

HPLC and Densitometric TLC Methods for Simultaneous Determination of Gemifloxacin with Some Co-administered Drugs in Human Plasma

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Abstract

Two chromatographic methods have been developed for determination of gemifloxacin in human plasma with three co-administered drugs, theophylline, warfarin and omeprazole. First method depends on reverse phase high performance liquid chromatography. The plasma sample was extracted using acetonitrile. The method was linear over the concentration range 0.05 to 6 µg/mL, 0.25 to 8 µg/mL, 0.1 to 10 µg/mL and 0.1 to 6 µg/mL of gemifloxacin mesylate, theophylline, warfarin and omeprazole, respectively. The mobile phase used was prepared by mixing acetonitrile and 0.02 mol L⁻¹ potassium dihydrogen phosphate buffer (pH adjusted to 2.5 using ortho phosphoric acid) in a ratio 10:90 with the addition of 1% TEA and flow rate 1 mL/min in isocratic mode and UV-detection at wavelength 254 nm. Second method depends on densitometric thin layer chromatography. The method was linear over concentration range 0.1 to 3 µg/ml, 0.5 to 6 µg/mL, 0.2 to 2.5 µg/mL and 0.1 to 1.5 µg/mL of gemifloxacin mesylate, theophylline, warfarin and omeprazole, respectively. The mobile phase was selected as mixture of dichloromethane, methanol and ammonia in the ratio of (7: 5.5: 3 v/v/v) for the development of plates. Densitometric analysis was carried out at wavelength 254 nm. The stability of gemifloxacin mesylate and the co-administered drugs in plasma was confirmed during three freeze-thaw cycles (-20°C).

Keywords: Gemifloxacin; Theophylline; Warfarin; Omeprazole; Plasma; HPLC; TLC

Introduction

Gemifloxacin mesylate (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 is a new fluoroquinolone antibacterial compound with enhanced affinity for bacterial topoisomerase IV and it is used for the treatment of respiratory and urinary tract infection. The compound has broad spectrum of activity against gram-positive and gram-negative bacteria [1]. Gemifloxacin mesylate is not official in any pharmacopoeia. The literature survey revealed that analytical methods reported for the estimation of gemifloxacin mesylate in human plasma were Spectrofluorimetric method [2], Densitometric TLC [3], HPLC-Tandam mass [4], HPLC [3,5,6]. No method has been reported for determination of gemifloxacin mesylate in human plasma with co-administered drugs by HPLC and densitometric TLC. Some fluoroquinolones have the potential to modify the kinetics of co-administered drugs via CYP inhibition and may be via inhibition of drug transport [7]. The co-administration of gemifloxacin and theophylline was well tolerated, with no clinically significant changes seen in vital signs. Adverse events were generally transient, mild to moderate in nature [8]. Co-administration of warfarin with fluoroquinolones generally needs caution because of a possible increased anticoagulant response [9]. A study was designed to demonstrate the lack of effect of steady-state concentrations of gemifloxacin on the pharmacodynamic effects of warfarin. There were no changes of clinical significance in vital signs [10]. A study was designed to demonstrate the effect of omeprazole on the pharmacokinetics of oral gemifloxacin in healthy volunteers. Following co-administration of gemifloxacin and omeprazole the AUC_{0-∞} and C_{max} for gemifloxacin increased on average by 10% and 11%, respectively, although neither t_{max} nor t_{1/2} appeared to be affected. However, none of these changes are considered to be clinically important and there were no recommendations to alter the dose of fluoroquinolones in the presence of omeprazole [11].

The proposed research work describes the estimation of gemifloxacin mesylate with three different co-administered drugs

using only one separation system that cover the expected concentration ranges of all of the studied drugs in human plasma, Figure 1. Other published work describes the determination of gemifloxacin with only one co-administered drug.

Material and Methods

Instrumentation

HPLC Knauer instrument (Germany) equipped with K-501 pump, Knauer injector and UV-detector K-2501. Data acquisition was performed on Eurochrom 2000 software. The analytical column employed was X-terra LC-18-DB, (25 cm×4.6 mm×5 µm). The working temperature was 25°C.

DESAGA CD 60 HPTLC densitometer connected to IBM compatible computer fitted with Proquant evaluation software for Windows. (Sarstedt-Gruppe, Germany) with precoated silica gel Plate 60F254 (20 cm×20 cm) 250 µm thicknesses (E. Merck, Darmstadt, Germany) was used as stationary phase. Sample application was done by using DESAGA AS30 HPTLC Applicator. (Sarstedt-Gruppe, Germany).

Linear ascending development was carried out in 25 cm×25 cm glass chamber. Evaluation of chromatogram was done by using peak areas.

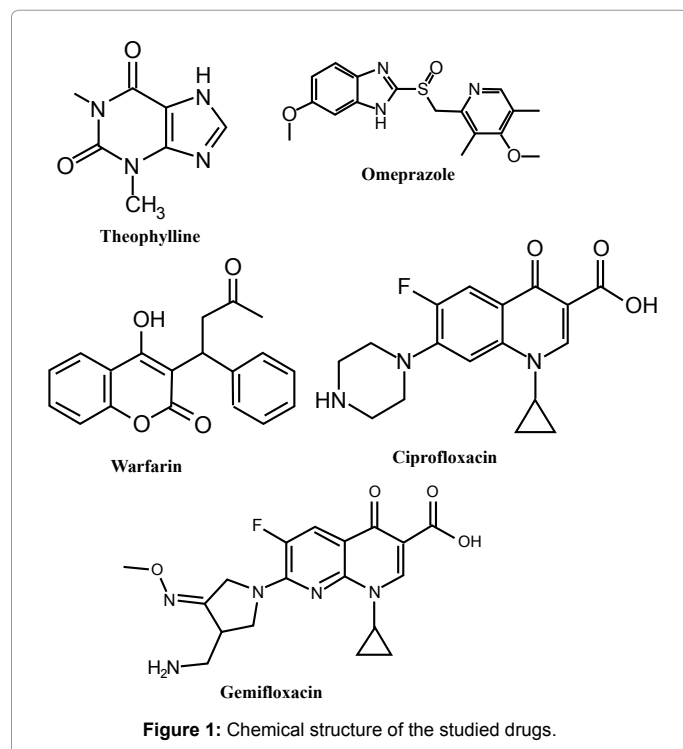
Rotatory vacuum evaporator, Buchi Rotavaper R-3000 (Germany).

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Sonicator (Crest Ultrasonics, New York). Syringe filters (Gelman, Sigma-aldrich).

Chemicals

Gemifloxacin mesylate (Mediphar pharmaceutical company, Cairo, Egypt), theophylline (GlaxoSmithKline, Cairo, Egypt), Warfarin (Nile Company for pharmaceuticals and chemical industries), omeprazole and ciprofloxacin (Al-hekma for pharmaceuticals) were received having 99.20%, 99.20%, 99.60%, 99.53 and 99.65% purity, respectively. The HPLC grade methanol, acetonitrile, ortho phosphoric acid, dichloromethane, ammonia and water were purchased from (Sigma GmbH, Germany). Analytical Reagent grade di-potassium hydrogen phosphate was used. Freshly isolated human plasma from collected blood used for research work was supplied by Vacsera, Cairo, Egypt.

