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Short communication

Reverse phase-HPLC and HPTLC methods for determination of gemifloxacin mesylate in human plasma

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ABSTRACT

Two simple, rapid, sensitive and economic chromatographic methods have been developed for determination of gemifloxacin mesylate in human plasma by using internal standard. First method depends on reverse phase high performance liquid chromatography. The plasma sample was extracted using chloroform:acetic acid (5.4:0.1, v/v). A concentration range from 30 to 600 ng/ml was used for calibration curve. The percent recovery of gemifloxacin mesylate was found to be 80.06–84.88. The mobile phase used consist of methanol:sodium acetate (1%):ortho phosphoric acid (65:35:0.5, v/v/v) with pH 2.1 and flow rate 0.8 ml/min in isocratic mode. The separation was carried out by UV-detector at wavelength 263 nm. Second method depends on high performance thin layer chromatography. The plasma sample was extracted using chloroform:acetic acid (5.9:0.1, v/v). A concentration range from 50 to 600 ng/spot was used for calibration curve. The percent recovery of gemifloxacin mesylate was found to be 80.01–86.17. The mobile phase used consists of ethyl acetate:methanol:ammonia (8.0:4.0:3.0, v/v/v). Densitometric analysis was carried out at wavelength 254 nm. The R_f values for gemifloxacin mesylate and linezolide were 0.33 ± 0.03 and 0.69 ± 0.03 respectively. The stability of gemifloxacin mesylate in plasma was confirmed during three freeze–thaw cycles (−20 °C), on bench during 12 h. The proposed method was validated statistically and by performing recovery study for determination of gemifloxacin mesylate in human plasma.

1. Introduction

Gemifloxacin mesylate (R, S)-7(3-aminomethyl-4-syn-methoxyimino-1-pyrrolidinyl)-1-cyclopropyl-6-fluro-1,4 dihydro-4-oxo-1,2 naphthyridine-3-carboxylic acid is a new fluroquinolone 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]. The literature survey revealed that analytical methods reported for the estimation of gemifloxacin mesylate include rapid determination in human plasma by HPLC–MS-MS [2,3] microchip electrophoresis [4]. Gemifloxacin mesylate is not official in any pharmacopoeia.

No method has been reported for determination of gemifloxacin mesylate in human plasma by HPTLC using liquid–liquid extraction (LLE). For HPLC the extracting solvent is different than that of reported method, chloroform:acetic acid (5.5:0.1, v/v) was used as extracting solvent.

The proposed research work describes the estimation of gemifloxacin mesylate in human plasma by RP-HPLC and HPTLC using hydrochlorothiazide and linezolide (Fig. 1) as an internal standards [5–11].

A widely used technique of quantitation involves the addition of an internal standard to compensate for various analytical errors. In this approach, a known compound of a fixed concentration is added to the known amount of samples to give separate peaks in the chromatograms to compensate for the losses of the compounds of interest during sample pretreatment steps. It must have a completely resolved peak with no interferences; it must not be present in the original sample. It must be stable, uncreative with sample components, column packing and the mobile phase. For RP-HPLC hydrochlorothiazide is used as an internal standard showing no significant interference at the retention time of the analyte. Retention time of hydrochlorothiazide and gemifloxacin mesylate was 4.6 and 6.1 min. For HPTLC linezolide was chosen as internal standard as it dose not interfere with the peak area of gemifloxacin mesylate and there was good resolution between gemifloxacin mesylate ($R_f = 0.33 \pm 0.03$) and linezolide ($R_f = 0.69 \pm 0.03$) [12].

