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# Development and validation of new microbiological analytical method foranalysis of Gemifloxacinmesylate in pharmaceutical formulation

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**ABSTRACT**

The gemifloxacinmesylate is a fourth-generation synthetic broad-spectrum fluorinated quinolone antibacterial agent for oral administration indicated for infections caused by gram positive and gram-negative micro-organisms. Although this drug is studied and researched regarding the antimicrobial activity, pharmacokinetics and pharmacodynamics, there are few studies regarding the development of analytical methodology for this antibiotic. This study describes the development and validation of a microbiological analytical method using the turbidimetric method for the determination of gemifloxacinmesylate in tablets, using *Staphylococcus epidermidis* NCIM 2493 as test microorganism and compared with an HPLC method which was optimized and partially validated according to ICH guidelines. The developed and validated method showed excellent results of linearity, precision and robustness, in the concentration range from 0.5 to 4.5µg/mL. The microbiological analytical method can be used for routine quality control analysis of gemifloxacinmesylate in dosage forms.

## INTRODUCTION

Gemifloxacin is a fourth-generation synthetic broad-spectrum fluorinated quinolone antibacterial agent for oral administration. It was discovered by Hong et al in 1997, and is present in two forms: either as free gemifloxacin base or as gemifloxacinmesylate salt. Gemifloxacin has a broad-spectrum activity against both gram-negative and gram-positive microorganisms.It acts by inhibiting DNA synthesis through the inhibition of both DNA gyrase and topoisomerase IV (TOPO IV), which are essential for cellular replication and bacterial growth. It is well known that *Streptococcus pneumoniae* has been showed mutations in both DNA gyrase and TOPO IV (double mutants), and they are resistant to most fluoroquinolones. Thus, gemifloxacin is considered the only fluoroquinolone which has the ability to inhibit both enzyme systems (dual targeting of both DNA Gyrase and TOPO IV) at therapeutically relevant drug levels in *S. pneumoniae*.It has the lowest MICs against *S. pneumoniae* when compared with ciprofloxacin, levofloxacin, gatifloxacin, and moxifloxacin. The MICs of gemifloxacin against *H. influenzae* and *M.catarrhalis* are comparable to or lower than those of other quinolones tested. [1-4]The structure of gemifloxacinmesylate is shown in fig 1.

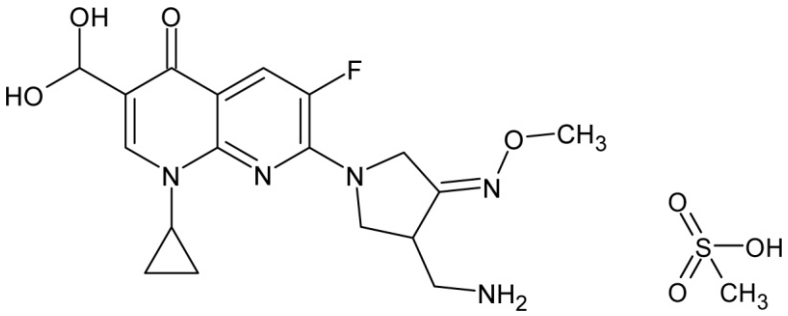


**Fig. 1 :** Structure of gemifloxacinmesylate

The potency of an antibiotic is estimated by comparing the inhibition of growth of sensitive micro-organisms produced by weighted portion of the sample. The literature review reveals that there were various analytical methodslike HPLC [5-8] and spectrophotometry methods [9,10]were reported for analysis of gemifloxacinmesylatein pharmaceutical dosage forms. There are microbiological methods available in the literature for other medicinal agents.[11-14] There were no microbiological analytical methods forthe estimation of gemifloxacinmesylate in formulations.

Biological methods are advantageousbecause the parameters that are measured withthese techniques and the properties for the drugused are the same. Thus, impurities and therelated substances do not interfere, maintainingthe precision of the analytical method.

