactericidal activity of GF is due to the presence of 4-oxo-3-carboxylic acid [5].

It is recently being approved by the US Food Drug Administration for the treatment of upper respiratory tract infections [6]. GF determination is not yet described in any pharmacopoeias. The literature survey reveals that few analytical method for this drug are reported which include HPLC methods for determination of GF in pharmaceutical preparations [7], in human plasma [8,9], in different rat tissues [10] and for GF enantiomeric separation [11,12]. Few spectrophotometric [13,14] and capillary electrophoresis [15-18] methods are reported for GF determination or its enantiomeric separation.

Our study presents five new methods for GF determination alone and in presence of its acidic degradation products, namely, HPLC, TLC, first derivative, first derivative of ratio spectra and second derivative of difference absorption spectrophotometric methods.

EXPERIMENTAL SECTION

2.1 Instrumentation

(1) HPLC Waters 600 liquid chromatograph (USA) with UV-Visible detector (2987 detector) operated at 267 nm at flow rate 1 ml/min using a Thermo BDS Hypersil C18 Column (250 mm×4.6 mm, 5µ m).
(2) Shimadzu TLC flying spot scanning densitometer (Japan) using plates of silica gel (20×20 cm, 0.5 mm).
(3) Shimadzu UV-1601 uv/vis spectrophotometer (Japan) with 1 cm matches quartz cell.
(4) Crest ultrasonic processor model 575DAE (USA).
(5) Jenway PH-meter model 3510 (UK).

2.2 Materials

(1) Pure sample of GF was kindly supplied and certified from Hikma Pharma, Cairo, Egypt to contain 99.7%.
(2) Factive® tablets B.N. 003 (Hikma Pharma,Cairo,Egypt) contain the equivalent of 320 mg gemifloxacin as the mesylate salt.
(3) Distilled water was produced in-house (Aquatron water still, A4000D, UK).
(4) HPLC grade acetonitrile from Scharlau (Spain), sodium citrate and citric acid from Finechem (Egypt) were used while all other chemicals and reagents were of analytical grade unless indicated otherwise.

2.3 Methods.

2.3.1 Preparation of degradation products

A 100.00 mg amount of intact GF was refluxed for 7 hours with 50 ml 2N HCl. The solution was cooled, neutralized using 2N NaOH and transferred into 100 ml volumetric flask. The volume was completed to the mark by distilled water to give a degradation product solution of concentration 1.00 mg/ml.

2.3.2 Preparation of standard solutions

(1) GF intact standard solution (100.00 µg/ml) in distilled water was prepared.
(2) GF degradation products standard solution (100.00 µg/ml) in distilled water was prepared.

2.3.3 Laboratory-prepared mixtures

2.3.3.1 HPLC method Aliquots equivalent to 10.00-90.00 µg of GF from its stock standard solution **(**100.00 µg/ml**)** were transferred into series of 10 ml volumetric flasks. Aliquots of degraded solution (100.00 µg/ml) equivalent to 10-90% of the intact solution were added to the same flasks.
2.3.3.2 TLC method Aliquots equivalent to 105.00-315.00 µg of GF from its stock standard solution **(**100.00 µg/ml**)** were transferred into series of 5 ml volumetric flasks. Aliquots of

degraded solution (100.00 µg/ml) equivalent to 10-70% of the intact solution were added to the same flasks.

2.3.3.3 Spectrophotometric methods (D^1 and DD^1) Aliquots equivalent to 42.00-126.00 µg of GF from its stock standard solution (100.00 µg/ml) were transferred into series of 10 ml volumetric flasks. Aliquots of degraded solution (100.00 µg/ml) equivalent to 10-70% of the intact solution were added to the same flasks.

2.3.3.4 Second derivative of difference absorption Spectrophotometric method (ΔD^2) Aliquots equivalent to 42.00-126.00 µg of GF from its stock standard solution (100.00 µg/ml) were transferred into series of 10 ml volumetric flasks. Aliquots of degraded solution (100.00 µg/ml) equivalent to 10-70% of the intact solution were added to the same flasks.

