

PERFORMANCE CHARACTERISTICS OF BIOASSAY UV-SPECTROPHOTOMETRY AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF GATIFLOXACIN IN TABLETS

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The microbiological bioassay, UV-spectrophotometry and HPLC methods for assaying gatifloxacin in tablets were compared. Validation parameters such as linearity, precision, accuracy, limit of detection and limit of quantitation were determined. Beer's law was obeyed in the ranges 4.0-14.0 $\mu\text{g/mL}$ for HPLC and UV-spectrophotometric method, and 4.0-16.0 $\mu\text{g/mL}$ for bioassay. All methods were reliable within acceptable limits for antibiotic pharmaceutical preparations being accurate, precise and reproducible. The bioassay and HPLC are more specific than UV-spectrophotometric analysis. The application of each method as a routine analysis should be investigated considering cost, simplicity, equipment, solvents, speed, and application to large or small workloads.

Keywords: fluoroquinolone; gatifloxacin; pharmaceutical analysis.

INTRODUCTION

Gatifloxacin (Figure 1), a third generation fluoroquinolone, is a potent antibacterial agent active against a wide range of Gram-positive and Gram-negative organisms including Mycobacteria.^{1,2} Chemically gatifloxacin is a (\pm)-1-cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-7-(3-methyl-1-piperazinyl)-4-oxo-3-quinolinecarboxylic acid sesquihydrated and this fluoroquinolone has high oral bioavailability (96%), and, therefore, oral and intravenous formulations are bioequivalent and interchangeable.³

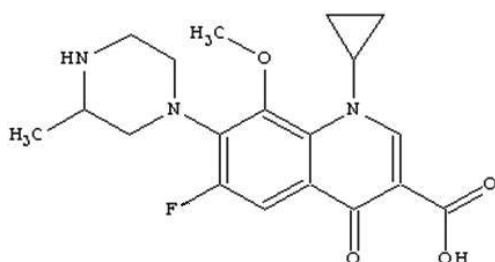


Figure 1. The chemical structure of gatifloxacin (CAS number 160738-57-8)

Although many chromatographic methods have been reported for the determination of fluoroquinolones,⁴⁻⁸ just few methods have been found in the literature for determination of fluoroquinolones in pharmaceutical formulations using spectrophotometry^{9,10} and bioassay.¹¹⁻¹⁴ More specifically, few reports about analytical methods of gatifloxacin in pharmaceutical formulations are available in the literature.¹⁵⁻²¹ In this paper, the results of three methods is compared with respect to accuracy, precision and speed. They included the bioassay (BA), the spectrophotometric analysis (SPA) and the high performance liquid chromatographic determination (HPLC). The proposed methods were applied to the determination of gatifloxacin in pharmaceutical formulation. No interference was observed in the assay of gatifloxacin from common excipients in levels found in pharmaceutical

formulation. These methods are validated by the statistical data. The proposed methods are simple and suitable for routine determination of gatifloxacin. The aim of this work was to determine the performance characteristics of these methods considering simplicity, cost, time and sensitivity to quantify gatifloxacin.

EXPERIMENTAL

Material and reagents

The gatifloxacin reference substance (assigned purity 99.99%) were generous gift from Bristol-Myers Squibb (Sao Paulo, Brazil), as well as gatifloxacin tablets. The tablets were claimed to contain 400 mg of drug and excipients. The GATX reference substance, as well as the tablets, was always kept protected from light. Methanol (HPLC grade), acetonitrile (HPLC grade) and acetic acid (analytical grade) were obtained from Merck®. Ultrapure water was obtained from a Milli-Q® Plus apparatus (Millipore®) and was used to prepare all solutions for the HPLC method and bioassay. Distilled water was used to prepare all solutions for the UV method.

