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SIMULTANEOUS DETERMINATION OF GEMIFLOXACIN AND DIURETICS IN BULK, PHARMACEUTICAL DOSAGE FORMS AND HUMAN SERUM BY RP-HPLC

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An isocratic reversed phase high-performance liquid chromatographic (RP-HPLC) method has been developed for the simultaneous determination of gemifloxacin and diuretics (hydrochlorothiazide and furosemide) in bulk, dosage formulations and human serum at 232 nm. Chromatographic separation was achieved on Purospher Start C_{18} (250 mm x 4.6 mm, 5 µm) column using mobile phase, methanol: water: acetonitrile (70:25:5 v/v/v) adjusted to pH 3.0 via phosphoric acid 85% having flow rate of 0.8 mL min^{-1} at room temperature. Calibration curves were linear over range of 0.5-10 µg mL^{-1} with a correlation coefficient ± 0.999. LOD and LOQ were in the ranges of 0.75-2.56 µg mL^{-1}. Intra and inter-run precision and accuracy results were 98.26 to 100.9.

Keywords: gemifloxacin; diuretics; HPLC.

INTRODUCTION

Gemifloxacin (GFX) is a fourth generation fluoroquinolone antibacterial compound with enhanced affinity for bacterial topoisomerase IV and is being used for the treatment of respiratory and urinary tract infections. The compound has a broad spectrum of activity against Gram-positive and Gram-negative bacteria.[1-3] It is particularly active against Gram-positive organisms including penicillin-, macrolide-, and quinolone-resistant *Streptococcus pneumoniae*,[4] 4-folds more potent than moxifloxacin against *S. pneumoniae*.[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.[6,7] Furthermore, the compound has shown potent activity against many organisms that cause urinary tract infections and bronchitis.[8]

Diuretics are widely used in the treatment of congestive heart failure and hypertension.[9, 10] Mostly increase urinary potassium excretion and thus can cause hypokalemia in patients with prolong use. Diuretics selected for study are hydrochlorothiazide (HCT) and furosemide (FUR) which are thiazide and loop diuretics, respectively.[11]

Literature survey revealed that few analytical methods have been reported for the estimation of GFX; they include high performance liquid chromatography tandem mass,[12,13] microchip electrophoresis,[14] chiral high-performance liquid chromatography,[15] and chiral counter-current chromatography.[16, 17] Simple and sensitive ion-pairing spectrophotometer methods have been described for the assay of gemifloxacin mesylate by Marothu *et al.*. [18] Barbosa *et al.* studied dissociation constants of series of compounds including diuretics and quinolones in several acetonitrile: water mixtures.[19]

Work done by Sudoh *et al.*[20] reveals that concomitant administration of furosemide and lomefloxacin increases the bioavailability of lomefloxacin by decreasing its rate of renal clearance. As quinolones, furosemide and hydrochlorthiazide,[21] are reported to be excreted in urine by the renal tubular anion transport system. Therefore, in present paper, we report a simple, easy, quick and inexpensive isocratic RP-HPLC method with ultraviolet detection at 232 nm for the simultaneous determination of GFX and two diuretics i.e. HCT and FUR. Simultaneous determination of both drugs is desirable as this would allow more efficient generation of clinical data and could be performed at more modest cost than separate assays. The method is equally valid for the determination in bulk materials, pharmaceutical dosage formulations and human serum. This method can be used for the quantitative analysis of diuretics and gemifloxacin alone or in combination. The low LOD and LOQ values merit the method for the determination of these drugs in clinical samples.

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EXPERIMENTAL

Materials and reagents

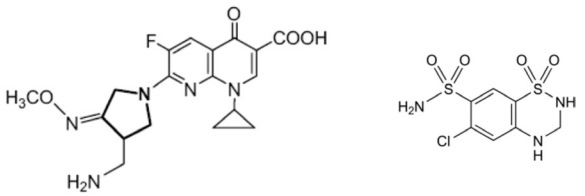
All chemicals and reagents were of analytical grade. Gemifloxacin (purity 99.82%) was a kind gift from PharmEvo (Pvt) Limited, Pakistan. Hydrochlorothiazide (purity 99.94%) and furosemide (purity 99.79%) were gifts from Zafa Pharmaceutical Laboratories (Pvt) Ltd and Sanofi Aventis (Pvt) Limited, Pakistan. HPLC grade acetonitrile, methanol and phosphoric acid were obtained from Tedia (USA) and Merck Darmstadt, Germany.