2. Materials and methods

2.1. Instrumentation

A HPLC Knauer with Chromgate software version 3.1 having binary pumps Smartline-1000-1 and Smartline-1000-2 and detector Smartline-UV-2600 variable wavelength programmable was used. The analytical column employed was Eurosphere-100 C18

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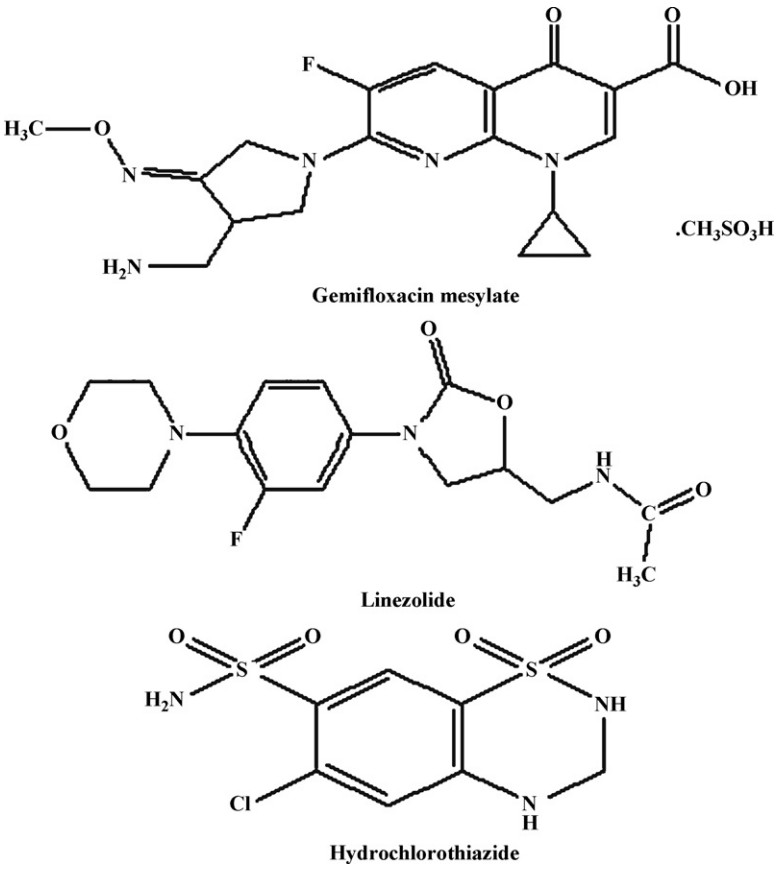

Fig. 1. Structure of gemifloxacin mesylate, linezolide (IS) and hydrochlorothiazide (IS).

(250 mm × 4.6 mm × 5 μ) supplied by Knauer, Berlin, Germany. The working temperature was 25 °C.

HPTLC Camag with precoated silica gel Plate $60F_{254}$ (20 cm × 10 cm) 250 μm thicknesses (E. Merck, Darmstadt, Germany) was used as stationary phase. Sample application was done by using Camag 100 μl syringe and Camag Linomat V applicator. The sample was sprayed in the form of narrow bands of 8 mm length at a constant rate 2 μl/s. Linear ascending development was carried out in 20 cm × 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland). The densitometric scanning was performed by using Camag TLC scanner III supported by win CATS software (V 1.4.2.8121 Camag). Evaluation of chromatogram was done by using peak areas.

2.2. Chemicals

Gemifloxacin mesylate and hydrochlorothiazide (Ajanta Pharma, Paithan, Maharashtra, India), linezolide (Matrix Pharma Ltd., Sinner, Maharashtra, India) were received having 99.80%, 98.70% and 100.1% purity respectively. They were used as such by without checking their purity. The HPLC grade methanol and water were purchased from Qualligens Fine Chemicals, Mumbai, India. Analytical Reagent grade sodium acetate and ortho phosphoric acid were used. HPLC grade ethyl acetate was purchased from s d Fine Chem. Ltd., Mumbai, India. Human plasma used for research work was supplied by Arpan Blood Bank, Nashik, Maharashtra, India.

2.3. Preparation of stock solution and working standard solution

Stock solutions 1.0 mg/ml each of gemifloxacin mesylate, hydrochlorothiazide and linezolide were prepared in methanol. The working standard solutions of 0.1 mg/ml of gemifloxacin mesylate and hydrochlorothiazide were prepared by further dilution of stock solutions with mobile phase for HPLC. For HPLTC 0.1 mg/ml of gemifloxacin mesylate and linezolide were prepared in methanol.