2.3.4 Procedure

2.3.4.1 Calibration Curves

2.3.4.1.1 HPLC method

Different aliquots of stock standard solution (100.00 µg/ml) equivalent to 5.00-150.00 µg of GF were transferred into a series of 10 ml volumetric flasks and the volume was completed by distilled water. These different concentrations were injected in Waters HPLC with 20 µl injection volume using Hypersil BDS C18 column (250mm×4.6 mm, 5µm) with 1 ml/min flow rate and 267 nm wavelength of detection. The mobile phase consisted of citrate buffer (adjusted to 2.5 pH by citric acid): Acetonitrile (70:30, v/v). The peak area of all concentrations are detected and plotted against the corresponding concentration and the linear regression equation was computed.

2.3.4.1.2 TLC method

Different aliquots of stock standard solution (100.00 µg/ml) equivalent to 100.00-350.00 µg of GF were transferred into a series of 5 ml volumetric flasks and the volume was completed by methanol .These different concentrations were applied in spot form to a TLC plate with 20µl spotting volume. The plate was developed to 16 cm after 3 hours of saturation using chloroform : methanol : toluene : diethylamine : water (33.6:33.6:16.8:10.8:6,by volume) as a developing system, dried and detect the spots under UV light at 254 nm and peak area was detected for all spots using TLC flying spot scanning densitometer. The peak area of all spots was plotted against the corresponding concentration and the linear regression equation was computed.

2.3.4.1.3 First derivative spectrophotometric method

Different aliquots of stock standard solution (100.00µg/ml) equivalent to 40.00-140.00 µg of GF were transferred into a series of 10 ml volumetric flasks and the volume was completed by distilled water. These different concentrations were scanned in the first order derivative spectra and the D^1 value at 258.6 nm was plotted against the corresponding concentration and the linear regression equation was computed.

2.3.4.1.4 First derivative of ratio spectra method

The same series of dilutions of the first order derivative method were prepared and scanned in the range of 200-400 nm (zero order), then divided by the spectrum of 5.00 µg/ml degradants.The resulting curves were then transformed into first order derivative with $\Delta\lambda = 4$ and scaling factor 10 and the DD^1 value at 285 nm was plotted against the corresponding concentration and the linear regression equation was computed.

2.3.4.1.5 Second derivative of difference absorption Spectrophotometric method

Different aliquots of stock standard solution (100.00 µg/ml) equivalent to 40.00-140.00 µg of GF were transferred into a series of 10 ml volumetric flasks (2 flasks for every concentration), one was completed to volume with 0.1 N HCl and the other with 0.1 N NaOH. For each concentration measure the difference absorbance of GF acidic solution in the sample cell against GF alkaline solution in the reference cell (ΔA). The second derivative curve of (ΔA) was computed (ΔD^2) at 288.2 nm and plotted against the corresponding concentration and the linear regression equation was computed.

2.3.4.2 Procedure for pharmaceutical preparation (Factive tablet)

Ten tablets were accurately weighed and finely powdered. A weight equivalent to 100.00 mg of GF was placed into 100 ml volumetric flask, 20 ml of distilled water was added and the sonication is done for 15 minutes then the volume was completed by distilled water to

obtain a solution of 1.00 mg/ml. The solution was filtered and subsequent dilutions from the filtrate were used for the determination of GF by HPLC, TLC and spectrophotometric methods. The same procedure was repeated applying the standard addition technique.

RESULTS AND DISCUSSION

GF contains imino, carbonyl and carboxylic acid groups in its structure, so it is susceptible to hydrolysis. Therefore stability indicating methods are required. The literature survey reveals only two stability indicating methods by HPLC [7] and capillary electrophoresis [18]. The present work describes new, simple, fast, validated, and economical HPLC, TLC, D^1, DD^1 and ΔD^2 methods for determination of GF in the presence of its degradation products. Our HPLC method is more sensitive than the published one [7].

