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Original Article

RAPID AND VALIDATED HPLC-UV METHOD FOR DETERMINATION OF GEMIFLOXACIN IN HUMAN URINE

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ABSTRACT

Objective: To develop and validate a simple and rapid reversed phase high performance liquid chromatographic (RP-HPLC) method for the determination of Gemifloxacin (GFX) in human urine.

Methods: GFX was isolated from urine samples after acidification using methylene chloride. Good chromatographic separation was achieved using C_{18} Ultrasphere (250 mm × 4.6 mm, 5 µm.) analytical column maintained at 25 °C. The mobile phase consisted of methanol and 0.1 M phosphate buffer pH 3 in the ratio of (48: 52, v/v), respectively. The analysis time was 10 min at a 1.0 ml/min flow rate. The UV detection was carried out at 272 nm.

Results: GFX has been eluted at 7.5 min. Linearity was obtained over a concentration range of 20-200 ng/ml ($r2$>0.999). The extraction recovery of GFX from urine samples was 60%. The proposed method demonstrated excellent intra-and inter-day precision and accuracy within 1.19% and 100.65 %, respectively. The limit of detection (LOD) was found to be 6.3 ng/ml.

Conclusion: Simple and accurate RP-HPLC method for determination of GFX in human urine was developed and validated. The method was successfully applied for determination of GFX in human urine samples from healthy volunteers up to 24 hours after oral administration of 320 mg gemi floxacin tablets.

Keywords: Gemifloxacin, RP-HPLC, Human urine.

INTRODUCTION

Since the introduction of nalidixic acid as the first quinolone; various structural modifications have been introduced, that resulted in production of second-, third-, and fourth-generation fluoroquinolones with improved antibacterial activity and pharmacological properties [1]. The US Food and Drug Administration (FDA) has been approved Gemifloxacin as a fourth generation fluoroquinolone [2]. Gemifloxacin [(R,S)-7-[(4Z)-3-(aminomethyl)-4-(methoxyimino)-1-pyrrolidinyl]-1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo1,8-naphthyridine-3-carboxylic acid] (fig. 1), is a fluoroquinolone antibacterial agent, it is active against both gram-positive and gram-negative bacteria [2-4]. Gemifloxacin used for the treatment of urinary tract and respiratory tract infections [5, 6]. It has been found that Gemifloxacin was four times more potent than moxifloxacin against *S. pneumonia* (including multi-drug resistant strains [MDRSP]) [7, 8]. Gemifloxacin has two mechanisms of action. Firstly; depends on blocking of bacterial DNA replication by binding to an enzyme called DNA gyrase (topoisomerase II), which allows the untwisting required to replicate one DNA double helix into two. Notably the drug has 100 times higher affinity for bacterial DNA gyrase than for mammalian ones. Secondly, The bactericidal action of Gemifloxacin results from inhibition of the enzymes topoisomerase II (DNA gyrase) and topoisomerase IV, which are required for bacterial DNA replication, transcription, repair, and recombination [9]. The important aspect of the routine use of any drug is to understand the correlations of drug levels with drug action. The accurately quantifying of the drugs in biological matrices such as blood, serum, urine, and tissue samples is important for therapeutic drug monitoring. Validated results are essential for accurate drug monitoring, clinical decisions and further treatment options. Accurate, reproducible, and specific method for the quantification of Gemifloxacin is compulsory. Therefore several methods have been developed for the analysis of Gemifloxacin in pharmaceutical preparations or human plasma such as spectrophotometric techniques [10–19], capillary electrophoresis [20], high performance liquid chromatography [21–29], LC-mass spectrometry [30–33], microchip electrophoresis [34], chemiluminescence [35] and potentiometry [36]. In view of the fact that more than 20% of Gemifloxacin is excreted unchanged in urine, so determination of Gemifloxcin in urine samples may be a useful analytical tool. However, as much as we know; there is no published method for determination of Gemifloxacin in human urine using reversed phase high performance liquid chromatographic method with UV-detector. Urine is a more readily available biological medium compared to plasma. It is easier to be obtained and less invasive. The aim of this work is to develop and validate a simple and rapid method for determination of Gemifloxacin in human urine.

