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GEMIFLOXACIN MESYLATE: UV SPECTROPHOTOMETRIC METHOD FOR QUANTITATIVE DETERMINATION USING EXPERIMENTAL DESIGN FOR ROBUSTNESS

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This study describes the validation of UV spectrophotometric method for quantitative determination of gemifloxacin mesylate (GFM) in tablets using methanol as solvent. The method was specific, linear, precise, exact and robust at 272 and 343 nm. The results confirmed that the method in both wavelengths is valid and useful to the routine quality control of GFM in coated tablets. The validate method was compared to liquid chromatography (HPLC), microbiological assay and visible (VIS) spectrophotometry, which were previously developed and validated to the same drug. There was not significative difference between the methods for GFM quantitation.

Keywords: experimental design; gemifloxacin mesylate; UV spectrophotometric method.

INTRODUCTION

Gemifloxacin mesylate (GFM, Figure 1), chemically ((*R*,*S*)-7-[(4*Z*)-3-(aminomethyl)-4-(methoxyimino)-1-pyrrolidinyl]-1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-1,8-naphthyridine-3-carboxylic acid methanesulfonate is a synthetic broad-spectrum antibacterial agent for oral administration.[1]

The drug has shown potent antibacterial activity against clinical isolates and reference strains in both in vitro studies and experimental models of infection in animals.[2,3]

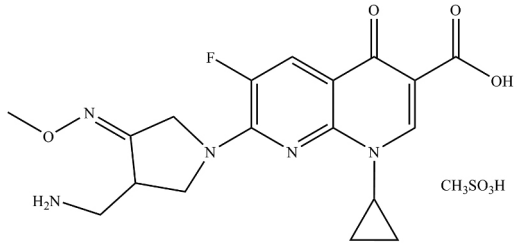

Figure 1. *Chemical structure of GFM*

The drug is approved by FDA only for respiratory indications: acute bacterial exacerbation of chronic bronchitis and community-acquired pneumonia, including that caused by known or suspected multidrug resistant strains of *S. pneumoniae,* because it has good penetration into respiratory secretions, such as the epithelial lining fluid and into alveolar macrophages, with adequate concentrations at the site of the infection.[4,5]

Although there are studies describing the determination of GFM in biological fluids by HPLC-MS,[6,7] HPLC-ESI-MS-MS,[8] HPLC and HPTLC[9] and in tablets by VIS spectrophotometry,[10,11] HPLC[12] and microbiological assay[13] there are no studies describing quantification methods by UV spectrophotometry. Then the main objective of this study was to develop a simple, fast and low cost UV spectrophotometry method, without extraction process, derivatization, evaporation step, and complexation agent, providing decrease in time and error in the quantitative determination of GFM in coated tablets.

The method described in this study was validated through the evaluation of the following analytical parameters: specificity, linearity, precision (repeatability and intermediate precision), accuracy and robustness.[14,15] Experimental design was used to evaluate method robustness.[16] Besides, the statistical comparison of the precision results with those obtained from the HPLC, microbiological assay and VIS spectrophotometry (previously validated for the same research group) was presented to show the suitability of the developed method.

EXPERIMENTAL

Reagents and chemicals

GFM reference standard (RS) was acquired by Toronto Research Chemicals, Inc. (Ontario, Canada) possessing 99.0% of purity. Factive® (Aché, Brazil) 320 mg of gemifloxacin was purchased in the market. The excipients ingredients contained in the dosage form (microcrystalline cellulose, crospovidone, titanium dioxide, magnesium stearate, hydroxypropyl methylcellulose, polyethylene glycol, and povidone) were all pharmaceutical grades and acquired from different suppliers. Methanol and ethanol were purchased from Tedia® (Fairfield, USA). Purified water was obtained using a Milli-Q Plus® (Millipore, Bedford, USA). The buffer solutions and HCl 0.1 M were prepared in agreement with USP 32 (2009).[15]

Instrumentation and conditions

Spectral and absorbance measurements were performed with an UV-Vis Shimadzu model UV 160A using 10 mm quartz cells and detection at 272 and 343 nm.