3.1 HPLC method

3.1.1 Method development

Various mobile phase systems were attempted for the proposed HPLC method for the separation and solvent polarity optimization. A mobile phase of citrate buffer (adjusted to pH 2.5 by citric acid): Acetonitrile (70:30, v/v) was used at 1 ml/min flow rate and 267 nm wavelength of detection. The buffer solution was filtered through 0.45 µm membrane filter and degassed for 30 minutes in an ultrasonic bath prior to its use. Analyses were performed at ambient temperature and the injection volume was 20 µL. Successful separation of the drug and its two degradation products were achieved by this method as presented in Fig. 1, where GF showed a peak at 4.1 minute and its degradation products at 3.1 and 4.9 minute, respectively. A linear calibration curve was obtained in the concentration range 0.50 – 15.00 µg/ml with mean percentage recovery 99.41 ± 1.73. The parameters of regression equation are shown in table 1.

3.1.2 System suitability tests

According to USP 2007 [19], system suitability tests are an integral part of liquid chromatographic methods in the course of optimizing the conditions of the proposed method. System suitability tests were used to verify that the resolution and reproducibility were adequate for the analysis performed. The parameters of these tests are column efficiency (number of theoretical plates) (N), height equivalent to theoretical plate (HETP), peak resolution (R), peak tailing (T), separation factor (relative retention) (α), and capacity factor (K). The results of these tests are listed in table 2.

3.2 TLC method

In this work, TLC densitometric method was used for the determination of GF in presence of its degradation products depending on the difference in R_f values [20]. Complete separation was obtained using chloroform: methanol: toluene: diethylamine: water (33.6:33.6:16.8:10.8:6, by volume) as a developing system with 20µl spotting volume and 260 nm wavelength of detection. The R_f values of the drug and its two degradation products were 0.69, 0.00 and 0.61 respectively. A linear calibration curve was obtained in the concentration range 0.40-1.40 µg/spot with mean percentage recovery 98.85 ± 0.83. The parameters of regression equation are shown in table 1.

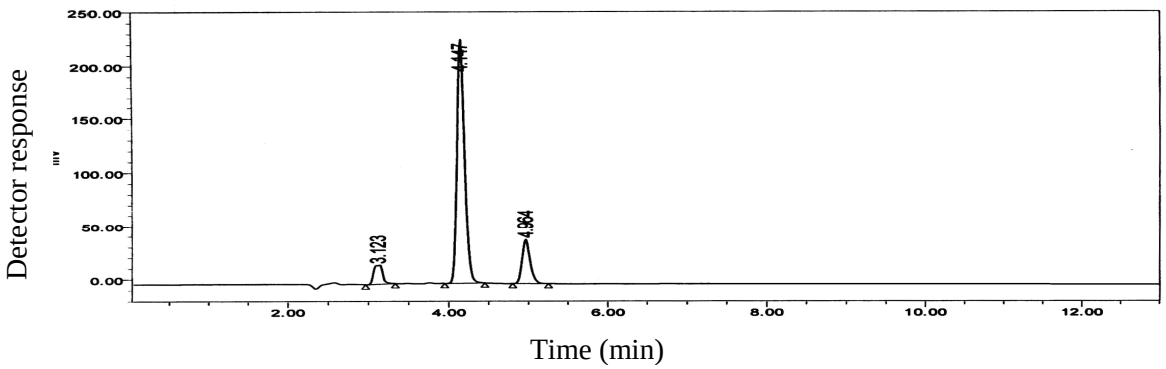


Fig.1. HPLC chromatogram of 10.00 µg/ml of intact GF (R_t = 4.1 min) and 10.00 µg/ml of its acidic degradation products (R_t = 3.1, 4.9 min).

