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HPTLC Methods for Determination of Gemifloxacin Mesylate in Rabbit Plasma

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Authors' contributions

This work was carried out in collaboration between all authors. Author ULN designed the study. Author BG performed the statistical analysis. Author SKP wrote the protocol and author SS wrote the first draft of the manuscript and performed the experimental work. Authors SKP and SS managed the literature searches. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aim: A new, simple, rapid, very sensitive and accurate high performance thin-layer chromatographic (HPTLC) method has been developed and validated for estimation of Gemifloxacin in rabbit plasma.
Study Design: Validation study.
Methodology: HPTLC was performed on silica gel $60F_{254}$ plates with ethanol: ethyl acetate: hexane, 2:7:1 (*v*/*v*), as mobile phase. Densitometry scanning was performed in absorbance mode at λ=254 nm.
Result: The R_F value was 0.21. The response was a linear function of concentration over the range 0.1–0.7µg mL^{-1} (r^2=0.996). A maximum recovery of drug from plasma was obtained by using chloroform and glacial acetic acid. Mean extraction recovery was 80%. Intra-day and inter-day precision (% RSD) of the assay were in the range 1.19–2.85% and accuracy was 1.7-5.66%
Conclusion: This method can be applied to pharmacokinetic studies in rabbit plasma.

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Keywords: HPTLC; Gemifloxacin; Rabbit plasma; Liquid-liquid extraction.

1. INTRODUCTION

Gemifloxacin mesylate is chemically 7-[(4Z)-3-(aminomethyl)-4-methoxyimino-pyrrolidin-1-yl]-1-cyclopropyl-6-fluoro-4-oxo-1, 4 dihydro -1, 8-napththyridine-3-carboxylic acid, [1] a fourth generation fluoroquinolones antibacterial agent. It is an oral broad spectrum quinolone antibacterial used in the treatment of acute bacterial exacerbation of chronic bronchitis and mild to moderate pneumonia. [2,3] As a class, fluoroquinolones act by preventing Deoxyribonucleic acid (DNA) synthesis through inhibition of bacterial type II topoisomerase enzymes (DNA gyrase and topoisomerase), enzymes that are essential for bacterial growth. [4,5] Gemifloxacin possesses a dual mechanism of action. It inhibits bacterial topoisomerase IV and gyrase enzymes resulting in interruption of bacterial DNA synthesis [6-8]. This drug is not official in any pharmacopeia. Literature survey revealed that analytical methods reported for the estimation of Gemifloxacin mesylate include rapid determination by HPLC-tandem mass spectrometry [9], microchip electrophoresis in Human plasma [10,11], HPTLC [12], RP-HPLC in human serum [13], RP-HPLC [14-16] and simple UV Spectrophotometric method [17-23], for tablet formulation. No methods for the estimation of the drug in Rabbit plasma has been reported by HPTLC.

2. EXPERIMENTAL

2.1 Instruments

A Camag Linomat IV sample applicator, a Camag TLC Scanner II controlled by Cats 3.15 version software and a Camag twin trough chamber were used. Merck HPTLC plates coated with silicagel 60 F 254 (0.2mm thickness) on aluminium sheets were used as the stationary phase.

2.2 Chemicals

Gemifloxacin mesylate (Sigma Aldrich, Mumbai, India.), was received having 99.80% purity. It was used as such, without checking its purity. HPLC grade methanol, ethanol, chloroform, Glacial acetic acid, ethyl acetate and water were purchased from Merck Specialities, Mumbai, India. Analytical Reagent grade sodium acetate and ortho phosphoric acid were purchased from Loba Chemicals, Mumbai, India. Rabbit plasma used for research work was procured from the department of pharmacology, Dadhichi college of Pharmacy, Sunder gram Cuttack.

2.3 Preparation of Standard Stock Solution

10mg of gemifloxacin mesylate was accurately weighed and dissolved in 10ml of methanol to get a concentration of 1000µg/ml.

2.4 Preparation of Plasma Sample

In a 15 ml centrifuge tube 1, 2, 3, 4, 5, 6, 7µl of working stock solution was added to drug-free plasma to provide calibration standards of (no gemifloxacin mesylate added) 100, 200, 300, 400, 500, 600, 700ng/ml. The quality control (QC) samples were prepared in plasma in

concentration range 200, 400, 600ng/ml. Protein precipitation and extraction was carried out by using chloroform 5.9ml and acetic acid 0.lml with a Remi mixer, and centrifuged at 10,000 rpm for 6min. The organic phase was evaporated under a stream of Nitrogen and the residue was reconstituted in the mobile phase.