2.4. Preparation of plasma sample

For HPLC, in a 15 ml centrifuge tube 0.3, 1, 2, 3, 4, 5, 6 μl of working stock solution of gemifloxacin mesylate was added to drug-free plasma to provide calibration standards of 0 (no gemifloxacin mesylate added) 30, 100, 200, 300, 400, 500, 600 ng/ml and 300 ng/ml of hydrochlorothiazide (internal standard) was kept constant. The quality control (QC) samples were prepared in plasma concentration range 100, 300 and 600 ng/ml. Protein precipitation and extraction was carried out by using chloroform 5.4 ml and acetic acid 0.l ml by vigorous vortex using remi mixer for 1 min and centrifuged at 10,000 rpm at 6 min. The organic phase was recovered and evaporated to dryness on hot plate. The residual mass was reconstituted with 0.1 ml mobile phase. The analysis was carried on RP-HPLC.

For HPTLC, in a 15 ml centrifuge tube 0.5, 2, 3, 4, 5, 6 μl of working stock solution was added to drug-free plasma to provide calibration standards of 0 (no gemifloxacin mesylate added) 50, 200, 300, 400, 500, 600 ng/ml and 400 ng of linezolide (internal standard) was kept constant. The quality control (QC) samples were prepared in plasma in concentration range 200, 400, 600 ng. Protein precipitation and extraction was carried out by using chloroform 5.9 ml and acetic acid 0.l ml on by vigorous vortex using remi mixer for 1 min and centrifuged at 10,000 rpm at 6 min. The organic phase was recovered and evaporated to dryness on hot plate. The residual mass was reconstituted with 0.1 ml methanol. The analysis was carried on HPTLC.

2.5. Chromatographic condition

Mobile phase used was mixture of methanol:sodium acetate (1%):ortho phosphoric acid (65:35:0.5, v/v/v). The pH of final mixture was adjusted to pH 2.1. The mobile phase was degassed prior to use under vacuum by filtration through Nylon 66 membrane of 47 mm size and 0.45 μm thickness. Flow rate of 0.8 ml/min was applied throughout analysis with 20 μl injection. The detector was set at 263 nm.

For HPTLC the mobile phase was selected as mixture of ethyl acetate, methanol and ammonia in the ratio of (8.0:4.0:3.0, v/v/v) for the development of plates. Time for chamber saturation was optimized to 20 min. The length of chromatographic development was 70 mm. The densitometric scanning was performed at 254 nm.

2.6. Method validation

The method was validated for sensitivity, selectivity, precision, accuracy, linearity, recovery and stability. The validation of the method was based on FDA guidelines and on standard bioanalytical method validation recommendation. The selectivity of method was investigated by analyzing six blank plasma samples. Each blank sample was tested for interference using proposed extraction procedure. Five replicate of three QC sample low, mid and high were used for the determination of precision and accuracy. Intra-day and inter-day precision were carried out. Precision and accuracies showed 15% relative standard deviation (RSD) from nominal values, at LLOQ these were both 20%.

The recovery of gemifloxacin mesylate was calculated by comparison of the peak areas of low, mid, and high quality control sample (100, 300 and 600 ng/ml respectively) prepared in plasma (extracted) with unextracted gemifloxacin mesylate in mobile phase for HPLC and for HPTLC by using peak areas of low, mid, high quality control sample (200, 400 and 600 ng/spot respectively) in similar manner.

Stability experiments were undertaken to detect degradation of gemifloxacin mesylate under certain condition. The stock solution stability of gemifloxacin mesylate was examined at room temperature for 6 h. Freeze–thaw stability was determined at two QC concentrations (low, high) after freezing (−20 °C) and thawing for three cycles and compared with nominal value. Bench-top stability was assessed for low and high QC samples by comparing with nominal value which stored at room temperature for 12 h. The effect of storage within the auto-sampler was assessed by comparing QC samples injected immediately after preparation with those left in auto-sampler for 48 h.