3.3 Derivative spectrophotometric methods

In this work, two different spectrophotometric techniques were applied for quantitative determination of GF; these techniques are first-derivative (D^1) and first-derivative of ratio spectra (DD^1). The zero-order spectra (D^0) of the drug and its degradation products showed a

severe overlap; as shown in Fig. 2. , which interfere with the analysis of the drug. The (D^1) was applied which intern allowed better resolution showing maximum absorbance of drug and zero for degradation at 258.6 nm as shown in Fig. 3. By the application of the first-derivative of ratio spectra (DD^1) GF can be quantitatively determined at 285 nm without any interference from its degradation products as shown in Fig. 4. Careful choice of the divisor and the working wavelength were of great importance so different concentrations of degradation products were tried as a divisor (2.50, 5.00, 10.00, 20.00 µg/ml), the best one was 5.00 µg/ml as it produced minimum noise and gave better results in accordance with selectivity.

A linear calibration curves were obtained for the two methods in the concentration range 4.00- 14.00 µg/ml with mean percentage recoveries 100.19 ± 1.44 and 100.26 ± 1.60 for D^1 and DD^1, respectively. The characteristic parameters of regression equations and correlation coefficients are given in table 1.

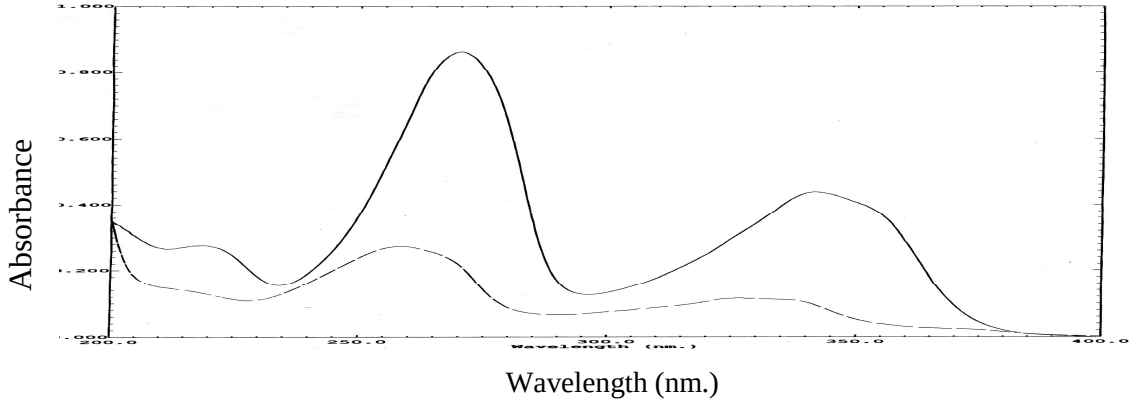


Fig.2. UV scanning of zero order of 10.00 µg/ml GF (—) and 10.00 µg/ml of its acidic degradation products (-----).

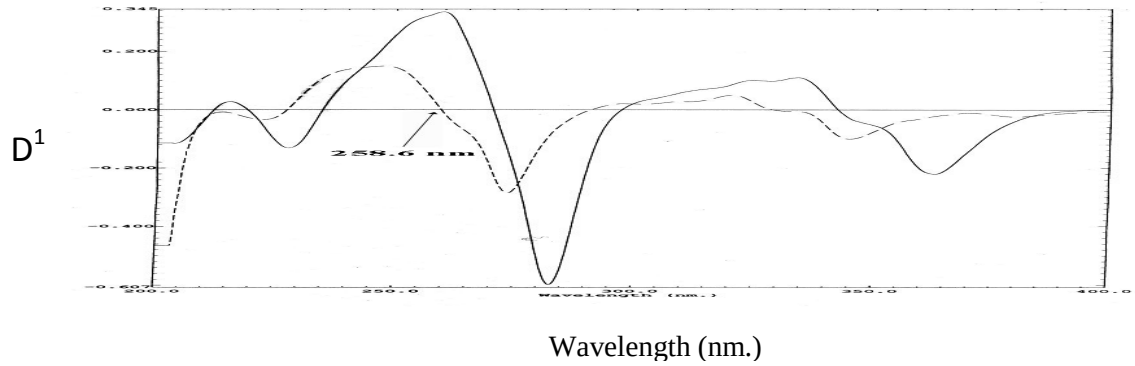


Fig.3. First-derivative spectra of 10.00 µg/ml GF (—) and 10.00 µg/ml of its acidic degradation products (-----).