Preparation of stock solutions and working standard solutions

Standard solutions preparation was conducted at room temperature. The solutions were protected from light with aluminum foil wrapping and stored at -20°C.

Stock solutions 1.00 mg/mL each of gemifloxacin mesylate, theophylline, warfarin omeprazole and ciprofloxacin were prepared in methanol.

The first working standard solutions of 0.1 mg/mL of gemifloxacin mesylate, theophylline, warfarin, omeprazole and ciprofloxacin hydrochloride were prepared by further dilution of stock solutions with mobile phase for HPLC and with methanol for TLC. Second working standard solutions (0.01 mg/mL) of gemifloxacin mesylate, theophylline, warfarin, omeprazole and ciprofloxacin hydrochloride were prepared by further dilution of stock solutions with mobile phase for HPLC and with methanol for TLC.

Preparation of plasma samples

For HPLC, in a stoppered centrifuge tube, an aliquot quantity of 500 μ L plasma was added and spiked with 50 μ L ciprofloxacin (internal standard 100 μ g/mL) and 450 μ L mixtures of gemifloxacin, theophylline, warfarin and omeprazole working solutions to provide concentrations of (0.05, 0.1, 0.5, 1, 2, 4, 6 μ g/mL), (0.25, 0.5, 1, 2, 4, 6, 8 μ g/mL), (0.1, 0.25, 0.5, 1, 2.5, 5, 10 μ g/mL) and (0.1, 0.25, 0.5, 1, 2, 4, 6 μ g/mL); respectively.

The quality control samples (QCs) were prepared in plasma concentration range (0.15, 1, 5 μ g/mL), (0.75, 4, 7 μ g/mL), (0.3, 2, 9 μ g/mL) and (0.3, 1, 4 μ g/mL) for gemifloxacin, theophylline, warfarin and omeprazole, respectively. Protein precipitation and extraction were carried out by using 1ml acetonitrile. The samples were sonicated for 10 minutes followed by vortex mixing for 5 minutes and centrifugation at 5000 rpm for 15 minutes. The organic layer was transferred to another centrifuge tube and evaporated to dryness at 40°C under vacuum. The residue was reconstituted in 0.1 mls mobile phase and 20 μ l injected into HPLC system.

For densitometric TLC, in a stoppered centrifuge tube, an aliquot quantity of 500 μ L plasma was spiked with 50 μ l ciprofloxacin (internal standard 100 μ g/mL) and 450 μ l mixtures of gemifloxacin, theophylline, warfarin and omeprazole. Different aliquots of gemifloxacin were added to provide concentrations of (0.1, 0.2, 0.4, 0.8, 1, 1.5, 2, 2.5, 3 μ g/mL). Different aliquots of theophylline were added to provide concentrations of (0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6 μ g/mL). Different aliquots of warfarin were added to provide concentrations of (0.2, 0.6, 1, 1.2, 1.6, 2, 2.5 μ g/mL). Different aliquots of omeprazole were added to provide concentrations of (0.1, 0.2, 0.4, 0.6, 0.8, 1, 1.2, 1.5 μ g/mL). The quality control samples (QCs) were prepared in plasma concentration range (0.3, 1.5, 2.5 μ g/mL), (1.5, 3, 5 μ g/mL), (0.6, 1, 2 μ g/mL) and (0.3, 0.8, 1.2 μ g/mL) for gemifloxacin, theophylline, warfarin and omeprazole, respectively. Protein precipitation and extraction were carried out as previously mentioned in HPLC method. The residue was reconstituted in 0.05 mLs methanol and 20 μ L were applied to TLC plates.

Chromatographic Conditions

For HPLC, the mobile phase used was prepared by mixing acetonitrile and 0.02 mol L⁻¹ potassium dihydrogen phosphate buffer (pH adjusted to 2.5 using ortho phosphoric acid) in a ratio 10:90 with the addition of 1% TEA. The mobile phase was freshly prepared and filtered by vacuum filtration through 0.45 μ m filter and degassed by ultrasound sonication for 50 minutes just prior to use. The samples were also filtered using 0.45 μ m syringe filters. The analysis was done under isocratic conditions at a flow rate 1 mL.min⁻¹ and at room temperature using UV detector at 254 nm.

For densitometric TLC, the mobile phase was selected as mixture of dichloromethane, methanol and ammonia in the ratio of (7: 5.5: 3 v/v/v) for the development of plates. The densitometric scanning was performed at 254 nm. The samples were also filtered using 0.45 μ m filters. Analysis was performed on precoated 20×20 cm silica gel 60 F₂₅₄ aluminum sheets (E.Merck). The plates were pre-washed with methanol and activated at 60°C for 5 min prior to chromatography. Samples were applied to the plates using a DESAGA AS30 Applicator (Germany). Spots were applied 1.5 cm apart from each other and 2 cm from the bottom edge. The chromatographic chamber was pre-saturated with the mobile phase for 45 min. the developing distance on TLC-plate was 180 mm.

Method Validation

The described methods were validated in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), recovery, selectivity, stability, precision and accuracy according to FDA guidelines regarding standard bioanalytical method validation recommendation [12]

Linearity: The analytical range to be validated was chosen on the basis of the expected plasma concentrations of the studied drugs [13-18]. The calibration curve was done for each analyte in the biological sample. The calibration curve should consist of a blank sample (matrix sample processed without internal standard), a zero sample (matrix sample processed with internal standard), and six to eight non-zero samples covering the expected range, including LLOQ that were prepared by adding the required volume of working solution of analyte to blank plasma. The plasma samples were subjected to the sample preparation procedure and injected into the LC or applied on TLC plates.

Plasma calibration curve was prepared by taking area ratio of analyte to internal standard as Y-axis and concentration of analyte ($\mu\text{g}/\text{mL}$) as X-axis.

Accuracy and precision: Accuracy and precision were determined for LQC, MQC and HQC (Low, Middle and High Quality Control) samples with LLOQ. Five replicates of each concentration were analyzed on the same day to determine the within-run accuracy and precision of the method. To confirm the between-run accuracy and precision five replicates of each concentration were analyzed at three separate days.

For HPLC, the used concentrations were (0.05, 0.15, 1 and 5 $\mu\text{g}/\text{mL}$), (0.25, 0.75, 4 and 7 $\mu\text{g}/\text{mL}$), (0.1, 0.3, 2 and 9 $\mu\text{g}/\text{mL}$), (0.1, 0.3, 1 and 4 $\mu\text{g}/\text{mL}$) for gemifloxacin, theophylline, warfarin and omeprazole, respectively.

For TLC, the used concentrations were (0.1, 0.3, 1.5 and 2.5 $\mu\text{g}/\text{mL}$) equivalent to (0.04, 0.12, 0.6, 1 $\mu\text{g}/\text{spot}$), (0.5, 1.5, 3 and 5 $\mu\text{g}/\text{mL}$) equivalent to (0.2, 0.6, 1.2, 2 $\mu\text{g}/\text{spot}$), (0.2, 0.6, 1 and 2 $\mu\text{g}/\text{mL}$) equivalent to (0.08, 0.24, 0.4, 0.8 $\mu\text{g}/\text{spot}$) and (0.1, 0.3, 0.8 and 1.2 $\mu\text{g}/\text{mL}$) equivalent to (0.04, 0.12, 0.32, 0.48 $\mu\text{g}/\text{spot}$) for gemifloxacin, theophylline, warfarin and omeprazole, respectively.