An Agilent liquid chromatograph 1200 Series (Santa Clara, CA, United States) with ChemStation manager system software was used. The chromatographic separation was performed in an Agilent Eclipse® XDB RP-18 column (150 x 4.6 mm I.D., 5 µm, Santa Clara, CA, United States). The mobile phase composed by a mixture of triethylamine 0.3% (pH adjusted to 3.0 with phosphoric acid 10%) and acetonitrile (80:20, *v/v*) at a flow-rate of 1.0 mL min^{-1} was operated at isocratic elution mode. GFM was determined by UV detection at 272 nm using photodiode-array.

The agar diffusion bioassay followed the 3×3 parallel line design (3 doses of standard and 3 doses of sample in each plate), according to the Brazilian (1988) and European Pharmacopoeias (2005).[17,18] The

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microorganism used was *Staphylococcus epidermidis* ATCC 12228 and the concentrations of reference and sample solutions were 0.5; 1.5 and 4.5 μg mL^{-1}. The layer base agar was composed by medium number 1. All experiments were performed in a biological safety cabinet and the infected material was decontaminated before discarded.

In the VIS spectrophotometric method, aliquots of GFM in methanolic solution (100.0 μg mL^{-1} in gemifloxacin) were transferred to separating funnels, added pH 3.3 potassium biphthalate buffer solution and 0.001 M bromocresol green solution. The extraction procedure was done using chloroform, allowing the separation of the phases and the organic layer was collected. The absorbance of this layer was measured at 417 nm against the blank solution.

Preparation of RS and sample solutions

GFM RS was accurately weighed and dissolved in a 100 mL volumetric flask with methanol to produce a concentration of 100.0 μg mL^{-1} in gemifloxacin. This solution was diluted appropriately in the same diluent to yield a final concentration of 6.0 and 12 μg mL^{-1} at 272 and 343 nm, respectively.

To prepare a sample solution, twenty tablets of Factive® were weighed and finely powdered. A quantity equivalent to 10.0 mg of gemifloxacin was transferred into a 100 mL volumetric flask with 60 mL methanol and kept in an ultrasonic bath for 30 min. The volume was completed with the same diluent and filtered. Aliquots of 3.0 mL of this solution were diluted in a 25 and 50 mL volumetric flasks to yield a final concentration of 6.0 μg mL^{-1} at 272 nm and 12.0 μg mL^{-1} at 343 nm.

The stability of RS and sample solutions was evaluated at room temperature (23 ± 1 °C) during 24 h using the HPLC method. The stability of these solutions was verified by observing any change in the chromatographic pattern and in the decrease of the response of the peak (area), which can indicate the degradation of the solutions.

Method validation

Different solvents were investigated to develop a suitable UV spectrophotometric method for the analysis of GMF in tablets. For selection of solvent the criteria employed was the easiness of sample preparation, solubility and stability of the drug, cost of solvent and applicability of the method.

The evaluation of the method specificity was performed by preparing a placebo containing the same excipients of the commercial product.

Three calibration curves were prepared with seven concentrations at 272 nm (2.0, 4.0, 6.0, 8.0, 10.0, 12.0 and 15.0 μg mL^{-1}) and at 343 nm (6.0, 8.0, 10.0, 12.0, 15.0, 20.0 and 25.0 μg mL^{-1}) of the GFM RS to evaluate the linearity. For each concentration the solutions were prepared in triplicate. The obtained absorbances were plotted against the respective concentrations of drug to obtain the analytical curves. The calculation of the regression line was employed by the method of least squares and the curves were validated by means of the analysis of variance (ANOVA).

The determination of precision was done through six sample solutions, at the same concentrations (6.0 and 12.0 μg mL^{-1} at 272 and 343 nm, respectively) under the same experimental conditions in the same day for intra-day precision (repeatability) and on 3 different days for inter-day precision (intermediate precision). The relative standard deviations (RSD) were determined.

The accuracy was determined by recoveries tests using three concentration levels by adding known amounts of RS to the samples. Portions of 3.0 mL of the sample stock solution (100.0 μg mL^{-1}) were transferred to 25 and 50 mL volumetric flasks and aliquots of 1.0, 2.0, and 3.0 mL GFM RS (100.0 μg mL^{-1}) were added. After this procedure, it was added methanol to a final concentration of 8.0, 10.0, and 12.0 μg mL^{-1} at 272 nm and 16.0, 20.0, and 24.0 μg mL^{-1} at 343 nm. The analyses were done in 3 replicates.