3. Result and discussion

3.1. 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 drug. Different solvents were tried for the extraction of gemifloxacin mesylate from human plasma. First 5 ml each of methanol, hexane and toluene were tried for the precipitation of plasma but the recovery was very less up to 40–50% because of less precipitation of protein from plasma. Ethyl acetate was also tried up to 5.5 ml. It gave 60–70% of recovery because of less precipitation of protein from plasma. At the last chloroform was tried and 75–80% of recovery was obtained. It was found that the addition of acetic acid (0.1 ml) increases the precipitation of protein and also the recovery which is reproducible and high as compare to other solvents. So chloroform and acetic acid was kept as final solvent for extraction.

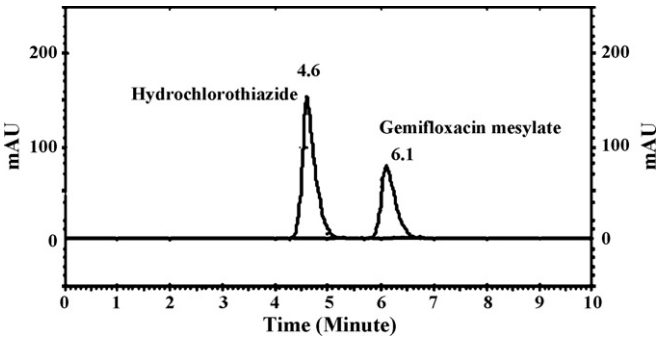

Fig. 2. Chromatogram of gemifloxacin mesylate 100 ng/ml (retention time = 6.1) and hydrochlorothiazide (internal standard) 300 ng/ml (retention time = 4.6).

3.2. Optimization of chromatographic condition

For HPLC the method demonstrated excellent chromatographic specificity with no endogenous plasma interference at the retention times of gemifloxacin mesylate and internal standard (hydrochlorothiazide), representative chromatogram of plasma spiked with gemifloxacin mesylate with internal standard (hydrochlorothiazide) are shown in Fig. 2. Gemifloxacin mesylate and IS were well resolved with good symmetry and retention time of 4.6 and 6.1 min. Analytical run time of 8 min was obtained which is less than other reported method.

Initially plain methanol and water was used but splitting of peak was observed for both the drugs. Then methanol and water was mixed in the ratio (50:50, v/v) in that broad peaks were observed with less resolution. The ratio of methanol and water was changed to (80:20, v/v). By using this mobile phase broad peaks with increased resolution were observed so again ratio was changed. Methanol and water were used in proportion of (65:35, v/v) respectively. Resolution was increased and broadening was minimised by addition of 0.5 ml ortho phosphoric acid and by changing the pH of the mobile phase from pH 2 to 2.5. At this situation both drugs were showing typical peaks at flow rate of 0.6/ml. A small negative peak was observed which was minimised by addition of sodium acetate (1%). So ratio of methanol:sodium acetate:ortho phosphoric acid (65:35:0.5, v/v/v) was selected as final mobile phase. For validation purpose flow rate of 0.6/ml, wavelength of detection 263 nm and working temperature 25 °C were used.

For HPTLC initially plane solvents like methanol, toluene, hexane, and ethyl acetate were tried. The spots were developed with methanol and ethyl acetate but tailing was observed. Then methanol and ethyl acetate in the ratio (8:2, v/v) was used but the distance traveled by the developed spots was high at solvent front and tailing was also observed. Then ethyl acetate and methanol in the ratio (2:8, v/v) was tried. In this condition R_f values were reduced and good resolution was obtained. The proportion of methanol was increased by 2 ml and the R_f of both drugs were satisfactory but peaks were not symmetrical and tailing was observed. The tailing was reduced by addition of ammonia. So acetic acid was replaced by using 3 ml ammonia. The symmetrical peaks were observed. Ultimately mobile phase used consisted of ethyl acetate:methanol:ammonia (8:4:3, v/v/v) which gave good resolution of peaks for both drugs gemifloxacin mesylate and internal standard (linezolide) in plasma at 254 nm. Fig. 3 shows the densitogram of spiked plasma with gemifloxacin mesylate and IS (linezolide). The R_f values for gemifloxacin mesylate and linezolide were 0.33 ± 0.03 and 0.69 ± 0.03 respectively. Well defined spots were obtained when plate was activated at 110 °C for 20 min and the chamber was saturated with the mobile phase for 20 min at room temperature.