Selectivity: The selectivity of the methods was investigated by analyzing six blank plasma samples. Each blank sample was tested for interference using proposed extraction procedure and the response of the endogenous compounds at the retention times of the studied drugs in plasma samples were compared with the response of LLOQ of the studied drugs.

Recovery: The extraction recovery of analytes was determined by measuring the peak areas of the drugs from the prepared plasma quality control samples. The peak areas of extracted LQC, MQC and HQC were compared to the absolute peak area of the unextracted samples in mobile phase for HPLC and in methanol for TLC containing the same concentration of the drug. To obtain good extraction efficiency the extraction recovery of gemifloxacin and its co-administered theophylline, warfarin and omeprazole was determined using five replicates of each QC samples.

Stability study: 4.6.5.1. Freeze and thaw stability: The stability of gemifloxacin together with co-administered theophylline, warfarin and omeprazole was determined after three freeze and thaw cycles. Five aliquots at each of the LLOQ, low, mid and high quality control concentrations were stored at -20°C for 24 hours and thawed unassisted at room temperature. When completely thawed, the samples were

refrozen for 24 hours under the same conditions. The freeze-thaw cycle were repeated two more times, and then analyzed.

Short term temperature stability: Five aliquots of each of the LLOQ, low, mid and high quality control concentrations were thawed at room temperature and kept at this temperature for 6 hours and then analyzed.

Long term stability: Long-term stability was determined by storing five aliquots of each of the LLOQ, low, mid and high concentrations of the studied drugs at -20°C for 6 weeks. The concentrations of all the stability samples were compared to the mean values for the standards at the appropriate concentrations from the first day of long-term stability testing.

Stock solutions stability: The stability of stock solutions of each of the studied drugs and the internal standards used were evaluated at room temperature for 10 hours. After completion of the desired storage time, the stability was tested by comparing the instrument response with that of freshly prepared solutions.

Post-preparative stability: The stability of the processed samples was examined by keeping five replicates of the LLOQ, low, mid and high plasma quality control samples at room temperature for approximately 24 hours. The stability was tested by comparing the instrument response with that of freshly prepared samples.

Results

HPLC-UV detection and densitometric TLC methods were suggested for the simultaneous determination of each of gemifloxacin with three co-administered drugs; theophylline, warfarin and omeprazole. This work aimed to develop highly selective and sensitive methods with a quantitation limits that cover the expected concentration ranges of all of the studied drugs in human plasma to be able to be used in pharmacological, bioavailability, bioequivalence or other clinical studies to obtain certain pharmacokinetic information.

Extraction procedure optimization

One of the most difficult parts during the method development was to achieve a high and reproducible recovery from the solvent which is used for extraction of the drugs. Different solvents were tried for the extraction of gemifloxacin, theophylline, warfarin and omeprazole from human plasma. Methanol was tried for plasma precipitation and extraction of the studied drugs. It was found that the recoveries of the gemifloxacin and internal standard were acceptable but it was below 70% for theophylline and warfarin. Other solvents were tried as chloroform, ethyl acetate and n-hexane. The use of acetonitrile raised the recoveries of all the studied drugs above 80%.

Optimization of chromatographic condition

For HPLC, Initially the use of acetonitrile and potassium dihydrogen phosphate buffer (pH=3.5) in a ratio 30:70, the peaks of gemifloxacin, ciprofloxacin (IS) and omeprazole were well resolved. The peaks of theophylline and warfarin were overlapped. This was treated by gradual increasing the ratio of the buffer solution in the mobile phase to obtain reasonable resolution. Gemifloxacin peak tailing was treated by the addition of 1% TEA. The final mobile phase used for the simultaneous determination of gemifloxacin and its co-administered theophylline, warfarin and omeprazole was mixture of acetonitrile and 0.02 mol L^{-1} potassium dihydrogen phosphate buffer (pH adjusted to 2.5 using ortho phosphoric acid) in a ratio 10:90 with the addition of 1% TEA. The average retention time (minutes) \pm SD, for 6 replicate injections of gemifloxacin, theophylline, warfarin, omeprazole and ciprofloxacin,

were found to be 10.457 ± 0.07 , 4.411 ± 0.05 , 5.478 ± 0.08 , 7.356 ± 0.04 and 3.308 ± 0.06 , respectively, as shown in figures 2 and 3.

For densitometric TLC, Different solvent systems were tried. Initially systems like mixtures of cyclohexane and ethanol were used

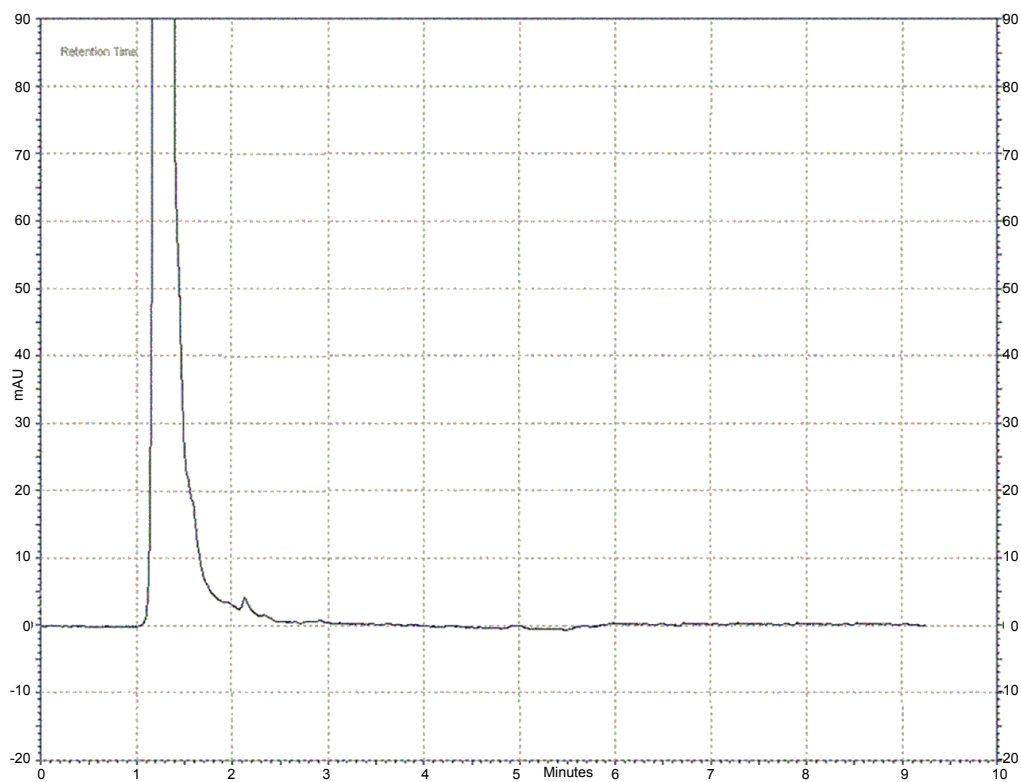


Figure 2: Chromatogram of blank human plasma sample.