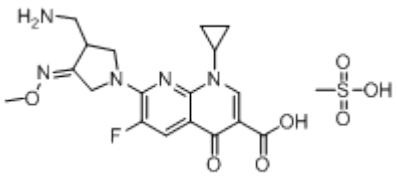

Fig. 1: Chemical structure of Gemifloxacin

MATERIALS AND METHODS

Reagents and chemicals

All the chemicals were of Analytical grade, and the solvents were of HPLC grade. High purity water was obtained by filtration of distilled water through 0.45 µm membrane filter (Millipore, Ireland) and it was used throughout the study. Gemifloxacin mesylate (Purity,>98%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Factive® tablet equivalent to 320 mg Gemifloxacin was obtained from local market. Blank human urine was collected from healthy volunteer's stored at-20 °C. Methanol was obtained from Tedia Company (1000 Tedia Way, Fairfield, OH 45014, USA). Phosphate buffer 0.1M was prepared from sodium dihydrogen phosphate and pH was adjusted by orthophosphoric acid. Anhydrous sodium di hydrogen phosphate monohydrate and orthophosphoric acid were purchased from El-Nasr Pharmaceutical Chemicals Company (ADWIC) (Cairo, Egypt).

Instrumentation

The HPLC system consisted of a Shimadzu LC-20AD Prominence liquid chromatogram equipped with a Rheodyne injector valve with

a 20 µl loop and a SPD-20A UV detector operated at 272 nm. C18 (Thermo, Beckman coulter™, USA) ultra sphere column (250 mm × 4.6 mm, 5 µm.),

Standards and quality control samples preparation

A 1.0 µg/ml stock solution was prepared in distilled water. Working solutions were prepared by serial dilutions of the stock solution. Calibration standards were prepared in human urine at 20, 30, 50, 90, 100, 110, 170 and 200ng/ml. Low, med and high quality control (QC) samples were prepared in human urine at concentration levels of 30, 100 and 170 ng/ml, respectively. All solutions were filtered through a 0.45 µm filters to eliminate any solid impurity. All samples were protected from light using aluminum foil and stored at-20 ºC until being used for analysis.

Liquid-liquid extraction (LLE) procedures

The urine standards/quality control samples were filtered using 0.45 µm filter to eliminate any solid impurities. Extraction of Gemifloxacin from human urine was achieved using one ml aliquot of each sample and five ml methylene chloride; then acidified with one ml (1N) hydrochloride. The samples were vortex mixed for 10 min, and centrifuged at 3500 rpm for 5 min at room temperature (RT). Four ml of methylene chloride from each sample was quantitavely transferred into a clean glass tube. Samples were then evaporated to dryness under a stream of nitrogen for about 5 min. The dried extracts were reconstituted in 0.2 ml (methanol: 0.1 M sodium dihydrogen phosphate (48:52 v/v), pH 3 prior to injection and a 20 µl was injected.

Chromatographic conditions

Isocratic elution using mobile phase consisted of a mixture of methanol and 0.1 M sodium dihydrogen phosphate (48:52 v/v), adjusted at pH 3 with orthophosphoric acid. The mobile phase was filtered through 0.45 µm Millipore membrane filter and degassed by sonication for 30 min before use. Detection was achieved at 272 nm. The column was operated at 25 °C. Run time was 10 min at a flow rate of 1.0 ml/min.

Validation

The resulting HPLC-UV method for determination of Gemifloxacin in human urine was validated according to the FDA guidelines [37]. Selectivity, linearity, accuracy, precision, recovery, stability and sensitivity experiments were performed. The calibration curves were evaluated based on back-calculated standard results.

Selectivity is the ability of a method to determine accurately the analyte of interest in the presence of other components in a sample matrix under the stated conditions of the test. To demonstrate the specificity of the analytical procedure, six different blank urine samples obtained from different healthy volunteers were analyzed for peaks interfering with the detection of the analytes.

The linearity of the method was determined at seven different concentrations that ranged from 20 to 200 ng/ml. Each concentration was injected three times.

The accuracy of an analytical procedure measures the closeness of measured values to the true values. It was evaluated as the percentage relative error between the measured mean concentrations and taken concentrations. Low, med and high QC samples at concentration levels of 30, 100 and 170 ng/ml, respectively, were analyzed through three validations.