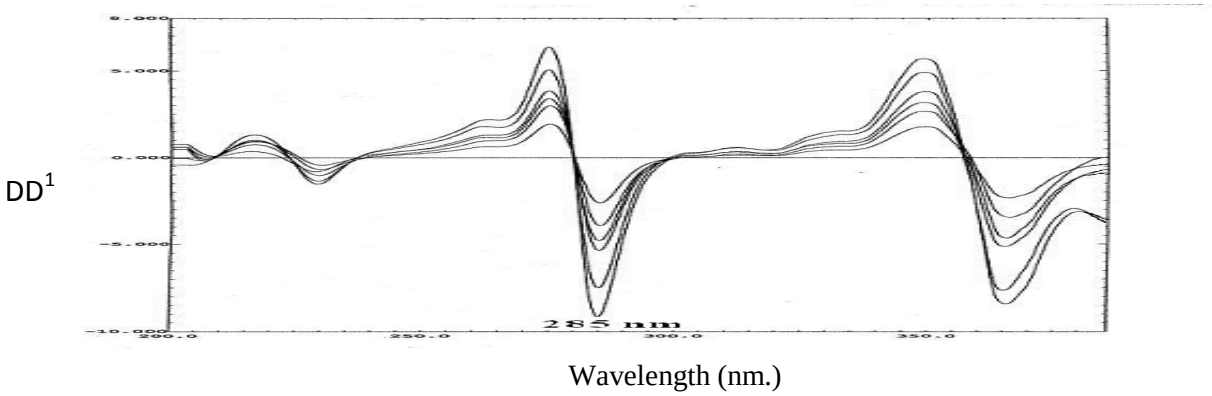
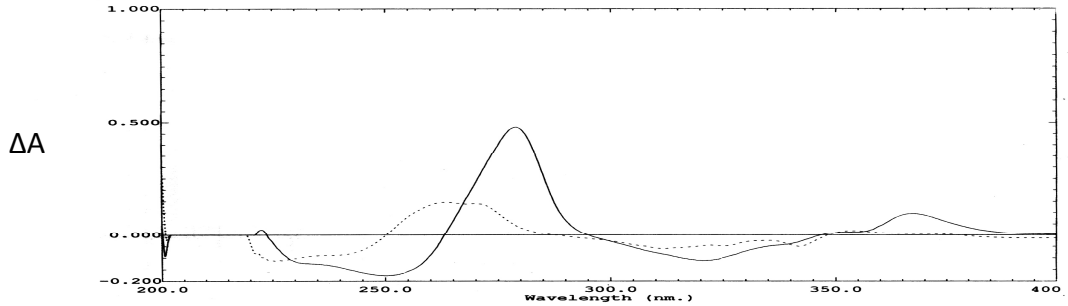


Fig.4. First derivative of the ratio spectra of 4.00 – 14.00 µg/ml GF at 285 nm using 5.00 µg/ml of its degradation products as the divisor.