Pharmaceutical dosage form

Gemixa™ (Gemifloxacin 320 mg tablets by Bosch Pharmaceuticals (Pvt) Ltd), Diuza™ (Hydrochlorothiazide 25 mg tablets by Zafa Pharmaceutical Laboratories (Pvt) Ltd**),** and Lasix™ (40 mg tablets from Sanofi Aventis Pakistan Limited), were purchased from the local pharmacies (Figure 1). All these drugs had an expiry of not less than 1 year at the time of study.

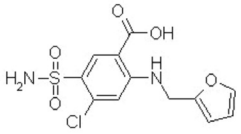
Instrumentation

The HPLC system consisted of an LC-10 AT VP Shimadzu pump, SPD-10AV VP Shimadzu UV visible detector, a Purospher Start C_{18} (250 x 4.6 mm, 5 µm) column was used for separation. The chromatographic system was integrated using a CBM-102 communication Bus



Gemifloxacin mesylate

Hydrochlorothiazide



Furosemide

Figure 1. *Chemical structures of Gemifloxacin mesylate, Hydrochlorothiazide and Furosemide*

Module Shimadzu with a Pentium™ IV PC loaded with Class GC software for data acquisition. Separation was carried out under isocratic elution with methanol: water: acetonitrile (70:25:5) as mobile phase. The pH of the mobile phase was adjusted to 3.0 with phosphoric acid (85%), sonicated by DGU-14 AM on-line degasser, and filtered through 0.45-micron membrane filter. The flow rate was 0.8 mL min^{-1}, the elution was monitored at 232 nm, and the injection volume was 20 µL.

Preparation of standard and sample solutions

Standard preparation

Stock standard solutions 100 ppm of GFX, HCT and FUR were prepared in 100 mL mobile phase as solvent. Working solutions were prepared separately by making serial dilutions from the standard solution to obtain concentration between 0.5-10, 0.125-2.5 and 0.025-0.5 µg mL^{-1} for GFX, HCT and FUR, respectively. These solutions were stored at 20 ºC. Once prepared, analyzed daily for inter and intra-day variations of the method. 20 µL of these solutions were injected into LC system and chromatographed.

Procedure for tablets

Twenty tablets of each formulation were powdered finely and an amount equivalent to 10 mg of GFX, HCT and FUR was weighed and then dissolved in the mobile phase. Solutions were then filtered through ordinary filter paper. The desired concentrations 0.5-10, 0.125-2.5 and 0.025-0.5µg mL^{-1} for GFX, HCT and FUR, respectively were obtained by accurate dilution, solutions were then sonicated. Finally, all the solutions were filtered through 45-µm Millipore filter, in order to separate out the insoluble excepients before chromatographed.

Procedure for human serum

Plasma samples, obtained from healthy volunteers, were collected and stored. To 1.0 mL of plasma, 9.0 mL of acetonitrile was added; the mixture was vortexed for 1 min and than centrifuged for 10 min at 10,000 rpm and the supernatant was filtered by 0.45-micron membrane filter. An aliquot of serum sample was fortified with GFX, HCT and FUR to achieve final concentration.

RESULTS AND DISCUSSION

Development and optimization of isocratic HPLC conditions

The aim of the present study was to develop a simple, isocratic, accurate and sensitive HPLC method for the simultaneous determination of GFX, HCT and FUR. A UV scan showed a maximal absorbance at or near 232 nm. Initial method development was conducted on a Purospher Start C_{18} (250 x 4.6 mm, 5 µm) column for separation at ambient temperature. This column provides efficient and reproducible separations of non-polar compounds while minimizing solvent usage. Initially various mobile phases were tested to obtain the best separation and resolution. The mobile phase consisting of methanol, water and acetonitrile in the ratio of 70:25:5, v/v/v found to have good resolution. The chromatographic conditions were optimized to achieve best separation and to get best resolution between analytes and to optimize chromatographic parameters like resolution, tailing factor and retention time. The optimized conditions were reached at pH 3.0, producing well resolved and sharp peaks for all drugs. The specificity of the method was established through the study of resolution factor of gemifloxacin peak. Peaks were identified using retention times compared with those of standards. For validation of analytical methods, the guidelines of the International Conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use,[22] and USP 2002,[25] were followed for the accuracy tests, precision, specificity, linearity, work strip and robustness of the method. Retention time of GFX was 2.8 min, HCT 3.4 min and FUR was 4.2 min, at a flow rate of 0.8 mL min^{-1}.