The purpose of this study was to develop and validatea microbiological method to determine the potencyof gemifloxacinmesylate in commercial tablets. The bioassayresults were compared to those obtained by HPLC, usingthe same samples.

## MATERIALAND METHODS

### Standards and Reagents:

Gemifloxacinmesylate reference standard was kindly supplied by Hetero Labs Limited, Hyderabad. Gemifloxacinmesylate tablets were purchased from local market with brand name Gemone. Methanol, sodium hydroxide (AR), formic acid were used for analysis along with HPLC grade water were purchased from SD fine chemicals, Hyderabad.

### Microbiological assay

### Preparation ofgemifloxacinmesylatereference standard solutions

A stock standard solution of 1000 µg/mL was preparedby taking 10 mg of reference standard of gemifloxacinmesylate into a flask and diluting with sterile distilled water to a total volume of10 mL. This solution was further diluted in sterile distilledwater to obtain working standard solutions with concentrations of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 µg/mLrespectively named as S1, S2, S3, S4, S5, S6, S7, S8 and S9 which were used in the bioassay.

### Preparation of gemifloxacinmesylate sample solutions

Sample solutions were prepared from gemifloxacin tablets. Not less than 20 tablets were weighed and portion of the tablet powder equivalent to about 10.0mg was transferred to a volumetric flask and the volume was completed with sterile distilled water to obtain a solution with a concentration of 1000µg/mL. From 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 µg/mLconcentration solutions were prepared identical to reference standard solutions and named as T1, T2, T3, T4, T5, T6, T7, T8 and T9 which were used in assay.All the solutions (standard and test) were prepared freshly before performing the test.

### Preparation of Mueller Hinton Broth

21grams of Muellerhinton broth was accurately weighed and transferred to 1000mL volumetric flask and volume was made up to 1000mL with distilled water. Boil to dissolve the medium. Sterilize by autoclaving at 15lbs pressure (121$^0$C) for 15minutes. Adjust the pH of the medium to 8.1 using 0.1M NaOH.

### Preparation of inoculums

The strain of *Staphylococcus epidermidis* NCIM 2493 was cultivated and maintained in nutrient agar medium at 4$^o$C. The strain was peeled into nutrient agar medium and maintained in an oven at 35$^0$C ±2$^0$C, for 21h before the assay, for the growth of *Staphylococcus epidermidis.*

The assay was performed by using turbidimetric method and results were analysed statistically by the linear parallel model and by means of regression analysis of variance.

A volume of 0.5 mL of standardized *Staphylococcus epidermidis* NCIM 2493 was added to 18 tubes containing 9mL of Mueller hinton broth. To nine among 18 test tubes 1mL of standard solutions each from respective concentrations (0.5 4.5 µg/mL) were added and named as S1, S2, S3, S4, S5, S6, S7, S8, and S9. To other nine test tubes 1mL of sample solutions each from respective concentrations (0.5 4.5 µg/mL) was added T1, T2, T3, T4, T5, T6, T7, T8, and T9. After that, all the tubes were incubated at a temperature of 35.0$^0$± 2.0$^0$C for 4 hours.After the incubation period, the multiplication of microorganisms was interrupted by the addition of 0.5ml of formaldehyde solution to each tube.

The Then, the spectrophotometer was reset by the test tube containing a negative control (9mol of MHB containing 0.5mol of the organism and 0.5mL of the formaldehyde solution) and the absorbance values were calculated for each tube at a wavelength of 530nm in a spectrophotometer.

### Method validation

The method was validated by evaluationof linearity, precision and accuracy, according to theprocedures described in ICH guidelines Q2 (R1).According to the ICH guidelines, the limits of detection and quantification are not required for this category of assay[15].

#### *Linearity*

The linearity of theassay for pure drug and drug product was assessedwith nine concentrations of the reference standard and samples weretested in the concentration rangeof 0.5µg/mL to 4.5µg/mL. Calibration curve for log10 of gemifloxacinmesylate concentration versus absorbance values was plotted and the obtained data were subjectedto regression analysis using the Least Squares Method.