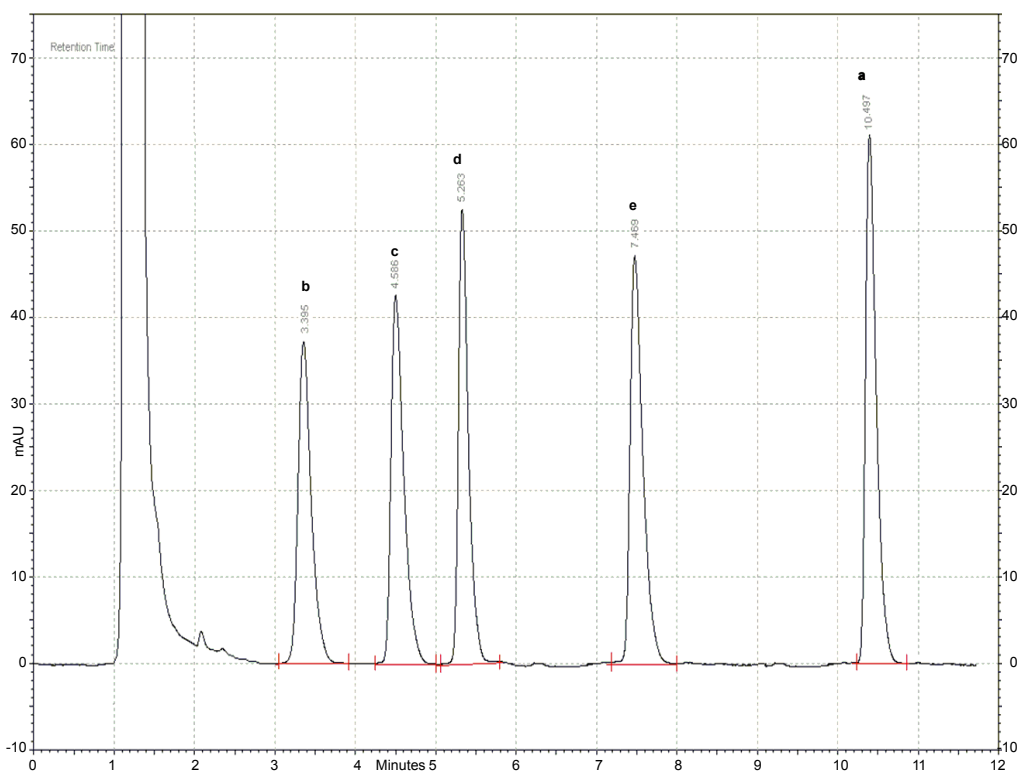


Figure 3: Chromatogram of human plasma sample spiked with (a) gemifloxacin, (b) ciprofloxacin, (c) theophylline, (d) warfarin and (e) omeprazole.

in varying ratios, but these systems showed excessive tailing and most peaks were too high near the solvent front. Other systems of mixtures of methanol and chloroform were tried. Better results were obtained but tailing was observed and peaks of theophylline and warfarin were still overlapped. Other mixture of dichloromethane, methanol and ammonia were tried and the best results were obtained by using dichloromethane, methanol and ammonia in the ratio of (7: 5.5: 3 v/v/v) for the development of plates.

The RF values were 0.16 ± 0.051 , 0.28 ± 0.042 , 0.53 ± 0.037 , 0.58 ± 0.022 and 0.74 ± 0.031 for ciprofloxacin, gemifloxacin, theophylline, warfarin and omeprazole, respectively, Figures 4 and 5.

Method validation

Linearity: For HPLC, the seven point calibration curves were

constructed by plotting the peak area ratio of each of gemifloxacin, theophylline, warfarin and omeprazole to ciprofloxacin (IS) versus their concentrations in plasma. The mean equations of calibration curves consisting of seven points are $y=0.3835C+0.0013$ for gemifloxacin mesylate with a correlation coefficient 0.9998, $y=15.412C+0.0498$ for theophylline with a correlation coefficient 0.9997, $y=0.0308C+0.0013$ for warfarin with a correlation coefficient=0.9997 and $y=0.1938C+8E-05$ for omeprazole with correlation coefficient 0.9996. Where y represents the ratios of peak area of each drug to that of IS and C represents the plasma concentration of each drug. The values of correlation coefficient confirmed that the calibration curves were linear over the concentration ranges 0.05-6 $\mu\text{g/mL}$, 0.25-8 $\mu\text{g/mL}$, 0.1-10 $\mu\text{g/mL}$ and 0.1-6 $\mu\text{g/mL}$ for gemifloxacin, theophylline, warfarin and omeprazole, respectively.

For densitometric TLC, the calibration curves were constructed

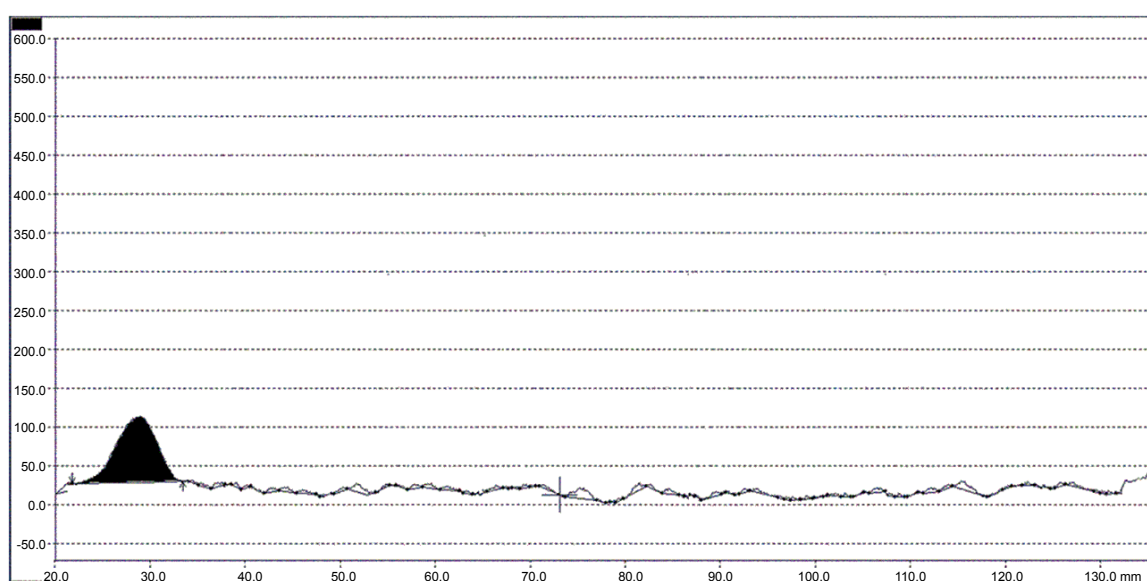


Figure 4: TLC densitogram of blank human plasma sample.