System suitability

It is an imperative module of method validation to make certain that the operational system is running appropriately throughout the analysis. The system was equilibrated with the initial mobile phase composition, followed by 10 injections of the same standard. These 10 consecutive injections were used to evaluate the system suitability on each day of method validation (Table 1).

Table 1. System suitability parameters of the proposed method for gemifloxacin, hydrochlorothiazide and furosemid

Drugs	Retention time (t_R)	Capacity factor (K')	Tailing factor (T)	Resolution (R)	Theoretical plates (N)	Separation factor(α)
GFX	2.8	2.4	1.34	1.47	1782	4.03
HCT	3.4	3.07	1.4	2.09	2621	1.28
FUR	4.2	3.95	1.5	2.6	3075	1.29

GFX: Gemifloxacin; HCT: Hydrochlorothiazide; FUR: Furosemide

Linearity

Linearity is generally reported as the variance of the slope of the regression line. Linearity was tested with known concentrations of GFX, HCT and FUR i.e. 0.5, 1.0, 2.5, 5 and 10, 0.125, 0.25, 0.625, 1.25 and 2.5 and 0.025, 0.05, 0.125, 0.25 and -0.5 µg mL^{-1}, respectively. Five runs were performed for every concentration. Injected concentrations versus area were plotted and the correlation coefficients were calculated which are shown in Table 2.

Accuracy

The accuracy of an analytical procedure measures the closeness of measured values to the true values. It was evaluated as percentage relative error between the measured mean concentrations and taken concentrations.[22-24] Minimal of 3 concentration levels covering the specified ranges were selected and three runs were performed for every concentration and then peak area was calculated as given in Table 3.

Table 2. Regression characteristics of the proposed method for gemifloxacin, hydrochlorothiazide and furosemide

Drugs	GFX	HCT	FUR
Conc. range (μg mL^{-1})	0.5-10	0.125-2.5	0.025-0.5
Correlation coefficient (r^2)	0.9992	0.9992	0.9992
Standard error of estimate	1.28	1.26	1.29
Standard error	0.82	0.81	0.83
Intercept	2.02	2.52	2.1
Slope	6020	5489	5529

GFX: Gemifloxacin; HCT: Hydrochlorothiazide; FUR: Furosemide

Table 3. Accuracy of the proposed method for gemifloxacin, hydrochlorothiazide and furosemide

Parameters	Conc. (μg mL^{-1}) Spiked	Conc. found			%Recovery		
		GFX	HCT	FUR	GFX	HCT	FUR
Assay in bulk	4.0	3.93	3.934	3.943	98.26	98.36	98.57
	5.0	4.968	4.968	4.965	99.37	99.37	99.3
	6.0	6.018	6.013	6.05	100.2	100.3	100.9
Assay in serum	4.0	3.94	3.94	3.951	98.46	98.46	98.62
	5.0	4.97	4.974	4.968	99.47	99.47	99.4
	6.0	6.02	6.02	6.057	100.3	100.3	100.9

GFX: Gemifloxacin; HCT: Hydrochlorothiazide; FUR: Furosemide

Intraday and inter-day precision

The precision of the method was investigated with respect to repeatability. For intra-day and inter-day precision, ten samples of five concentrations were analyzed on the same day and after one day (Table 4). Generally acceptable repeatability of the results with in one day and day-to-day was observed.[22-24] The precision of the method was analyzed as % RSD throughout the linear range of concentrations.