#### *Precision*

The intraday precision of the method wasevaluated by analyzing six replicates of gemifloxacinmesylate solutions, at 100% test concentration (3µg/mL). Similarly, the interday precision wasevaluated at three concentrations levels of 2.0, 2.5 and 3.0µg/mL ($n$ = 12)on three different days. Theconcentration of gemifloxacinmesylatein the capsule samples wasdetermined and the relative standard deviation (RSD) wascalculated.

#### *Accuracy*

Accuracy was determined by adding known amounts of the gemifloxacinmesylate reference standard(3.0µg/mL) to a samplesolution at three different concentrations (2.0, 2.5 and 3.0µg/mL) corresponding to 80, 100 and 120% of the test concentration.At each concentration, solutions were preparedin triplicate and the recovery percentage of gemifloxacinmesylate was determined.

### Assay of gemifloxacinmesylate tablets using turbidimetric method

20 tablets were taken and powdered. Tablet powder quantity equivalent to 100mg of the drug was transferred in 100mL clean dry volumetric flask and 20mL of steriledistilled water was added and sonicated to dissolve it completely and make volume up to the mark with the sterile water (1000µg/mL stock solution). From the above solution 100µg/mL was prepared. From the 100µg/mL solution 1.5µg/mL test concentration solution was prepared. From this solution add 1mL to 10 mL test tube containing MHB and 0.5 mL of organism.

The tube was incubated at a temperature of $35.0^0 \pm 2.0^0$C for 4 hours. After the incubation period, the multiplication of microorganisms was interrupted by the addition of 0.5mL of formaldehyde solution Then, the spectrophotometer was reset by the test tube containing a negative control (9mL of MHB containing 0.5 mL of the organism and 0.5mL of the formaldehyde solution) and the absorbance values were calculated for each tube at a wavelength of 530nm in a spectrophotometer.

### HPLC assay method for gemifloxacinmesylate

A previously developed and validated HPLC method was selected as a comparative method for the determination of gemifloxacinmesylate in tablets.[16] The method was performed in isocratic mode and the mobile phase consisted of methanol and 7% formic acid (60:40v/v). The chromatographic separation was carried out on an Enable C18 analytical column (250 × 4.6 mm: 5 mm) at a flow rate of 1.2mL/min. The peak areas were defined as analytical signs, with detection at 262nm.

### Comparison of methods

The results of the analysis obtained by the microbiological method in this study and chromatographic method were compared statistically using student t- test, at a level of significance of 5% using graph pad prism software. The agreement between the two methods was evaluated by Pearson's coefficient (*r*) and the two curves must be parallel and linear over the working range chosen.

## RESULTS

A new microbiological analytical method was developed for quantitative analysis ofgemifloxacinmesylate in tablets. To develop and validate this bioassay strain of Staphylococcus epidermidis was found tobe appropriate micro-organism allowing quantitation of gemifloxacinmesylate as it showsgood linearity. The calculation procedure assumes a direct relationship between theabsorbance and the logarithm of the dose.The parameters tested to establish the conditions were described and shown in the table….