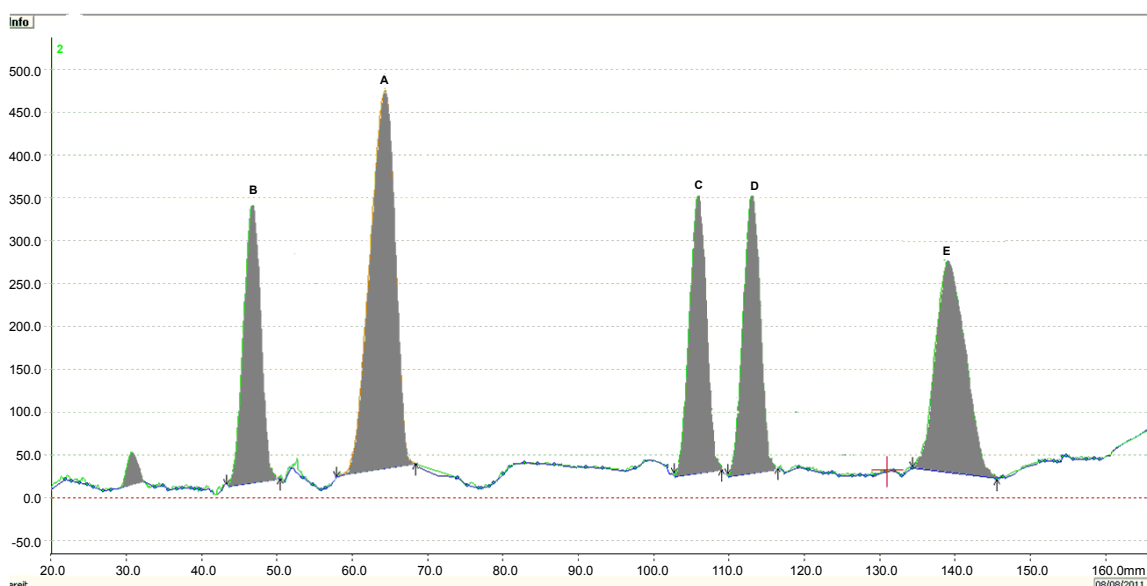


Figure 5: TLC densitogram of human plasma sample spiked with (A) gemifloxacin, (B) ciprofloxacin, (C) theophylline, (D) warfarin and (E) omeprazole.

by plotting the peak area ratio of each of gemifloxacin, theophylline, warfarin and omeprazole to ciprofloxacin (IS) versus their concentrations in plasma. The mean equations of calibration curves consisting of seven points $y=1.1053C-0.0374$ for gemifloxacin mesylate with a correlation coefficient of 0.9998, $y=0.227C+0.012$ for theophylline with correlation coefficient of 0.9995, $y=0.5007C+0.00563$ for warfarin with correlation coefficient=0.9991 and $y=1.915C-0.0146$ for omeprazole with correlation coefficient of 0.9993. Where y represents the ratios of peak area of each drug to that of IS and C represents the plasma concentration of each drug. The calibration curves were linear over the concentration ranges 0.1-3 µg/mL, 0.5-6 µg/mL, 0.2-2.5 µg/mL and 0.1-1.5 µg/mL for gemifloxacin, theophylline, warfarin and omeprazole; respectively.

Accuracy and precision: The accuracy of the method expressed in term of bias (percentage deviation from true value). The mean value should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20%. The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV [12]

Precision of the method was determined by repeatability (intraday) and intermediate precision (inter-day) and accuracy for set of quality control (QC) sample (low, mid, high) in replicate (n=5). The inter-day and intra-day precision and accuracy for pazufloxacin, cefoperazone and sulbactam sodium were evaluated by assaying the QC samples (low, mid, high) (n=5) in (%CV). In HPLC assay for gemifloxacin, the intra-run precision (CV %) was found to be in the range of 1.041-5.187% and the inter-run precision was 1.571-4.591%. For theophylline, the intra-run precision (CV %) was found to be in the range of 0.668-1.748% and the inter-run precision was 0.799-3.061%. For warfarin, the intra-run precision (CV %) was found to be in the range of 1.272-5.625% and the inter-run precision was 1.672-6.122%. For omeprazole, the intra-run precision (CV %) was found to be in the range of 1.208-4.947% and the inter-run precision was 1.972-5.414%. The accuracy values (%RE) were within the ranges of -0.70-1.50% for gemifloxacin, -1.6-1.729% for theophylline, -4-1.75% for warfarin and -5.8-1.4 for omeprazole.

The above values were within the acceptable range, they show that the HPLC method is accurate and precise.

For densitometric TLC for gemifloxacin, the intra-run precision (CV %) was found to be in the range of 1.751-6.186% and the inter-run precision was 1.849-5.119%. For theophylline, the intra-run precision (CV %) was found to be in the range of 2.717-6.695% and the inter-run precision was 3.273-5.556%. For warfarin, the intra-run precision (CV %) was found to be in the range of 2.176-4.725% and the inter-run precision was 3.290-5.380%. For omeprazole, the intra-run precision (CV %) was found to be in the range of 1.408-3.720% and the inter-run precision was 1.779-4.384%. The accuracy% values (%RE) were found to be in the range of -3.667-0.2% for gemifloxacin, -6.4-0.133% for theophylline, -7-0.333 for warfarin and -7--1.167 for omeprazole. The low percent coefficient of variation (%CV) and (%RE) were within the acceptable limit. The results of inter-day, intra-day precision and accuracy for gemifloxacin, theophylline, warfarin and omeprazole are shown in Table 1.

Selectivity: Selectivity was assessed to show that the intended analytes are measured and that their quantitation is not affected by the presence of the biological matrix. Figures 2-5 showed the typical chromatograms and densitograms of blank plasma and gemifloxacin with its co-administered drugs spiked with plasma

Recovery: Absolute recovery was calculated by comparing peak areas obtained from freshly prepared sample extracted with unextracted standard solutions of the same concentration. Recovery data was determined in triplicates at three concentrations (low, mid, high) as recommended by the FDA guidelines [12]. The average recovery of gemifloxacin, theophylline, warfarin and omeprazole for RP-HPLC, determined at the three concentrations (low, mid, high concentration) of each were found to be 89.555, 90.824, 89.574 and 88.471, respectively. For TLC the average recovery using the three concentrations for gemifloxacin, theophylline, warfarin and omeprazole were found to be 89.967, 89.050, 89.079 and 90.208, respectively as shown in table 2.

Sensitivity: Sensitivity of the method is defined as the lowest concentration that can be measured with an acceptable limit of accuracy and precision which is lower than 20% [12]. For HPLC, LLOQ were