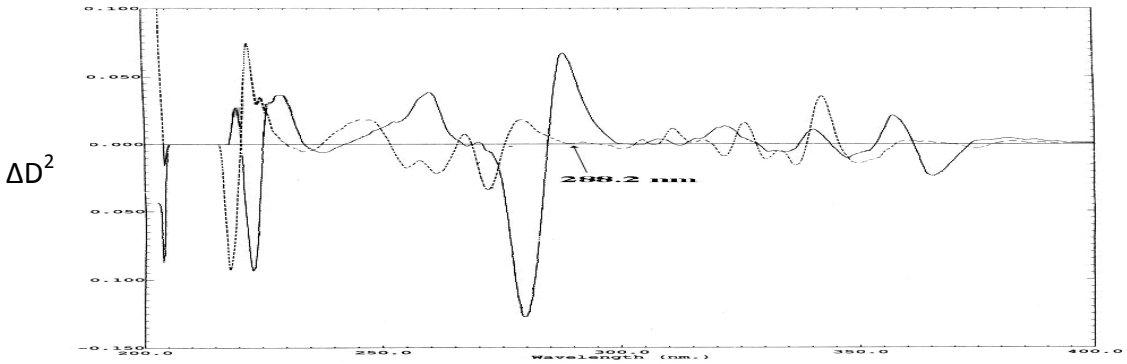
3.4 Second derivative of difference absorption Spectrophotometric method

In this work, a spectrophotometric technique was applied for quantitative determination of GF. The difference absorption spectra (ΔA) of intact and degraded GF acidic solutions against their alkaline solutions showed a severe overlap; as shown in Fig. 5. , which interfere with the analysis of the drug. The (ΔD^2) was applied which intern allowed better resolution showing maximum absorbance of drug and zero for degradation at 288.2 nm as shown in Fig. 6. A linear calibration curve was obtained in the concentration range 4.00- 14.00 µg/ml with mean percentage recovery 98.48 ± 0.70. The parameters of regression equation are shown in table 1.



Wavelength (nm.)

Fig.5. UV scanning of difference absorption spectra (ΔA) of intact (—) and degraded (-----) GF 10 .00 µg/ml in 0.1N HCl against intact and degraded GF 10 .00 µg/ml in 0.1N NaOH.



Wavelength (nm.)

Fig.6. UV scanning of the second derivative of difference absorption spectra (ΔD^2) of intact (—) and degraded (-----) GF 10 .00 µg/ml in 0.1N HCl against intact and degraded GF 10 .00 µg/ml in 0.1N NaOH.

3.5 Methods Validation

3.5.1 Linearity and Range

Linearity range for GF estimation were found to be 0.50 - 15.00 µg/ml for the HPLC method, 0.40 - 1.40 µg/spot for TLC method, and 4.00 - 14.00 µg/ml for the three spectrophotometric methods.

3.5.2 Precision

Precision of the method was made by analysis of three independent drug preparations. The determination of RSD% value obtained from three assay values in order to calculate intra-day variations. These studies were repeated for three different days for the determination of inter-day variation. The RSD% values for intraday and interday variation study were found to be satisfactory (not more than 2) which confirm a suitable precision of the proposed methods as shown in table 1.

3.5.3 Accuracy

Accuracy was established across the specified range of the analytical procedure and the mean percentage recovery ± S.D. was calculated for the five proposed methods. Satisfactory recoveries with small S.D. were obtained, as shown in table. 1. The accuracy was assessed also

by standard addition technique. The results obtained were reproducible with low S.D. as shown in table. 3. As shown from the data in tables 1, 3, excellent recoveries were obtained at each concentration level.

Table 1.The characteristic parameters of regression equations and correlation coefficients of the five proposed methods for the determination of Gemifloxacin.