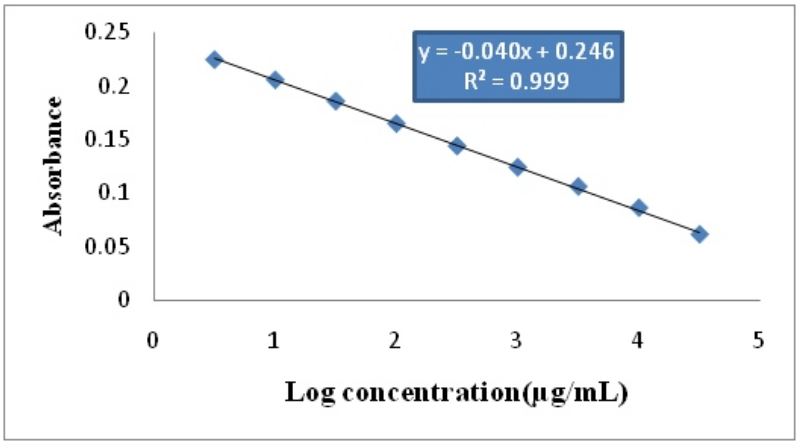


**Fig. 2 :** Calibration curve of pure gemifloxacinmesylate

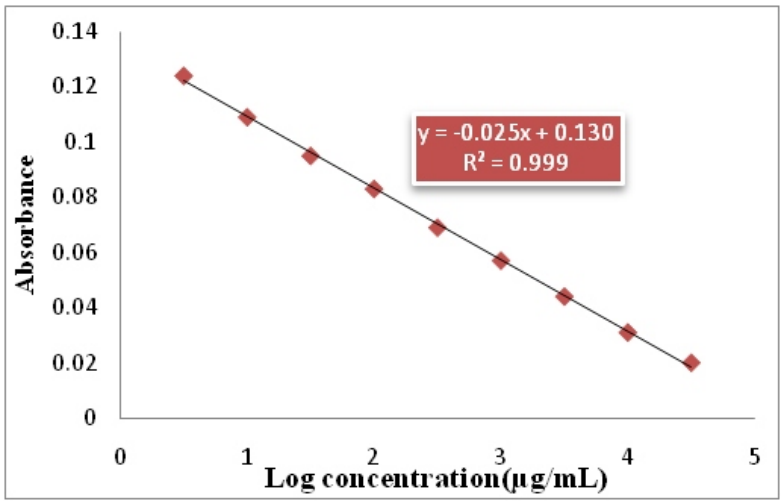


**Fig. 3 :** Calibration curve of gemifloxacinmesylate in drug product

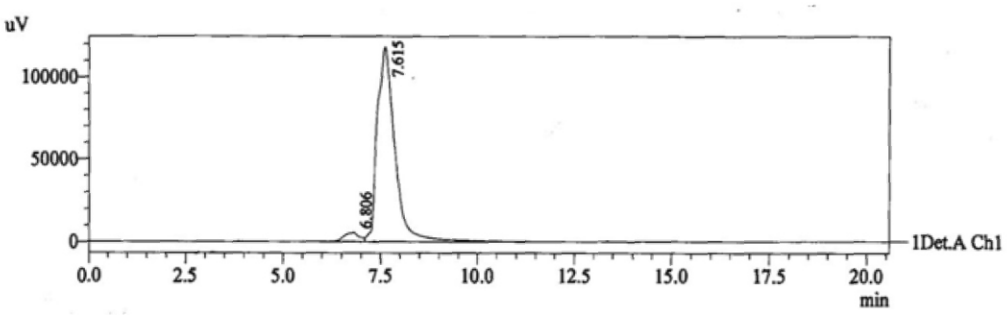


**Fig. 4 :** Chromatogram of assay of gemiflxacinmesylate tablets by HPLC

**Table 1 :** Calibration curve values for both drug substance and drug product.

| S.No | Concentration (µg/ml) | Absorbance*± SD(drug substance) | Absorbance*± SD(drug product) |
|---|---|---|---|
| 1 | 0.5 | 0.225± 0.001 | 0.124 ±0.001 |
| 2 | 1 | 0.206 ± 0.007 | 0.109 ±0.001 |
| 3 | 1.5 | 0.186 ±0.002 | 0.095 ±0.002 |
| 4 | 2 | 0.165 ±0.001 | 0.083 ±0.001 |
| 5 | 2.5 | 0.144± 0.001 | 0.069± 0.001 |
| 6 | 3 | 0.124 ±0.001 | 0.057 ±0.003 |
| 7 | 3.5 | 0.106 ±0.001 | 0.044± 0.001 |
| 8 | 4 | 0.086 ±0.001 | 0.031 ±0.001 |
| 9 | 4.5 | 0.061± 0.001 | 0.020 ±0.001 |
| | Slope | 0.040 | 0.025 |
| Correlation coefficient | | 0.999 | 0.999 |

* Average of three determinations.