The robustness tests using experimental design in the GFM assay provided an effective approach as part of the method validation. Robustness testing was performed in order to evaluate the susceptibility of measurements due to deliberate variations in analytical conditions.

The factors were examined in an experimental design of 8 experiments, which was selected as a function of the number of factors to investigate (3). This designs applied, so-called two-level screening designs, allows screening a relatively large number of factors in a relatively small number of experiments.

The factors and the levels investigated in robustness evaluation are summarized in Table 1. The construction of the experimental designs was performed in accordance to Plackett–Burman described in Heyden and collaborators.[16] The first row in the experimental design was copied from model described and the following rows were obtained by cyclical permutation of one position (i.e. shifting the line by one position to the right) compared to the previous row. This procedure was repeated until all but one line is created and the last row must consist only of minus signs. For each of the 8 experiments, two solutions were performed for each solution: GFM RS and drug product in each wavelength.

Table 1. Factors and levels investigated in the robustness test

Factors	Nominal	Level (-1)	Level (+1)
Solvent (brand)	Tedia	Tedia	Nuclear
Time of Shaking (ultrasonic bath) (min)	30	28	32
Wavelength (nm)	272 / 343	269 / 340	275 / 346

After determination of the number of real factors to be examined, the remaining columns in the design were defined as dummy factors, which is an imaginary factor that has no physical meaning.

A half-normal probability plot for the effects in combination with the dummy factors was used to estimate the error and identify significant effects.[16]

For each factor its resulting effect was calculated according to the Equation 1:

$$E_x = \frac{\sum Y(+)}{N/2} - \frac{\sum Y(-)}{N/2} \quad (1)$$

where: E_X is the effect of X on response Y (GFM concentration); $\Sigma Y(+)$ and $\Sigma Y(-)$ are the sums of the responses where X is at the extreme levels (+) and (-), respectively, and N is the number of experiments of the design.

The effect of the estimate experimental error $(SE)_e$ allows concluding what is significant from dummy factors (Equation 2). This value was used to perform the statistical test.

$$(SE)_e = \sqrt{\frac{\sum E^2_{dummy}}{n_{error}}} \quad (2)$$

where: ΣE^2error is the sum of squares of the n_{error} dummy.

The utilization of the dummy factors to calculate the $(SE)_e$ was possible because the number of degrees of freedom (4 dummy factors) was suitable to test the effects, showing the power of the *t*-test to detect any significance of the studied factors. The statistical interpretation provides to the user a numerical limit value that allows defining what is significant and what is not. This limit value to identify statistically

significant effects is usually derived from the *t*-test statistic, in accordance to the Equation 3:

$$t = \frac{|E_x|}{(SE)_e} \quad (3)$$

An effect is considered significant at a given α level if t calculated > *t* critical.[16]

Methods comparison

In order to compare the developed UV spectrophotometry method at 272 and 343 nm with well characterized procedures, HPLC, microbiological and VIS spectrophotometric methods previously validated, the precision results of these methods were statistically analyzed using ANOVA, which indicates if there is significant difference between the methods at 5% significant level.

RESULTS AND DISCUSSION

The UV-VIS method is very used in the quality control of pharmaceutical products due to the potential of the great majority of the drugs of absorbing energy in these wavelengths. The absorption of UV-VIS radiation occurs through the excitation of electrons within the molecular structure to a higher energy state. Although the selectivity depends on the chromophore of the drug, the method presents a series of applications: quantification of drugs in formulations where there is no interference from excipients, pka determination, release of drugs from formulations with time in dissolution testing, to monitor the reaction kinetics of drug degradation and to identity drugs starting from UV spectrum.[19]

The development of a simple, rapid, sensitive, and accurate UV spectrophotometric method for the routine quantitative determination of samples reduces unnecessary tedious sample preparations and the cost of analysis.

Considering the solubility and stability, the following solvents were used as diluent of the GFM RS and the sample solutions: water, ethanol and methanol. The first solvent studied was water. Although the solubility tests showed that GFM RS was very soluble in water, the preliminary studies showed a low percentage of recuperation of the drug from tablets (± 75%). The utilization of the phosphate buffer solutions (pH 5.0, 6.0 and 7.0) was not possible due to the low solubility of the drug at room temperature.