Parameters	HPLC method	TLC method	D^1 method	DD^1 method	ΔD^2 method
Calibration range	0.50-15.00 µg/ml	0.40 – 1.40 µg/spot	4.00-14.00 µg/ml	4.00-14.00 µg/ml	4.00-14.00 µg/ml
Limit of detection(LOD)	0.04 µg/ml	0.07 µg/spot	0.18 µg/ml	0.58 µg/ml	0.24µg/ml
Limit of quantitation(LOQ)	0.15 µg/ml	0.22 µg/spot	0.59 µg/ml	1.93 µg/ml	0.80µg/ml
Slope (b)	10.59	2.1243	0.0335	0.6379	0.0062
Standard deviation of the slope (Sb)	0.1059	0.0136	0.0009952	0.0176	0.00014
Confidence limit of the slope	10.59 ± 0.2722	2.1243 ± 0.0378	0.0335 ± 0.002557	0.6379 ± 0.0489	0.0062 ± 0.000389
Intercept (a)	-4.932	0.60429	-0.0035	-0.0557	-0.0002
Standard deviation of the intercept (Sa)	0.9288	0.0131	0.009494	0.1667	0.00135
Confidence limit of the intercept	-4.932 ± 2.387	0.60429 ± 0.0364	-0.0035 ± 0.02439	-0.0557 ± 0.4634	-0.0002 ± 0.00375
Correlation coefficient	0.9995	0.9998	0.9978	0.9983	0.9990
Standard error of estimation	1.3969	0.0114	0.008327	0.14617	0.001178
Intra-day % RSD *	0.12-0.65	1.08-1.32	0.75-1.18	0.71-1.90	0.44-1.87
Inter-day % RSD *	0.24-0.81	0.34-1.71	1.41-1.42	0.60-1.92	1.15-1.53
Accuracy	99.41 ± 1.73	98.85 ± 0.83	100.19 ± 1.44	100.26 ± 1.60	98.48 ± 0.70

** The interday and the intraday (n = 3), average of three concentrations (4, 7, 12 µg/ml) for HPLC, (0.4, 0.8, 1.2 µg/spot) for TLC, (6, 8, 10 µg/ml) for D^1, (4, 8, 9 µg/ml) for DD^1 and (6, 8, 12 µg/ml) for ΔD^2 .*

Table 2. System suitability tests for HPLC method for the determination of GF in bulk and in pharmaceutical dosage forms

Parameter	Obtained value	Reference value
Resolution (R)	5.94	R > 0.8
Tailing factor (T)	1.22	< 1.5 – 2 (T=1 for a typical symmetric peak)
Capacity factor (K)	3.15	1 – 10 acceptable
Relative retention (α)	1.48	> 1
No. of theoretical plates (N)	9510.72	Increase with the efficiency of the separation
HETP	2.63×10^{-3}	The smaller the value, the higher the column efficiency

Table3. Application of standard addition technique to the analysis of GF by the five proposed methods

Method	GF Dosage Form	Found (a)%	Conc. Added *	Conc. Found *b	Recovery %	Average recovery (mean ± SD)
HPLC	Factive tab. (b.n.003)	99.85 ± 0.96	1.00 2.00 4.00 6.00 7.00	0.99 1.98 3.97 6.00 7.06	99.00 99.00 99.25 100.00 100.86	99.62 ± 0.80
TLC	Factive tab. (b.n.003)	100.97 ± 0.87	0.40 0.60 0.80	0.40 0.61 0.80	100.00 101.67 100.00	100.56 ± 0.96
D[1]	Factive tab. (b.n.003)	100.43 ± 0.66	4.00 6.00 8.00	4.01 5.93 7.95	100.25 98.83 99.38	99.49 ± 0.72
DD[1]	Factive tab. (b.n.003)	100.33 ± 1.61	4.00 6.00 8.00	4.01 5.98 8.02	100.25 99.67 100.25	100.06 ± 0.33
ΔD[2]	Factive tab. (b.n.003)	99.72 ± 0.48	4.00 6.00 8.00	3.99 5.92 7.86	99.75 98.67 98.25	98.89 ± 0.77

a….Average of six determinations.
b….Average of three determinations.
**…..µg/ml except for TLC method µg/spot*

Table4. Determination of GF in laboratory-prepared mixtures by the five proposed methods.