The precision of the method was investigated with respect to repeatability. For intra-day and inter-day precision and acceptable repeatability of the results within one day and day-to-day was observed. The precision of the method was analyzed as % RSD throughout the linear range of concentrations.

Extraction recovery from human urine was determined by comparing the analyte responses of pre-extraction spiked samples at three sets of low, mid, and high quality control concentrations to those of post-extraction spiked samples. Each concentration repeated three times.

The stability of Gemifloxacin in urine samples was investigated by preparing spiked samples at two different concentrations (30 and170 ng/ml) three times. Freeze/thaw stability was calculated by analyzing low quality control samples (30 ng/ml) and high quality control samples (170 ng/ml) over three freeze/thaw cycles (n = 3). Samples were frozen at – 20 ºC for at least 24 hours for the first cycle; thawed at room temperature and then frozen at –20 ºC for at least 12 hours for each freeze/thaw cycle. Stability in thawed matrix was evaluated by analyzing low quality control (30 ng/ml) and high quality control (170 ng/ml) samples (n =3) from –20 °C storage. Thawed matrix was allowed to sit at room temperature for 4 hours and 24 hours before extraction. Long term stability was investigated at low and high quality control sample levels after storing for 30 days at-20 °C.

To investigate the effect of variable endogenous matrix components from multiple individuals, quality control samples (n = 3) at a concentration of 30 ng/ml of Gemifloxacin were spiked into six different human blank urine samples from six different individuals, extracted and analyzed as described above.

The sensitivity of the analytical technique was expressed as the limit of detection (LOD) which was determined as the lowest concentration of analyte that produce peak height at 3: 1 the noise level and the limit of quantitation (LOQ) which was determined as the lowest concentration of analyte that produce peak height at least at 10: 1 the noise level and could be determined with adequate precision of 20% and accuracy of 80–120%.

Robustness of the method was accomplished by designed modifications made to the method parameters such as composition, flow rate and pH of the mobile phase.

The unchanged portion of GFX that excreted in urine will be tremendously higher than the validated calibration curve. The ability to analyze urine samples originally above the upper limit of the calibration range was validated by analyzing three replicates of a 100 ng/ml QC sample as 10000-fold dilutions with blank urine.

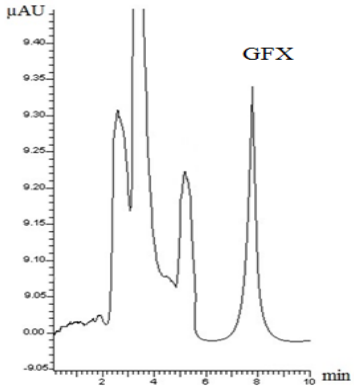

Fig. 2: HPLC-UV chromatogram of (200 ng/ml) of Gemifloxacin spiked urine sample

RESULTS AND DISCUSSION

Method development

HPLC method coupled to UV detection was developed and fully validated for the determination of Gemifloxacin with satisfactory accuracy and precision. This method was applied for the analysis of the studied drug in spiked human urine using the rapid and simple procedure. The method was optimized to obtain acceptable theoretical plates and a good separation with sufficient sensitivity and suitable peak symmetry. RP-C_{18}column was suitable for separation of GFX from other endogenous components in human urine with adequate resolution. The chromatographic conditions were optimized through several trials to achieve optimum resolution, high sensitivity, and symmetrical peak shape for Gemifloxacin. Different organic modified such as methanol and acetonitrile with different percentages of phosphate buffer were tested. An isocratic mobile phase with a mobile phase consists of methanol and phosphate buffer pH 3 (48:52, v/v) showed

excellent peak shape and chromatographic resolutions from endogenous matrix component peaks (fig. 2); repeated injections showed no co-elution of any interfering peak with target analyte peak. The elution of the GFX was achieved at 7.5 min and a run time of 10 min. Precision of retention time was examined to evaluate the system suitability. Intra-day repeatability (n=5) and inter-day precision (n=11) were evaluated. The optimized chromatographic conditions showed excellent peak shape and repeatability in addition to rapid determination within 10 min.