Table 4. Inter and intra-day precision of the proposed method for gemifloxacin, hydrochlorothiazide and furosemide (n=6)

Drug	Concentration (μg mL^{-1})	Formulation (%RSD)		Serum (%RSD)
		Intra-day	Inter-day variations	Intra-day
GFX	0.5	0.02	0.03	0.08
	1	0.03	0.04	0.04
	2.5	0.09	0.05	0.02
	5	0.06	0.07	0.09
	10	0.08	0.07	0.92
HCT	0.125	0.02	0.05	0.07
	0.25	0.05	0.04	0.06
	0.625	0.01	0.03	0.09
	1.25	0.07	0.05	0.09
	2.5	0.06	0.04	0.8
FUR	0.025	0.02	0.03	0.06
	0.05	0.1	0.07	0.12
	0.125	0.16	0.05	0.03
	0.25	0.06	0.06	0.09
	0.5	0.02	0.02	0.03

GFX: Gemifloxacin; HCT: Hydrochlorothiazide; FUR: Furosemide

Robustness

Robustness of the method was accomplished by designed modifications made to the method parameters such as composition, flow rate, pH of the mobile phase, detection wavelength, injection volume and column temperature (Table 5) and it was found that the %R.S.D values did not exceed more than 2%.[22-24]

Table 5. Robustness of the proposed method

Factors	Level	t_R	k	T
pH				
2.8	-2	2.6	2.45	1.32
3	0	2.8	2.4	1.34
3.2	2	3	2.35	1.36
Flow rate (mL min $^{-1}$)				
0.6	-2	3	2.35	1.36
0.8	0	2.8	2.4	1.34
1.2	2	2.6	2.45	1.32
Percentage of methanol in mobile phase (v/v)				
65	-5	2.9	2.3	1.36
70	0	2.8	2.4	1.34
75	5	2.6	2.6	1.32

Limit of detection and quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) of this method were determined from the known concentrations of GFX, HCT and FUR. The LOD and LOQ for this assay were calculated from three and ten times the noise level of the response, respectively. Which are given in Table 6.

Table 6. LOD and LOQ of the proposed method for gemifloxacin, hydrochlorothiazide and furosemide

Drugs	Conc. (μg mL^{-1})	LOD (μg mL $^{-1}$)	LOQ (μg mL $^{-1}$)
GFX	0.5-10	0.77	2.35
HCT	0.125-2.5	0.85	2.56
FUR	0.025-0.5	0.84	2.55

GFX: Gemifloxacin; HCT: Hydrochlorothiazide; FUR: Furosemide; LOD: Limit of Detection; LOQ: Limit of Quantification

Specificity and selectivity

The specificity of the chromatographic method was determined to ensure separation of gemifloxacin and diuretics as shown in Figure 2. Specificity was also determined by screening four different samples of controlled human serum, which were free from interfering endogenous plasma components. Solutions of placebo, gemifloxacin and diuretics were prepared and then injected to check for interference from common excepients**.**

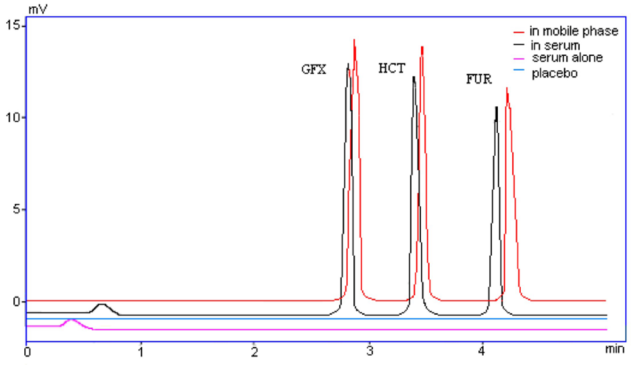


Figure 2. *Chromatograms of GFX (50 ppm), HCT (12.5 ppm) and FUR (25 ppm) in mobile phase, human serum, human serum alone and placebo at 232 nm*

Ruggedness

The ruggedness was established by determining GFX, HCT and FUR in dosage formulation and in human serum using same and different chromatographic system and same column by different analysts on different days. The assay results indicated that the method was capable with high precision (Table 4).

CONCLUSION

A simple and reliable HPLC method for monitoring GFX, HCT and FUR in human serum and pharmaceutical dosage formulation has been developed. A fully validated RP-HPLC procedure for the assay of these drugs in bulk, tablets and human serum is described for the first time. Hence, it can be recommended for the routine quality control of these drugs, low volume of blood or plasma is needed. Simplicity of the separation procedure; shorter run time and the low volume of injection make this method suitable for quick and routine analysis. The intra-run and inter-run variability and accuracy results were also in acceptable limit. In addition, this method has the potential application to clinical research of drug combination, multi-drug pharmacokinetics and interactions.

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