3. METHODS VALIDATION

The method was validated for sensitivity, selectivity, precision, accuracy, linearity, recovery and stability. The method was validated basing on FDA guidelines and on standard bio-analytical method validation recommendation [24]. The selectivity of method was investigated by analyzing seven blank plasma samples. Each blank sample was tested for interference using proposed extraction procedure. Three replicate of three QC samples low, mid and high were used for the determination of precision and accuracy. Intra-day and inter-day precision were carried out. RSD values for Precision and accuracies were in between 1.19 to 2.85% from nominal values.

Recovery studies of gemifloxacin mesylate was calculated by comparing the peak areas of low, mid, and high quality control sample (200, 400 and 600ng/ml) prepared in plasma and extracted in mobile phase. Stability experiments were undertaken to detect degradation of gemifloxacin mesylate under certain condition. The stock solution of gemifloxacin mesylate was examined and was stable at room temperature for 6h. Freeze–thaw stability was determined at two QC concentrations (low, high) after freezing (−20ºC) 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 when stored at room temperature for 12h.

4. RESULTS AND DISCUSSION

4.1 Optimization of Extraction Procedure

The most important part in the method development was to obtain a high and reproducible recovery for extraction of the drug. Various solvents were tried for the extraction of drug from plasma. Initially 5ml each of ethanol, hexane and diethyl ether were tried for the precipitation of plasma but the recovery was very low due less precipitation of protein from plasma, with ethyl acetate the recovery of the drug increased moderately to 60–70%. With chloroform 75–80%, good recovery was obtained. It was found that the addition of 0.1N glacial acetic acid (0.5ml) increases protein precipitation and also the recovery. So chloroform and acetic acid was kept as final solvent for extraction.

4.2 Optimization of Chromatographic Condition

Various solvents like methanol, toluene, hexane, and ethyl acetate were tried in different ratios. With ethanol and ethyl acetate in the ratio (4:1) good spots were obtained, but tailing was observed, the Rf value was also very high. The proportional of ethyl acetate was increased and a small amount of hexane was added to decrease the polarity of the solvent system, thus decreasing the Rf value and tailing. Finally the solvent system consisted of ethanol: ethyl acetate: Hexane in the ratio of 2:7:1 (v/v/v) which gave a good peak in plasma at 254nm. The Rf values for gemifloxacin mesylate was 0.21. Well defined spot was obtained when the plate was activated at 120ºC for 20 min and the chamber was saturated with the mobile phase for 30 min at room temperature.

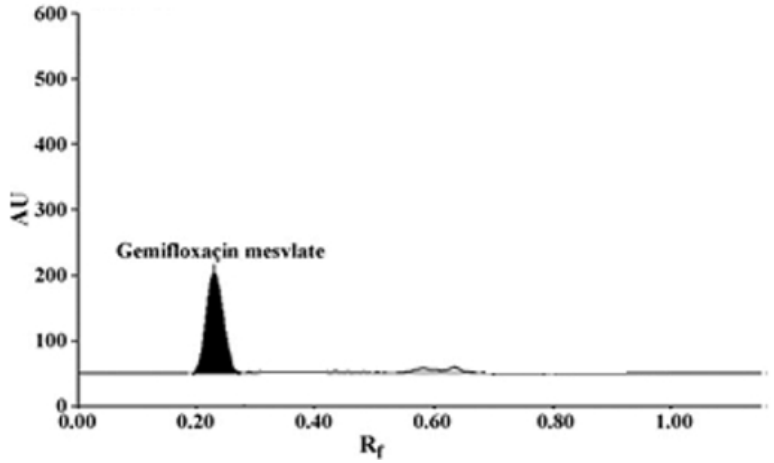

Fig. 1. Chromatogram of gemifloxacin in rabbit plasma at 100ng/spot

4.3 Calibration Curves

The seven point calibration curve was constructed by plotting the peak area of gemifloxacin mesylate versus concentration in plasma. The correlation coefficient (r^2) was 0.996 and linearity was found over the range 100 to 700ng per spot. Regression equations for standard curve was Y=19.37x+510.5. The lower limit of quantification was defined as lowest concentration in the calibration curve. Thus gemifloxacin can be determined at LLOQ of 0.1µl/ml.