| Method | Gemifloxacin mesylate | | | | Theophylline | | | | Warfarin | | | | Omeprazole | | | | |
|---------------------|-----------------------|----------|-------|-------|-----------------------|----------|-------|--------|-----------------------|----------|-------|--------|-----------------------|----------|-------|--------|--------|
| | Concentration (µg/ml) | SD (n=5) | CV% | %RE | Concentration (µg/ml) | SD (n=5) | CV% | %RE | Concentration (µg/ml) | SD (n=5) | CV% | %RE | Concentration (µg/ml) | SD (n=5) | CV% | %RE | |
| RP-HPLC intraday | 0.05 | 0.0025 | 5.187 | -3.60 | 0.25 | 0.0043 | 1.748 | -1.6 | 0.1 | 0.0054 | 5.625 | -4 | 0.1 | 0.0047 | 4.947 | -5 | |
| | 0.15 | 0.004 | 2.400 | 0.00 | 0.75 | 0.005 | 0.668 | -0.267 | 0.3 | 0.007 | 2.034 | -1.667 | 0.3 | 0.006 | 1.993 | 0.333 | |
| | 1 | 0.012 | 1.206 | -0.50 | 4 | 0.054 | 1.343 | 0.55 | 2 | 0.025 | 1.272 | -1.7 | 1 | 0.012 | 1.268 | -0.7 | |
| | 5 | 0.052 | 1.041 | 0.30 | 7 | 0.112 | 1.603 | -0.2 | 9 | 0.204 | 2.262 | 0.222 | 4 | 0.084 | 2.087 | 0.625 | |
| interday | 0.05 | 0.0023 | 4.591 | 0.20 | 0.25 | 0.004 | 1.639 | -2.4 | 0.1 | 0.006 | 6.122 | -2 | 0.1 | 0.0051 | 5.414 | -5.8 | |
| | 0.15 | 0.003 | 2.000 | 0.00 | 0.75 | 0.006 | 0.799 | 0.133 | 0.3 | 0.005 | 1.672 | -0.333 | 0.3 | 0.006 | 2.007 | -0.333 | |
| | 1 | 0.031 | 3.054 | 1.50 | 4 | 0.090 | 2.264 | -0.6 | 2 | 0.036 | 1.769 | 1.75 | 1 | 0.020 | 1.972 | 1.4 | |
| | 5 | 0.078 | 1.570 | -0.70 | 7 | 0.218 | 3.061 | 1.729 | 9 | 0.196 | 2.165 | 0.578 | 4 | 0.132 | 3.313 | -0.4 | |
| TLC | | | | | | | | | | | | | | | | | |
| | intraday | 0.1 | 0.006 | 6.186 | -3 | 0.5 | 0.032 | 6.695 | -4.4 | 0.2 | 0.006 | 3.125 | -4 | 0.1 | 0.002 | 2.083 | -4 |
| | | 0.3 | 0.013 | 4.498 | -3.667 | 1.5 | 0.058 | 3.862 | 0.133 | 0.6 | 0.016 | 2.658 | 0.333 | 0.3 | 0.004 | 1.408 | -5.333 |
| | | 1.5 | 0.026 | 1.751 | -1 | 3 | 0.084 | 2.953 | -5.167 | 1 | 0.021 | 2.176 | -3.5 | 0.8 | 0.013 | 1.656 | -1.875 |
| | | 2.5 | 0.062 | 2.545 | -2.56 | 5 | 0.133 | 2.717 | -2.08 | 2 | 0.092 | 4.725 | -2.65 | 1.2 | 0.043 | 3.720 | -3.667 |
| | interday | 0.1 | 0.005 | 4.990 | 0.2 | 0.5 | 0.026 | 5.556 | -6.4 | 0.2 | 0.009 | 4.839 | -7 | 0.1 | 0.004 | 4.301 | -7 |
| | | 0.3 | 0.015 | 5.119 | -2.333 | 1.5 | 0.061 | 4.144 | -1.867 | 0.6 | 0.023 | 3.879 | -1.167 | 0.3 | 0.005 | 1.779 | -6.333 |
| | | 1.5 | 0.030 | 2.009 | -0.467 | 3 | 0.092 | 3.273 | -6.3 | 1 | 0.033 | 3.290 | 0.3 | 0.8 | 0.016 | 2.062 | -3 |
| | | 2.5 | 0.046 | 1.849 | -0.48 | 5 | 0.162 | 3.401 | -4.72 | 2 | 0.102 | 5.380 | -5.2 | 1.2 | 0.052 | 4.384 | -1.167 |

CV%: coefficient of variation
%RE: percent relative error

Table 1: Accuracy and precision of gemifloxacin mesylate, Theophylline, Warfarin and Omeprazole in human plasma.

| Method | Gemifloxacin mesylate | | | Theophylline | | | Warfarin | | | Omeprazole | | |
|---------|-----------------------|------------|-------|-----------------------|------------|-------|-----------------------|------------|-------|-----------------------|------------|-------|
| | Concentration (µg/ml) | Recovery % | CV% | Concentration (µg/ml) | Recovery % | CV% | Concentration (µg/ml) | Recovery % | CV% | Concentration (µg/ml) | Recovery % | CV% |
| RP-HPLC | 0.05 | 90.000 | 6.667 | 0.25 | 90.000 | 5.333 | 0.1 | 87.000 | 8.046 | 0.1 | 87.000 | 9.195 |
| | 0.15 | 92.000 | 5.797 | 0.75 | 92.667 | 5.899 | 0.3 | 91.333 | 7.664 | 0.3 | 88.333 | 9.811 |
| | 1 | 89.500 | 2.570 | 4 | 89.800 | 2.840 | 2 | 87.050 | 3.561 | 1 | 90.400 | 6.416 |
| | 5 | 86.720 | 4.935 | 7 | 90.829 | 6.055 | 9 | 92.911 | 4.329 | 4 | 88.150 | 3.318 |
| TLC | 0.1 | 88.000 | 6.818 | 0.5 | 86.400 | 7.176 | 0.2 | 87.000 | 4.598 | 0.1 | 91.000 | 7.692 |
| | 0.3 | 88.333 | 3.396 | 1.5 | 93.000 | 4.659 | 0.6 | 88.667 | 4.323 | 0.3 | 87.000 | 4.215 |
| | 1.5 | 90.133 | 2.663 | 3 | 91.500 | 3.716 | 1 | 89.600 | 5.134 | 0.8 | 92.625 | 4.318 |
| | 2.5 | 93.400 | 2.484 | 5 | 85.300 | 7.550 | 2 | 91.050 | 5.052 | 1.2 | 93.583 | 5.788 |

CV%: Coefficient of Variation %

Table 2: Results of recovery of gemifloxacin, theophylline, warfarin and omeprazole in human plasma.