Instrumentation and analytical conditions

HPLC analysis as performed on a Varian 2550 chromatograph equipped with a variable-wavelength detector (set at 287 nm) and a spectra physics integrator and Rheodine injection valve with a 20- μL loop. The analytical column was a Phenomenex Luna (250 mm x 4.6 mm i.d., 5 μm particle size) column. The mobile phase used was aqueous 5% acetic acid-methanol-acetonitrile (70:15:15, v/v/v). All analyses were done under isocratic conditions at a flow-rate of 1.0 mL/min and at room temperature. The sensitivity was 0.5 AUFS and the chart speed was 0.5 cm/min. The HPLC system was operated at ambient temperature ($25 \pm 1^\circ\text{C}$). The mobile phase was filtered by a membrane filter (Supelco) 0.45 μm x 47 mm and degassed with a helium sparge for 15 min. UV method was performed on a UV-vis spectrophotometer, UV-1601 (Shimadzu®) with the signal at 287.0 nm spectrum and using 1.0 cm quartz cell. The bioassay was performed with antibiotic media Grove-Randall number 1 and number 11 (Merck), the indicator organism was *Bacillus subtilis* ATCC 9372 at

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37 °C overnight diluted in nutrient broth 1:50 inoculated to the surface of each plate. Six 8 mm diameter cylinders were used and 200 µL of each sample as added to each cylinder.

Preparation of standard solutions

HPLC method

For the calibration curve, a stock solution of 100 µg/mL was prepared in 100 mL volumetric flask by dissolving 10 mg GATX reference standard in ultrapure water. Appropriate aliquots of the stock solution were diluted with ultrapure water, yielding concentrations of 4.0, 6.0, 8.0, 10.0, 12.0, and 14.0 µg/mL. Triplicate injections of each concentration were performed. The recoveries were determined at four concentration levels, by adding known amounts of reference substance in the beginning of the process. Twenty tablets were weighed to obtain the average tablet weight. The tablets were ground up and 1800.00 mg (representing three times the average weight of each tablet) were transferred to a 500 mL volumetric flask; 250 mL water were added and the flask was shaken for 20 min by a mechanical shaker, followed by addition of ultrapure water to volume. Aliquots of 1 mL of this solution were transferred to a 100 mL volumetric flask followed by making up to volume with water to give a stock solution with concentration 240 µg/mL. Portions of 1.0 mL of this stock solution were transferred to 25 mL volumetric flasks where 0.1, 0.2, 0.4 and 0.6 mL of GATX reference solution (100 µg/mL), equivalent to 10.0, 10.4, 11.2 and 12.0 µg/mL of GATX, were added. After this procedure, dilutions were made with water to give final concentrations of 104.1, 108.3, 116.6 and 125.0%, respectively, of the sample concentrations used in the assay.

UV method

For the calibration curve, solutions of gatifloxacin powder in water (100 µg/mL) were prepared by accurately weighing 10 mg of gatifloxacin to a 100 mL volumetric flask, adding 50 mL water and shaken for 20 min in ultrasonic bath, followed by adding distilled water to make up the volume. The gatifloxacin standard solution was diluted in 100 mL volumetric flasks with distilled water to give a range of solutions with final concentrations of 4 to 14 µg/mL. The absorbance value of each solution was performed at 287 nm. For the recoveries were determined by adding known amounts of gatifloxacin reference substances (2.0, 4.0 and 6.0 µg/mL) to the samples at beginning of the process. A recovery exercise was then performed.

Bioassay

The standard solution in water (100 µg/mL) was diluted in potassium phosphate buffer pH 6.0 and assayed at concentrations of 4.0, 8.0 and 16.0 µg/mL. The bioassay plates were incubated at measured with calipers. A standard curve was prepared by measuring the zone diameters of three concentrations (4.0; 8.0 and 16.0 µg/mL) of Gatifloxacin. Accuracy was determined by adding known amounts of GATX reference substance to the samples at the beginning of the process. Accurately weighted amounts of tablets equivalent of 10 mg GATX were placed in three 100 mL volumetric flask. 0.5, 1.0 and 1.5 mL of Gatifloxacin reference solution (100 µg/mL) were added. Water (50 mL) was added and the flasks were shaken for 10 min. This was followed by making up to volume with water. The dilutions were made in potassium phosphate buffer, pH 6.0, to give a final concentration of 105.0, 110.0 and 115.0%, respectively. The solutions were applied the cylinder plate assay described above. The percentage recovery of GATX reference added was calculated using the formula proposed by the AOAC.²²

Preparation of sample solutions

Bioassay

An amount of powder equivalent to 10 mg of gatifloxacin was transferred to a 100 mL volumetric flask with 50 mL water and shaken for 10 min. This was followed by making up to volume with water. The dilutions were made with potassium phosphate buffer pH 6.0 to give a final concentration of 4.0, 8.0 and 16.0 µg/mL.