Method validation

A reversed phase liquid chromatographic method coupled with UV detector was developed to provide a rapid analysis of GFX. The most important advantage of LC method is the achievement of good resolution with acceptable peak symmetry in a reasonable analysis time. However, fully validated method is also mandatory to ensure the validity of the method for accurate and precise determination and monitoring GFX in human urine samples.

Specificity

The proposed method showed good specificity in blank urine samples from six different individuals. There was no significant chromatographic peak (greater than 20% of the mean response at the lower limit of quantification) detected at the expected retention times of the target analyte. No co-elution with endogenous matrix component peaks was observed after several injections (more than 50 injections)

Linearity and correlation coefficient

The average correlation coefficient from three validation runs was>0.999 which was evaluated by the least-squares regression method. Precision of the calibrators (measured as the percent relative standard deviation %RSD) of back calculated standards was within 3.3%. Accuracy of the residuals (measured as the percent difference from the nominal concentration %DFN) was within ±5.0%. LLOQ was established as the lowest concentration on the calibration curve with accuracy and precision within±20% (20 ng/ml), LOD was found to be 6.3 ng/ml and linearity was obtained over the concentration range of (20 ng/ml to 200 ng/ml). Sensitivity of the proposed method makes the method comparable to other more advanced hyphenated techniques such as LC-MS which may be not available in all research laboratories.

The high r^2value gave an indication of the good linearity, and the low values of standard deviations of the intercept and the slope gave an indication of the significant validity of the calibration points used for constructing the calibration curve.

Precision and accuracy

The precision and accuracy at the low, medium and high concentration of GFX in urine were evaluated as shown in table 1. Intra-and inter-day precision (relative standard deviations % RSD) was within 1.42 and 1.19%, respectively. Intra-and inter-day accuracy was less than±3%.

Table 1: Intra-and inter-day precision and accuracy calculated from quality control samples for GFX

QC level	Conc. ng/ml	Inter-day (n=11)			Intra-day (n=5)		
		mean±SD	Precision RSD	Accuracy %DFN	mean±SD	Precision %RSD	Accuracy %DFN
Low	30.00	29.58±0.002	0.007	-1.38	29.32±0.001	0.0030	-2.26
Medium	100.0	97.46±0.19	0.199	-2.54	97.38±0.002	0.0020	-2.62
High	170.0	171.0±2.03	1.190	0.65	171.82±2.43	1.42	1.07

Recovery

LLE is known to provide clean extracts in addition to simplicity and low cost in comparison to solid phase extraction (SPE). Optimization of the transfer of analytes from urine matrix to the organic layer is essential to obtain the highest possible extraction recovery; different extracting solvents and pHs were investigated. The aim of sample clean-up procedures, not only high extraction recovery, but also cleanliness of the extracts which can be confirmed by the elution profiles of unknown endogenous component peaks in blank samples. On the other hand, absence of co-elution and interferences are another advantages of the proposed method. The extraction recovery of the analyte from urine matrix using the optimized liquid-liquid extraction method was 58, 56 and 60%, for 30, 100 and 170 ng/ml, respectively.

Stability studies

All stability experiments showed precision within 2.0% and accuracy within±0.5 % (table 2)

Dilution integrity

The intra-assay precision and accuracy for the diluted QC pools and over the curve diluted samples showed was within the acceptable ranges.

Inter-subject variability

Variable endogenous matrix components from different donors may significantly affect precision and accuracy of the results. Validity of the method in different matrix sources should be investigated during method development and validation. Inter-subject variability experiments showed acceptable accuracy and precision within±4.6 % (table 3). The proposed chromatographic conditions showed excellent resolution in different urine sources.