| | Gemifloxacin | | | Theophylline | | | Warfarin | | | Omeprazole | | |
|------------------|------------------------------------|--------------------|-------|------------------------------------|--------------------|-------|------------------------------------|--------------------|-------|------------------------------------|--------------------|-------|
| | Added plasma concentration (µg/ml) | Found* (Mean ± SD) | CV% | Added plasma concentration (µg/ml) | Found* (Mean ± SD) | CV% | Added plasma concentration (µg/ml) | Found* (Mean ± SD) | CV% | Added plasma concentration (µg/ml) | Found* (Mean ± SD) | CV% |
| Freeze-thaw | 0.05 | 0.0502 ± 0.003 | 6.374 | 0.25 | 0.243 ± 0.005 | 1.893 | 0.1 | 0.098 ± 0.005 | 5.306 | 0.1 | 0.094 ± 0.006 | 6.064 |
| | 0.15 | 0.1489 ± 0.003 | 1.746 | 0.75 | 0.7493 ± 0.006 | 0.774 | 0.3 | 0.3011 ± 0.005 | 1.594 | 0.3 | 0.2966 ± 0.006 | 1.922 |
| | 1 | 1.003 ± 0.023 | 2.333 | 4 | 3.965 ± 0.069 | 1.735 | 2 | 1.969 ± 0.036 | 1.854 | 1 | 0.9885 ± 0.021 | 2.165 |
| | 5 | 5.035 ± 0.114 | 2.264 | 7 | 7.115 ± 0.174 | 2.446 | 9 | 8.885 ± 0.205 | 2.307 | 4 | 4.114 ± 0.132 | 3.209 |
| Short term | 0.05 | 0.0485 ± 0.003 | 5.979 | 0.25 | 0.2504 ± 0.004 | 1.518 | 0.1 | 0.101 ± 0.005 | 4.752 | 0.1 | 0.098 ± 0.005 | 5.408 |
| | 0.15 | 0.1503 ± 0.004 | 2.728 | 0.75 | 0.752 ± 0.007 | 0.904 | 0.3 | 0.312 ± 0.004 | 1.346 | 0.3 | 0.3025 ± 0.004 | 1.190 |
| | 1 | 1.008 ± 0.034 | 3.383 | 4 | 4.088 ± 0.091 | 2.228 | 2 | 2.052 ± 0.064 | 3.099 | 1 | 1.013 ± 0.019 | 1.935 |
| | 5 | 5.113 ± 0.201 | 3.931 | 7 | 7.066 ± 0.217 | 3.071 | 9 | 9.032 ± 0.265 | 2.934 | 4 | 4.052 ± 0.196 | 4.837 |
| Long term | 0.05 | 0.0492 ± 0.003 | 7.114 | 0.25 | 0.246 ± 0.004 | 1.707 | 0.1 | 0.095 ± 0.005 | 6.000 | 0.1 | 0.095 ± 0.005 | 5.895 |
| | 0.15 | 0.1496 ± 0.005 | 3.208 | 0.75 | 0.748 ± 0.009 | 1.229 | 0.3 | 0.298 ± 0.005 | 1.778 | 0.3 | 0.2963 ± 0.007 | 2.396 |
| | 1 | 0.989 ± 0.029 | 2.963 | 4 | 3.966 ± 0.078 | 1.979 | 2 | 1.974 ± 0.071 | 3.602 | 1 | 0.9932 ± 0.023 | 2.366 |
| | 5 | 4.989 ± 0.236 | 4.730 | 7 | 6.982 ± 0.306 | 4.383 | 9 | 8.963 ± 0.301 | 3.358 | 4 | 3.942 ± 0.223 | 5.657 |
| Post preparative | 0.05 | 0.0501 ± 0.003 | 6.786 | 0.25 | 0.247 ± 0.004 | 1.781 | 0.1 | 0.097 ± 0.005 | 5.567 | 0.1 | 0.101 ± 0.006 | 6.039 |
| | 0.15 | 0.1501 ± 0.004 | 2.798 | 0.75 | 0.752 ± 0.006 | 0.838 | 0.3 | 0.305 ± 0.006 | 1.869 | 0.3 | 0.301 ± 0.004 | 1.194 |
| | 1 | 0.985 ± 0.028 | 2.883 | 4 | 4.011 ± 0.0902 | 2.249 | 2 | 1.996 ± 0.046 | 2.305 | 1 | 1.011 ± 0.019 | 1.939 |
| | 5 | 4.988 ± 0.102 | 2.049 | 7 | 7.062 ± 0.141 | 1.997 | 9 | 9.05 ± 0.185 | 2.044 | 4 | 4.023 ± 0.096 | 2.399 |

• Mean of five determinations

Table 3: Stability study of gemifloxacin, theophylline, warfarin and omeprazole in human plasma by the proposed HPLC method.

| | Gemifloxacin | | | Theophylline | | | Warfarin | | | Omeprazole | | |
|------------------|------------------------------------|--------------------|-------|------------------------------------|--------------------|-------|------------------------------------|--------------------|-------|------------------------------------|--------------------|-------|
| | Added plasma concentration (µg/ml) | Found* (Mean ± SD) | CV% | Added plasma concentration (µg/ml) | Found* (Mean ± SD) | CV% | Added plasma concentration (µg/ml) | Found* (Mean ± SD) | CV% | Added plasma concentration (µg/ml) | Found* (Mean ± SD) | CV% |
| Freeze-thaw | 0.1 | 0.095 ± 0.006 | 6.316 | 0.5 | 0.477 ± 0.032 | 6.709 | 0.2 | 0.187 ± 0.006 | 3.209 | 0.1 | 0.093 ± 0.003 | 3.226 |
| | 0.3 | 0.285 ± 0.016 | 5.614 | 1.5 | 1.395 ± 0.056 | 4.014 | 0.6 | 0.574 ± 0.013 | 2.265 | 0.3 | 0.283 ± 0.005 | 1.767 |
| | 1.5 | 1.442 ± 0.032 | 2.219 | 3 | 2.805 ± 0.092 | 3.280 | 1 | 0.936 ± 0.046 | 4.915 | 0.8 | 0.781 ± 0.016 | 2.049 |
| | 2.5 | 2.415 ± 0.062 | 2.567 | 5 | 4.855 ± 0.202 | 4.161 | 2 | 1.885 ± 0.105 | 5.570 | 1.2 | 1.174 ± 0.036 | 3.066 |
| Short term | 0.1 | 0.097 ± 0.006 | 6.186 | 0.5 | 0.489 ± 0.026 | 5.317 | 0.2 | 0.192 ± 0.005 | 2.604 | 0.1 | 0.096 ± 0.003 | 3.125 |
| | 0.3 | 0.302 ± 0.014 | 4.636 | 1.5 | 1.450 ± 0.062 | 4.276 | 0.6 | 0.591 ± 0.018 | 3.046 | 0.3 | 0.291 ± 0.007 | 2.405 |
| | 1.5 | 1.486 ± 0.029 | 1.952 | 3 | 2.936 ± 0.102 | 3.474 | 1 | 0.942 ± 0.032 | 3.397 | 0.8 | 0.792 ± 0.017 | 2.146 |
| | 2.5 | 2.501 ± 0.058 | 2.319 | 5 | 4.962 ± 0.165 | 3.325 | 2 | 1.925 ± 0.101 | 5.247 | 1.2 | 1.184 ± 0.028 | 2.365 |
| Long term | 0.1 | 0.096 ± 0.005 | 5.208 | 0.5 | 0.478 ± 0.029 | 6.067 | 0.2 | 0.182 ± 0.008 | 4.396 | 0.1 | 0.094 ± 0.002 | 2.128 |
| | 0.3 | 0.281 ± 0.018 | 6.406 | 1.5 | 1.433 ± 0.081 | 5.652 | 0.6 | 0.584 ± 0.016 | 2.740 | 0.3 | 0.286 ± 0.009 | 3.147 |
| | 1.5 | 1.466 ± 0.036 | 2.456 | 3 | 2.856 ± 0.122 | 4.272 | 1 | 0.926 ± 0.062 | 6.695 | 0.8 | 0.783 ± 0.018 | 2.299 |
| | 2.5 | 2.425 ± 0.048 | 1.979 | 5 | 4.725 ± 0.205 | 4.339 | 2 | 1.900 ± 0.092 | 4.842 | 1.2 | 1.173 ± 0.034 | 2.899 |
| Post preparative | 0.1 | 0.101 ± 0.007 | 6.931 | 0.5 | 0.493 ± 0.021 | 4.260 | 0.2 | 0.196 ± 0.009 | 4.592 | 0.1 | 0.097 ± 0.004 | 4.124 |
| | 0.3 | 0.301 ± 0.011 | 3.654 | 1.5 | 1.485 ± 0.055 | 3.704 | 0.6 | 0.592 ± 0.011 | 1.858 | 0.3 | 0.289 ± 0.011 | 3.806 |
| | 1.5 | 1.495 ± 0.034 | 2.274 | 3 | 2.896 ± 0.065 | 2.244 | 1 | 0.965 ± 0.052 | 5.389 | 0.8 | 0.766 ± 0.025 | 3.264 |
| | 2.5 | 2.496 ± 0.006 | 2.644 | 5 | 4.905 ± 0.094 | 1.916 | 2 | 1.922 ± 0.102 | 5.307 | 1.2 | 1.154 ± 0.041 | 3.553 |