The stability of GMF in acid solution was evaluated to verify if any spontaneous degradation occurs when the samples were prepared. The studies done by HPLC method showed the instability of the drug in acid solution. Then the utilization of this solvent was discarded.

The preliminary results with ethanol demonstrated that the drug presented two wavelengths of maximum absorption (272 and 343 nm), however, the method did not show repeatability during the preliminary tests.

After these preliminary tests, the use of methanol as diluent was verified to develop the analytic method. The results were adequate and the percentage of gemifloxacin found in the tablets was around 100% in both wavelengths used (Figure 2).

The stability of the drug in methanolic solution was also evaluated. The studies done by HPLC method showed the stability of the drug in methanol for 24 h in these conditions.[12]

The specificity test demonstrated that there was not interference of the excipients in the drug determination in both wavelengths. The UV spectrums obtained through the analysis of the GFM placebo solution did not present any interference at 272 and 343 nm.

Linearity was observed over the concentrations range of 2.0 to 15.0 μg mL^{-1} at 272 nm and 6.0 to 25.0 μg mL^{-1} at 343 nm, with significantly high values of correlation coefficient. The validity of the assay was verified by means of ANOVA and according to it, there are linear regression and there are not deviation from linearity (α = 0.05, Table 2).

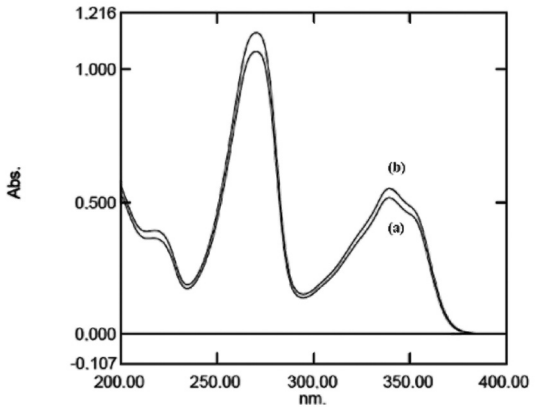

Figure 2. *UV-absorption spectra of 12.0 μg mL^{-1} concentration of GFM commercial sample (a) and GFM RS solutions (b) in methanol*

Table 2. Statistical data of the regression equations to analysis of GFM

Regression analysis	λ 272 nm	λ 343 nm
Linearity range (μg mL^{-1})	2 – 15	6 – 25
Slope	0.10422	0.04804
Intercept	0.00012	0.00666
Correlation coefficient (r)	0.99993	0.99991
Lack of fitting (critical F-value)[a]	2.80 (2.96)	2.71 (2.96)
Linear regression F-value (critical F-value)[a]	207296.2 (4.6)	151786.2 (4.6)

[a] Theoretical value of *F* is based on one-way ANOVA test at α = 0.05 level of significance.

The Student's *t*-test was performed to verify the significance of the experimental intercept in the regression equation. According to the results, it is not significantly different from the theoretical zero value with a significance level of 5% ($p > 0.05$) at λ 272 nm. At 343 nm the experimental intercept was significative ($p < 0.05$), but it was less than 2% of the absorbance obtained for the studied concentration (12 μg mL^{-1}) of GFM RS, therefore there is no interference on the validation, as postulated by Carr and Wahlich.[20]

The experimental values obtained for the determination of the analytical method precision (repeatability) are presented in Table 3. The low relative standard deviations (RSD) obtained for the repeatability and intermediary precision (1.62 and 1.53% at 272 and 343 nm, respectively) showed the good precision of the method in both wavelengths.

The accuracy was calculated in relation of the percentage of recovery of the known added amount of GFM RS to the samples. The accuracy of the method ranged from 98.64 to 100.68% at 272 nm and 99.10 to 101.32% at 343 nm. These values showed the good accuracy of the purposed method.