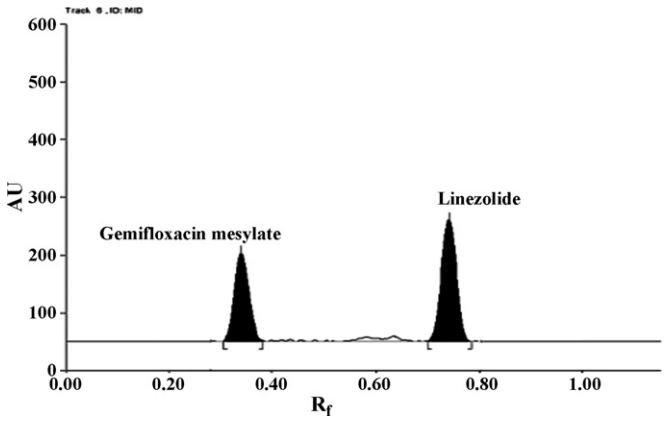

Fig. 3. Densitogram of gemifloxacin mesylate 400 ng/spot ($R_f = 0.33 \pm 0.03$) and linezolide (IS) 400 ng/spot ($R_f = 0.69 \pm 0.03$).

3.3. Calibration curves

The seven point calibration curve was constructed by plotting the peak response ratio of gemifloxacin mesylate to IS versus concentration of gemifloxacin mesylate in plasma. For HPLC the mean equation of calibration curve consisting of seven point is $y = 0.00430x + 0.0341$. Where y represents the ratios of peak area of gemifloxacin mesylate to that of IS and x represents the plasma concentration of gemifloxacin mesylate. Correlation coefficient 0.9989 confirmed that the calibration curve was linear over the concentration range 0.3–6 μl/ml. For HPTLC correlation coefficient is 0.9952 and linearity was found over the range 0.5–6 μl/ml. Regression equations for standard curve was $y = 0.173x - 75.09$. The lower limit of quantification was defined as lowest concentration in the calibration curve. The gemifloxacin can be determined at LLOQ 0.3 μl/ml for RP-HPLC and 0.5 μl/ml for HPTLC.

3.4. 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 [13]. The recovery of gemifloxacin for RP-HPLC, determined at the three concentrations 100, 300, 600 ng/ml (low, mid, high concentration) were found to be 82.42, 84.88 and 80.06%. For HPTLC recovery at the three concentrations 200, 400, 600 ng/spot were found to be 86.17, 84.75 and 80.01% (Table 1).

3.5. Precision and accuracy

Precision of the method was determined by repeatability (intra-day) 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 the gemifloxacin evaluated by assaying the QC samples (low, mid, high) ($n = 5$) in (%RSD). In this assay the intra-run precision was found to be in the range of 1.29–5.88% and the inter-run precision was 0.98–2.60%. The accuracy was within 1.10–2.67%. The above values were within the acceptable range, it shows that the methods are accurate and precise. For HPTLC intra-run precision was found in the range of 3.5–9.2% and the inter-run precision was 1.75–4.5% and the accuracy was within the range 0.83–19.1%. The low percent relative standard deviation (%RSD) and percent relative error (%RE) were within the acceptable limit. The results of inter-day, intra-day precision and accuracy for the gemifloxacin mesylate are shown in Table 2.

Table 1
Result of recovery of gemifloxacin mesylate in human plasma.

Method	Concentration	Recovery (%)	RSD (%)
RP-HPLC	100 (ng/ml)	82.42	4.89
	300	84.88	1.90
	600	80.06	1.94
HPTLC	200 (ng/spot)	86.17	2.560
	400	84.75	1.678
	600	80.01	2.843

RSD: relative standard deviation.