• Mean of five determinations

Table 4: Stability study of gemifloxacin, theophylline, warfarin and omeprazole in human plasma by the proposed TLC method.

found to be 0.05, 0.25, 0.1 and 0.1 µg/mL. LOD values were found to be 0.0165, 0.0825, 0.033 and 0.033 µg/mL for gemifloxacin, theophylline, warfarin and omeprazole, respectively. For TLC, LLOQ were found to be 0.1, 0.5, 0.2 and 0.1 µg/mL. LOD values were found to be 0.033, 0.15,

| | Gemifloxacin | | Theophylline | | Warfarin | | Omeprazole | |
|------------|----------------------|--------------------------------|----------------------|---------------------------------|----------------------|---------------------------------|----------------------|---------------------------------|
| | Proposed HPLC method | Reported method ⁽³⁾ | Proposed HPLC method | Reported method ⁽¹³⁾ | Proposed HPLC method | Reported method ⁽¹⁵⁾ | Proposed HPLC method | Reported method ⁽¹⁷⁾ |
| Mean | 98.088 | 98.784 | 98.228 | 100.022 | 98.035 | 99.845 | 97.968 | 98.816 |
| SD | 2.1625 | 2.3580 | 1.0535 | 1.5622 | 2.1709 | 2.6743 | 2.4952 | 2.8113 |
| n | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Variance | 4.6764 | 5.5602 | 1.1099 | 2.405 | 4.7128 | 7.1519 | 6.2260 | 7.9034 |
| t (2.447)* | 0.44 | | 1.9 | | 1.05 | | 0.46 | |
| F (9.28)* | 1.189 | | 2.167 | | 1.518 | | 1.2694 | |

*The values between parentheses are the corresponding theoretical values of t and F at the 95% confidence level.

Table 5: Statistical comparison between the results obtained by the proposed HPLC method and the reported methods for the analysis of gemifloxacin, theophylline, warfarin and omeprazole in human plasma.

| | Gemifloxacin | | Theophylline | | Warfarin | | Omeprazole | |
|------------|----------------------|--------------------------------|----------------------|---------------------------------|----------------------|---------------------------------|----------------------|---------------------------------|
| | Proposed HPLC method | Reported method ⁽³⁾ | Proposed HPLC method | Reported method ⁽¹³⁾ | Proposed HPLC method | Reported method ⁽¹⁵⁾ | Proposed HPLC method | Reported method ⁽¹⁷⁾ |
| Mean | 97.790 | 98.784 | 98.922 | 100.022 | 97.346 | 99.845 | 96.896 | 98.816 |
| SD | 1.1709 | 2.358 | 1.4535 | 1.5622 | 2.2999 | 2.6743 | 1.4392 | 2.8113 |
| n | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Variance | 1.3710 | 5.5602 | 2.1127 | 2.4405 | 5.285 | 7.1519 | 2.0713 | 7.9034 |
| t (2.447)* | 1.11 | | 1.03 | | 1.42 | | 1.85 | |
| F (9.28)* | 4.056 | | 1.155 | | 1.352 | | 3.816 | |

* The values between parentheses are the corresponding theoretical values of t and F at the 95% confidence level.

Table 6: Statistical comparison between the results obtained by the proposed TLC method and the reported methods for the analysis of gemifloxacin, theophylline, warfarin and omeprazole in human plasma.

| Parameter | Gemifloxacin | | Theophylline | | Warfarin | | Omeprazole | |
|---------------------------------|--------------|---------|--------------|---------|----------|---------|------------|---------|
| | HPLC | TLC | HPLC | TLC | HPLC | TLC | HPLC | TLC |
| Capacity factor (K') | 3.56 | 4.16 | 2.67 | 5.44 | 4.35 | 4.07 | 3.66 | 3.17 |
| Selectivity factor (α) | 1.44 | 1.08 | 1.36 | 1.24 | 1.76 | 1.56 | 1.08 | 1.56 |
| Resolution (R) | 3.83 | 2.65 | 2.08 | 1.78 | 2.55 | 1.98 | 4.55 | 3.78 |
| Theoretical plates (N) | 3589.56 | 2654.77 | 4554.76 | 2564.78 | 3786.88 | 3007.78 | 5641.67 | 3211.67 |
| Tailing factor (t) | 1.33 | 1.45 | 1.15 | 1.55 | 1.25 | 1.33 | 1.03 | 1.15 |

Table 7: System suitability results of the HPLC and TLC methods for determination gemifloxacin, theophylline, warfarin and omeprazole in spiked human plasma.

0.06 and 0.033 $\mu\text{g/mL}$ for gemifloxacin, theophylline, warfarin and omeprazole, respectively.

Stability study: In stock solution stability the studied drugs with their internal standards samples were left at room temperature for 10 h. Comparison of the results with freshly prepared stock solution showed that there was no significant difference between response of freshly prepared solutions and samples of the studied drugs after 10 h.

Freeze–thaw stability was determined after three freeze–thaw cycles for five replicate of LLOQ, low, mid and high QC samples. The samples were stored at -20°C temperature for 24 h. Then thaw at room temperature. No significant difference between freeze–thaw samples and freshly prepared samples was observed.

Statistical analysis: Statistical comparison between the results of the proposed HPLC and TLC methods to those obtained by applying reported methods [3,13,15,17] showed that the calculated t and F values are less than the theoretical ones, indicating that there was no significant difference between the results obtained from the proposed methods and those obtained from the reported methods shown in tables 5 and 6.

System suitability testing: The results of the system suitability tests represented in table 7 assured the ability of the proposed methods for the routine analysis of the studied drugs.

Discussion

Using the proposed methods it was found that acetonitrile was the appropriate solvent for plasma precipitation and extraction that lead to high recovery % (above 70%). For HPLC, the chromatographic conditions, especially the composition of mobile phase, were optimized to achieve a good resolution and symmetric peak shapes for the analytes and the internal standard, as well as a short analytical time. For TLC, The proposed TLC method is based on the difference between Rf values of gemifloxacin mesylate and its co-administered drugs which differ in their polarities and consequently in their migration rates on TLC plates. The chromatographic conditions were optimized by spotting the drug with its co-administered drugs on TLC plates and developed in different solvent systems to achieve best separation. The proposed HPLC and TLC methods were accurate and precise as the accuracy % was not more than 15% even at LLOQ and the precision was not exceed 15% even also at LLOQ. Also the proposed methods were highly selective as there was no significant interference observed at the retention times of the analytes in the biological matrix. The result of stability experiments shows that no significant degradation occurred at ambient temperature for 6 h for short term stability, at -20°C for 6weeks for long term stability and for the post preparative stability for 24 h after comparing with freshly prepared sample. Results of stability for both RP-HPLC and TLC methods are shown in tables 3 and 4.

Conclusion

The proposed RP-HPLC and Densitometric-TLC methods for the estimation of gemifloxacin mesylate in human plasma in presence of three co-administered drugs; theophylline, warfarin and omeprazole, are selective and sensitive. Sensitivity of the methods are suitable for handling various therapeutic plasma levels of the mentioned drugs. The methods are economical and faster than earlier published methods. In future these methods can be used for bioequivalence and bioavailability studies or any other pharmacokinetic studies.

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