**Table 2 :** Assay of gemifloxacinmesylatetablets byHPLC and turbidimetric methods

| Parameters | Method | |
|---|---|---|
| | HPLC | Microbiological method |
| Average Gemifloxacinmesylate content % | 97.3 | 99.7 |
| | 98.1 | 98.8 |
| | 97.1 | 97.6 |
| Mean | 97.6 | 98.6 |

*Validation results*

Linearity data obtained were analyzed by the least squares and parallelism was done by analysis of variance (ANOVA).The value of the correlation coefficient (r) 0.999 is considered highly significant for this method. A parallel-line model has been chosen, in which two curves areconstructed, one of them for gemifloxacinmesylate RS and the other for the sample oftablet, and these two curves must be parallel and linear over the working range chosen.These parameters must be verified by validity tests, considering a given probability, whichis usually $p = 0.05$. The tests performed in this study were validated through the analysisof variance (ANOVA). Through this analysis, it was found that there was no deviation inthe linearity and parallelism of two curves ($p < 0.05$), i.e. $p < 0.0001$log concentration (µg/ml) versus absorbance plots showed good linearity between 0.5to 4.5 µg/ml concentration range and shown in the fig. 2 and 3.

Accuracy of the method was determined at three different concentration levels. The accuracy of the method was confirmed by reporting the % recovery values were ontained in the range 98-102.Precision of the method was established by reporting repeatability and intermediate precision.The repeatability study was performed for the % RSD for gemifloxacinmesylate was found to be 1.78% indicating the method is precise.The intra-day and inter day precision results are obtained for three replicates of three concentrations of gemifloxacinmesylate. The samples were analysed at different sessions of the day and on different days and the samples didn't show any variations in the measured absorbance values and the calculated %RSD values are low, indicating that the method was precise.

*HPLC method*

The linearity of the method was established by performing linear regression analysis for the calibration curve constructed between concentration and peakarea in the concentration of 0.5µg/mL to 4.5µg/mL. The respective chromatogram is shown in the fig.4

*Comparison of methods*

Assay results of gemifloxacinmesylate calculated by both methods are shown in table 2. The results obtained by the microbiological and HPLC methods were statistically compared using the Student's *t*-test.The difference between microbiological method and HPLC was considered to be extremely statistically significant at a level of 5%, indicating rejection of null hypothesis.

## DISCUSSION

The development and validation of analytical methods for the determination of active ingredients in medicines is important for quality control. When choosing an analytical method for use in routine quality control, the analyst must consider cost, complexity, required time, availability of equipment and reagents, purity, quantity of the sample and the generation of residues.[15]The development and validation of analytical methods to determine potency, such as microbiological assays, have recently received considerable attention, mainly from regulatory agencies, because of their importance in pharmaceutical analysis. Microbiological assays have the potential to prevent the possible loss of activity. For this reason, a microbiological assay was proposed as a suitable method for the determination of gemifloxacinmesylate.The potency of an antibiotic may be demonstrated under suitable conditions by comparing the inhibition of growth of a microorganism caused by known concentrations of the antibiotic and a reference standard.

## CONCLUSION

The quantification of antibiotic components by instrumental methods such as HPLC and UV spectrophotometry are precise, but they cannot provide true indication of biological activity. Themicrobiological analytical method developed and validated for determination of gemifloxacinmesylate in tablets demonstrated linearity, precision and accuracy. Microbiological methods do not require any specialized equipment. Turbidimetric methods are faster than the agar diffusion methods. Although the biological assay methods have a high variability, the obtained results demonstrated that the proposed method can be useful for determination of this drug in pharmaceutical dosage forms, as an acceptable alternative method for the quality control analysis of gemifloxacinmesylate.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ACKNOWLEDGMENT

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