3.6. Sensitivity and selectivity

Selectivity or specificity should be assessed to show that the intended analytes are measured and that their quantitation is not affected by the presence of the biological matrix. For RP-HPLC Fig. 2 shows the typical chromatograms of gemifloxacin mesylate spiked with plasma. There was no significant interference observed at the retention time of the analyte. In HPTLC method by LLE as shown in Fig. 3, there is no any interference of the biological matrix in the quantitation of gemifloxacin mesylate there was no changes in retention time of gemifloxacin mesylate, the method is selective. 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% [14]. The accuracy and precision at lower limit of quantitation (LLOQ) analyzed by using five replicate ($n = 5$) of the sample at the LLOQ concentration. The accuracy is determined by %RE at this LLOQ concentration. The lower limit of quantitation which could be detected was found to be 30 ng/ml with CV = 1.70, %RE = 0.5 and %RSD 2.97 for HPLC and for HPTLC lower limit of quantitation was found to be 50 ng/spot with CV = 2.1, %RE = 2.3 and % SD 8.66 which is within acceptable limit.

3.7. Analysis speed

For RP-HPLC run time of analysis is 8 min so less time required for analysis. In case of HPTLC 18 spot can be applied on one plate so less time consuming.

3.8. Stability

In stock solution stability the low and high QC sample were thawed and left at room temperature for 6 h. Comparison of the results for QC sample (low and high) with freshly prepared stock

Table 2
Precision of gemifloxacin mesylate in human plasma.

Method	Concentration	SD ($n = 5$)	%RSD	% relative error
RP-HPLC				
Intra-day	100 (ng/ml)	0.027	5.88	2.54
	300	0.028	1.99	1.10
	600	0.034	1.29	1.14
Intra-day	100	0.026	2.60	2.67
	300	0.057	4.06	2.11
	600	0.012	0.98	1.23
HPTLC				
Intra-day	200 (ng/spot)	0.040	9.20	19.1
	400	0.022	2.54	11.9
	600	0.041	3.50	0.83
Inter-day	200	0.020	4.54	18.1
	400	0.015	1.75	10.9
	600	0.041	3.42	1.19

SD: standard deviation, RSD: relative standard deviation.

Table 3
Stability study of gemifloxacin mesylate in human plasma.

Parameters	RP-HPLC			HPTLC		
	Concentration (ng/ml)	SD ($n = 3$)	CV (%)	Concentration (ng/spot)	SD ($n = 3$)	CV (%)
Stability, short term (6 h)						
Low	100	0.027	5.87	200	0.019	2.88
High	600	0.034	1.20	600	0.029	3.40
Freeze–thaw						
Low	100	0.035	9.16	200	0.012	2.17
High	600	0.056	2.27	600	0.033	3.30
Bench top (12 h)						
Low	100	0.031	7.48	200	0.032	3.10
High	600	0.109	4.56	600	0.021	2.29
Post-preparative						
Low	100	0.021	5.62	200	0.013	2.90
High	600	0.033	1.28	600	0.036	3.60

solution showed that there was no significant difference between response of freshly prepared solution and sample of gemifloxacin mesylate after 6 h. Freeze–thaw stability was determined after two freezes–thaw cycles for three replicate of low and high QC sample for RP-HPLC and HPTLC. The samples were stored at −20 °C temperature for 24 h. Then thaw at room temperature. No significant difference between freeze–thaw sample and freshly prepared sample was observed. The result of stability experiments shows that no significant degradation occurred at ambient temperature for 12 h for bench-top stability. And also for the auto-sampler stability for 48 h after comparing with freshly prepared sample. Standard solution of gemifloxacin mesylate and IS was stable for 15 days at 4 °C. Results of stability for both RP-HPLC and HPTLC methods are shown in Table 3.

4. Conclusion

The proposed RP-HPLC and HPTLC methods for the estimation of gemifloxacin mesylate in human plasma are selective and sensitive. Sensitivity of the method is suitable for handling various plasma levels of the drug. The method is economical and faster than earlier published methods. In future these methods can be used for bioequivalence study.

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