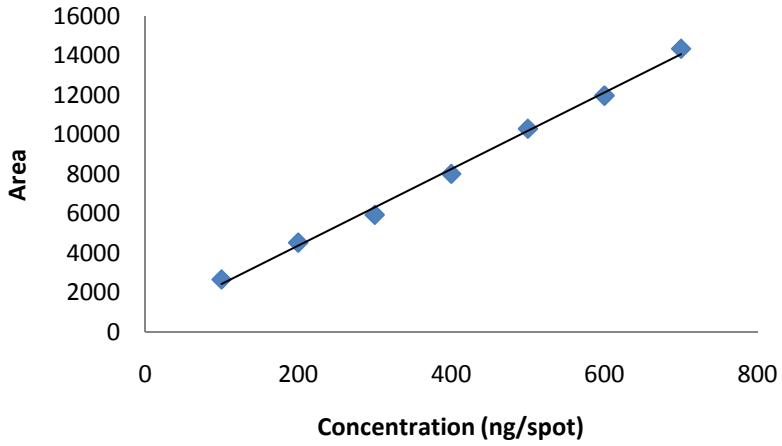

Fig. 2. Calibration curve of gemifloxacin in rabbit plasma

4.4 Precision and Accuracy

Precision of the method was determined by repeatability (intraday) and intermediate precision (inter-day) and accuracy for a set of quality control (QC) sample (low, mid, high) (n=5). Intra-run precision was found in the range of 1.19 to 1.87(% RSD) and the inter-run

precision was 1.37–2.85% (%RSD) and the accuracy was within the range 1.7–5.66% (%RE). The criteria for acceptability of the data included accuracy within ±15% deviation (SD) from the nominal values and a precision of within ±15% relative standard deviation (RSD). The percent relative standard deviation (%RSD) was within the acceptable limit. The results of inter-day, intra-day precision and accuracy for the gemifloxacin mesylate are shown in Table 1.

Table 1. Accuracy and pression data of the analysis of rabbit plasma

	Intra day				Inter day			
Spiked Conc. (ng/spot)	**Mean (ng/spot)**	**SD**	**%RSD**	**%Accuracy**	**Mean (ng/spot)**	**SD**	**%RSD**	**%Accuracy**
200	192	3.60	1.87	96	192	2.64	1.37	96
400	389.3	6.02	1.54	97.3	377.33	10.78	2.85	94.3
600	589.6	7.02	1.19	98.26	574.33	11.01	1.91	95.66

4.5 Sensitivity and Selectivity

Selectivity or specificity should be assessed to show that the intended analyte are measured and that their quantitation is not affected by the presence of the biological matrix. There was no significant interference observed at the retention time of the analyte. In this method by liquid liquid extraction (LLE) as shown in Fig. 1, there is no interference of the biological matrix in the quantitation of gemifloxacin mesylate and there were also no changes in retention time of gemifloxacin mesylate. Thus 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% [24]. The accuracy and precision at lower limit of quantitation (LLOQ) were 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 100ng/spot.

4.6 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 (five replicates) at three concentrations (low, mid, high) as recommended by the FDA guidelines [24]. Recovery at the three concentrations 200, 400, 600ng/spot were found to be 86.67, 84.75 and 83.01% (Table 2).

Table 2. Result of recovery of gemifloxacin

Concentration (ng/spot)	Mean	SD	% RSD	% Accuracy
200	222.6	2.6	2.9	86.67
400	410	2.6	3.2	84.75
600	691.6	2.5	2.71	83.01

4.7 Stability

Low and high QC sample were thawed and left at room temperature for 6h. Comparison of the results for QC sample (low and high) with freshly prepared stock solution showed that there was no significant difference between response of freshly prepared solution and

sample of gemifloxacin mesylate after 6h. Freeze–thaw stability was determined after two freezes–thaw cycles for three replicates of low and high QC sample. The samples were stored at −20ºC temperature for 24 hours, and then thawed 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 12h for bench-top stability. (Table 3)

Table 3. Stability data of gemifloxacin in rabbit plasma

Parameters	Conc. (ng/spot)	Mean (n=3)	SD (n=3)	% RSD
Stability, short term (6h)	100	191	1.732	0.9
	700	484	7	1.44
Freeze–thaw	100	179.3	8.08	4.5
	700	479	3	0.62
Bench top (12h)	100	176.3	4.50	2.5
	700	469.3	4.61	0.98

5. CONCLUSION

A new, simple, rapid very sensitive and accurate high performance thin-layer chromatographic (HPTLC) method has been developed and validated for estimation of Gemifloxacin in Rabbit plasma. HPTLC was performed on silica gel 60F $_{254}$ plates with ethanol: ethyl acetate: hexane, 2:7:1 (*v*/*v*), as mobile phase. Densitometry scanning was performed in absorbance mode at λ=254nm. The R_F value was 0.21. The response was a linear function of concentration over the range 0.1–0.7µg mL $^{-1}$ (r^2=0.996). A maximum recovery of drug from plasma was resulted using chloroform and acetic acid as extracting solvent in comparisons to other organic solvents. Mean extraction recovery was >82%. Intra-day and inter-day precision of the assay were in the range 1.19 to 2.85 (% RSD) and accuracy was within 94.3 to 98.26%. This method can be applied to pharmacokinetic studies in Rabbit plasma.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

COMPETING INTERESTS

Authors have declared that no competing interests exists.

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