The results of the robustness experiments are presented in Table 4. They are expressed in concentration of the drug in relation to the nominal dose, calculated using standard solution in the same experimental condition. The effects of the factors in analysis, the error estimated starting from the factors dummy and the value of *t* - calculated are also showed in Table 4. The analysis of the results

Table 3. Repeatability values obtained for GFM coated tablets by UV spectrophotometry at 272 nm and 343 nm

Sample (n)	Repeatability (λ 272 nm)			Repeatability (λ 343 nm)		
	Day 1	Day 2	Day 3[a]	Day 1	Day 2	Day 3 [a]
1	100.29	97.35	98.11	99.47	98.46	99.28
2	97.24	97.18	100.37	97.58	97.09	100.84
3	97.35	94.77	100.48	97.01	96.28	99.07
4	100.98	96.81	97.91	100.41	96.28	98.36
5	98.26	96.85	98.35	97.01	97.08	100.94
6	99.32	97.60	97.70	98.63	99.79	97.58
Mean (%)	98.91	96.76	98.24	98.35	97.50	98.77
RSD (%)	1.57	1.05	1.91	1.42	1.41	1.98

[a] Analyst B

Table 4. The selected Plackett-Burman design, results of the experiments and effects (Ex) of the factors

Exp	Sonic	Dummy	Dummy	λ (nm)	Dummy	Solvent	Dummy	Conc. GFM_{272}	Conc. GFM_{343}
1	+	+	+	-	+	-	-	5.96	12.08
2	-	+	+	+	-	+	-	5.66	11.21
3	-	-	+	+	+	-	+	5.80	11.42
4	+	-	-	+	+	+	-	5.72	11.56
5	-	+	-	-	+	+	+	5.87	11.54
6	+	-	+	-	-	+	+	5.72	11.30
7	+	+	-	+	-	-	+	5.97	12.08
8	-	-	-	-	-	-	-	5.84	11.93
E_{x272}	0.05	0.10	-0.06	-0.06	0.04	-0.15	0.05		
$(SE)_{e272}$	0.065								
$t_{calc\ 272}$	0.73			0.9		2.26			
E_{x343}	0.23	0.18	-0.27	-0.15	-0.02	-0.48	-0.11		
$(SE)_{e343}$	0.173								
$t_{calc\ 343}$	1.34			0.84		2.76			
t_{crit}	2.776								

Sonic = shaken in ultrasonic bath; λ = Wavelength; Conc. GFM272 and Conc. GFM343 = concentrations obtained in the experiments at 272 nm and 343 nm, respectively; Ex272 and Ex343 = effects obtained at 272 nm and 343 nm, respectively; (SE)e 272 and (SE)e343 = estimate experimental error at 272 nm and 343 nm, respectively.

of the robustness study demonstrated that the factors in analysis did not present significant effect on the quantitation of the GFM, indicating the robustness of the UV spectrophotometric method in both wavelengths.

The statistical comparison between the UV spectrophotometry at 272 and 343 nm, HPLC, microbiological assay and VIS spectrophotometry methods was performed through ANOVA using the mean experimental values obtained in the precision of the methods. The test did not show statistical difference between the techniques ($F_{calc} = 2.39 < F_{crit} = 2.49$, $p > 0.05$), showing also the capacity of this developed method to quantify the drug GFM in coated tablets with accuracy and precision in comparison with biological methods, that compares the growth inhibition of sensitive microorganisms produced by known concentrations of the antibiotic to be examined and a RS[15] and physico-chemical methods.

CONCLUSIONS

The results indicated that the UV spectrophotometric method presents linearity, precision, accuracy, specificity and robustness at 272 and 343 nm. Besides, there is no significative difference between the previously validated methods by HPLC, microbiological assay and VIS spectrophotometry and UV method at 272 and 343 nm, which confirm that the UV method in both wavelengths is adequate and useful to the routine quality control of GFM in pharmaceutical dosage forms.

The results indicated that the UV spectrophotometric method presents linearity, precision, accuracy, specificity and robustness at 272 and 343 nm. Besides, there is no significative difference between the previously validated methods by HPLC, microbiological assay and VIS spectrophotometry and UV method at 272 and 343 nm, which confirm that the UV method in both wavelengths is adequate and useful for quantitative determination of GFM in coated tablets. Moreover, the validated method is an excellent alternative for routine analysis of GFM, because it shows low cost and it is faster than other validated methods.

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