Method	Sample number	%Degradation	Standard conc.(µg/ml except for TLC µg/spot)	Degradation conc. (µg/ml except for TLC µg/spot)	Recovery %	Average recovery (mean ± SD)
HPLC	1 2 3 4 5 6 7 8 9	10 20 30 40 50 60 70 80 90	9.00 8.00 7.00 6.00 5.00 4.00 3.00 2.00 1.00	1.00 2.00 3.00 4.00 5.00 6.00 7.00 8.00 9.00	97.67 101.38 98.86 97.83 100.80 100.00 101.00 100.50 101.00	99.89 ± 1.42
TLC	1 2 3 4 5 6 7	10 20 30 40 50 60 70	1.26 1.12 0.98 0.84 0.70 0.56 0.42	0.14 0.28 0.42 0.56 0.70 0.84 0.98	101.59 98.21 97.96 97.62 98.57 100.00 100.00	99.14 ± 1.44
D[1]	1 2 3 4 5 6 7	10 20 30 40 50 60 70	12.60 11.20 9.80 8.40 7.00 5.60 4.20	1.40 2.80 4.20 5.60 7.00 8.40 9.80	97.14 101.88 99.59 97.14 100.00 98.21 100.00	99.14 ± 1.73
DD[1]	1 2 3 4 5 6 7	10 20 30 40 50 60 70	12.60 11.20 9.80 8.40 7.00 5.60 4.20	1.40 2.80 4.20 5.60 7.00 8.40 9.80	101.75 99.12 101.12 100.48 100.57 101.25 101.67	100.85 ± 0.91
ΔD[2]	1 2 3 4 5 6 7	10 20 30 40 50 60 70	12.60 11.20 9.80 8.40 7.00 5.60 4.20	1.40 2.80 4.20 5.60 7.00 8.40 9.80	97.22 100.54 101.73 97.98 101.29 100.89 101.67	100.19 ± 1.83

3.5.4 LOD and LOQ

The LOD and LOQ of the proposed methods were determined using calibration standards. LOD and LOQ were calculated as 3 σ/S and 10 σ/S respectively where S is the slope of the calibration curve and σ is the standard deviation of the response. LOD and LOQ values of the five proposed methods were assessed and given in table 1. These low values indicated the good sensitivity of the proposed methods.

3.5.5 Selectivity

The five proposed methods were applied for the determination of the drug in laboratory prepared mixture with its acidic degradation products. The mean percentage recovery ± S.D. of

intact in the laboratory mixtures for these methods were calculated. They are successfully applied for the determination of GF in the presence of its acidic degradation products up to 90% for HPLC method and up to 70% for TLC and spectrophotometric methods as shown from data in table 4.

3.5.6 Statistical analysis of the results

A statistical analysis of the results obtained by the proposed methods and the manufacturer method [21] was carried out. The test ascertained that there was no significant difference among the methods with respect to t-test and F-ratio as shown in table. 5.

Table5. Statistical comparison between the results obtained by the five proposed methods for the analysis of Gemifloxacin and the manufacturer method.

Statistical term	HPLC method	TLC method	D^1 method	DD^1 method	ΔD^2 method	manufacturer method**
Mean	99.41	98.85	100.19	100.26	98.48	99.93
S.D.	1.73	0.83	1.44	1.60	0.70	1.27
S.E.	0.71	0.37	0.59	0.65	0.31	0.57
Variance	3.00	0.69	2.07	2.56	0.49	1.61
N	6	5	6	6	5	5
t-test	0.57 (2.26)*	1.59 (2.31)*	0.32 (2.26)*	0.38 (2.26)*	2.23 (2.31)*	
F-ratio	1.86 (6.26)*	2.33 (6.39)*	1.29 (6.26)*	1.59 (6.26)*	3.29 (6.39)*	

** Figures in parenthesis are the theoretical t and F values at confidence limit 95%.*

*** HPLC method according to company file.*

CONCLUSION

The proposed five methods are simple, rapid, accurate and precise and can be used for the analysis of GF in pure form and in pharmaceutical dosage form (either alone or in the presence of its degradation products). The sample recovery for all five methods was in good agreement with their respective label claims which suggested no interference of formulation additives in estimation.

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