Monitoring Gemifloxacin in human urine after oral administration

Blank urine was collected from three healthy volunteers free from Gemifloxacin and stored at −20 ° C. Non-smokers and healthy volunteers participated in the study after giving informed written consent. After an overnight fast, the volunteers reported to the study centre received a single dose of Factive® tablet (labelled to contain Gemifloxacin mesylate equivalent to 320 mg Gemifloxacin per tablet) in 150 ml water. Urine samples were collected from each volunteer after: 0 (pre-dose), 2, 4, 6, 8, 12 and 24 h. For each collection period, 10 ml aliquot was retained and stored at −20 °C. 0.1 ml of each sample was diluted to 1000 ml with blank urine. 0.1 ml of each sample was analyzed as in section 2, 3, 2; endogenous matrix component peaks were chromatographically resolved from target peak (fig. 3). As shown in fig. 4; the proposed method was found to be useful for accurate determination and monitoring of the unchanged GFX in urine samples after oral administration as well as any further pharmacokinetics and toxicity studies.

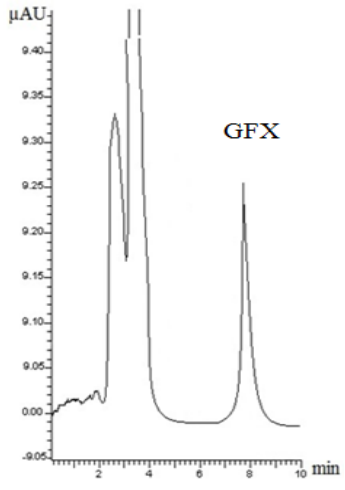

Fig. 3: HPLC-UV chromatogram of Gemifloxacin in human urine sample collected after 24 hours of oral administration

Table 2: Precision and accuracy calculated from stability experiments for GFX

Experiment (n=3)	Concentration (ng/ml)	Mean±SD (ng/ml)	Precision (%RSD)	Accuracy (%DFN)
Freeze/thaw stability	30.00	29.31±0.280	0.960	-0.030
(3cycles,-20 ºC)	170.0	169.63±0.58	0.580	-0.0020
Thawed matrix stability	30.00	29.70±0.550	1.850	-0.0090
(4 hours at RT)	170.0	169.10±0.84	0.499	-0.0050
Thawed matrix stability	30.00	28.84±0.500	1.740	-0.390
(24 hours at RT)	170.0	169.09±1.06	0.630	-0.0050
Long term stability	30.00	27.48±0.250	0.910	-0.080
(1 month at-20 ºC)	170.0	168.57±0.86	0.510	-0.0080

Table 3: Inter-subject variability experiment results of six different urine samples fortified with Gemifloxacin

Individual source	Concentration (ng/ml)	Mean (±SD) (ng/ml)	Precision (%RSD)	Accuracy (%DFN)
1	30.00	28.71±0.64	2.24	-0.040
2	30.00	29.69±1.18	3.98	-0.010
3	30.00	31.28±1.42	4.56	0.040
4	30.00	28.87±0.93	3.23	-0.040
5	30.00	28.89±0.56	1.93	-0.040
6	30.00	29.09±1.03	3.53	-0.030

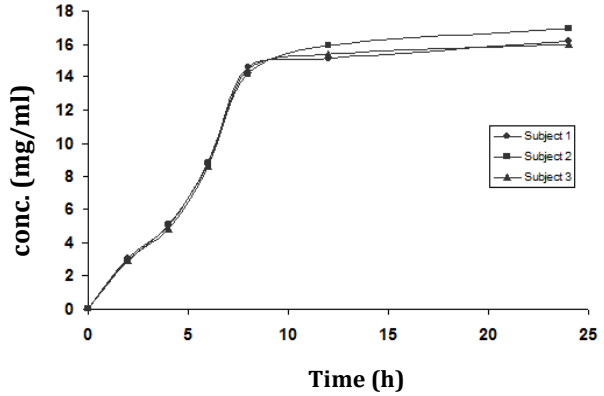

Fig. 4: The concentration-time curve of Gemifloxacin in urine from healthy subjects after oral administration of 320 mg Gemifloxacin tablet

CONCLUSION

A simple, precise and reproducible HPLC-UV method for determination of Gemifloxacin in human urine has been developed. This study provided a simple and low cost liquid–liquid extraction method compared with other methods such as solid-phase extraction. The sensitivity of the assay was satisfied and the method was successfully applied to the analysis of Gemifloxacin in human urine samples after oral administration of Gemifloxacin capsules. The method was fully validated with sufficiently precise, accurate results.

CONFLICT OF INTERESTS

Declared None

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