Spectrophotometric analysis

To analyze the concentration of gatifloxacin in tablets, each tablet was weighed to obtain the average tablet weight. The tablets were ground up and 1800.00 mg (representing three times the average weight of each tablet) were transferred to a 1000 mL volumetric flask; 500 mL water were added and shaken for 20 min in ultrasonic bath, followed by adding distilled water to make up the volume. Aliquots of 5 mL of this solution were transferred to 100 mL volumetric flask and distilled water added to volume to give an estimated concentration of 60 µg/mL. An aliquot of the 10 mL of this solution was transferred to a 100 mL volumetric flask and water added to make up the volume in order to give a final concentration of 6 µg/mL. This solution as prepared six times and the absorbance of each determined at 287 nm.

HPLC

To analyze the concentration of gatifloxacin in tablets, twenty tablets were weighed to get the average tablet weight the samples of the powdered tablets. A quantity of the powdered tablets containing 1200.0 mg of GATX was transferred to 500 mL volumetric flask with 250 mL water and shaken for 20 min in ultrasonic bath, followed by adding water to make up the volume. This sample solution had a concentration of 2400 µg/mL. Aliquots of 10 mL of this solution was transferred to a 200 mL volumetric flask and water added to make up the volume (concentration of 120 µg/mL). An aliquot of the 5 mL of this solution was transferred to a 50 mL volumetric flask and water added to make up the volume in order to give a final concentration of 12 µg/mL. The solution was homogenized and filtered. All determinations were conducted in triplicate.

Method validation

The methods were validated according to the International Conference on Harmonization²⁴ and AOAC International²² guidelines for validation of analytical methods. Tests *F* were used to compare the proposed methods.

Linearity

The calibration curve was obtained at six concentrations levels of GATX solutions (4.0–14.0 µg/mL for HPLC and UV method). For bioassay, the calibration curve was obtained at three concentrations levels (4.0 – 16.0 µg/mL). The linearity was evaluated by the least square regression method with triplicate determinations at each concentration level.

Precision

The precision of the assay was determined intra-day and inter-day on three different days. Repeatability was calculated from six replicate injections of sample solution of gatifloxacin in the same equipment at a concentration of 12 µg/mL of the intended test concentration value on the same day. The experiment was repeated by assaying freshly prepared solution at the same concentration additionally on two consecutive days to determine intermediate precision. The precision

of the assay method was evaluated by carrying out six independent assays of gatifloxacin test sample against a qualified reference standard and calculated the % R.S.D. of assay.

Accuracy

The recoveries were determined by adding known amounts of gatifloxacin reference substances to the samples at the beginning of the process.²³ A recovery exercise was then performed.

Detection Limit (LOD) and Quantification Limit (LOQ)

The LOD and LOQ of gatifloxacin by the proposed methods were determined using calibration standards. LOD and LOQ were calculated as $3.3 \sigma/S$ and $10 \sigma/S$, respectively, where S is the slope of the calibration curve and σ is the standard deviation of y-intercept of regression equation ($n = 9$).

RESULTS AND DISCUSSION

HPLC method

The development of the HPLC method for the determination of drugs has received considerable attention in recent years because of their importance in routine quality control analysis. A reversed-phase HPLC method was proposed as a suitable method for the estimation of gatifloxacin in pharmaceutical dosage form. The chromatographic conditions were adjusted in order to provide a good performance of the assay. Figure 2 shows a typical chromatogram obtained from the analysis of a standard and sample solution of gatifloxacin using the proposed method. As shown in this figure, GATX was eluted forming symmetrical peak, well separated from the solvent front. The retention time observed (5 min) allows a rapid determination of the drug, which is important for routine analysis. The calibration curves for gatifloxa-

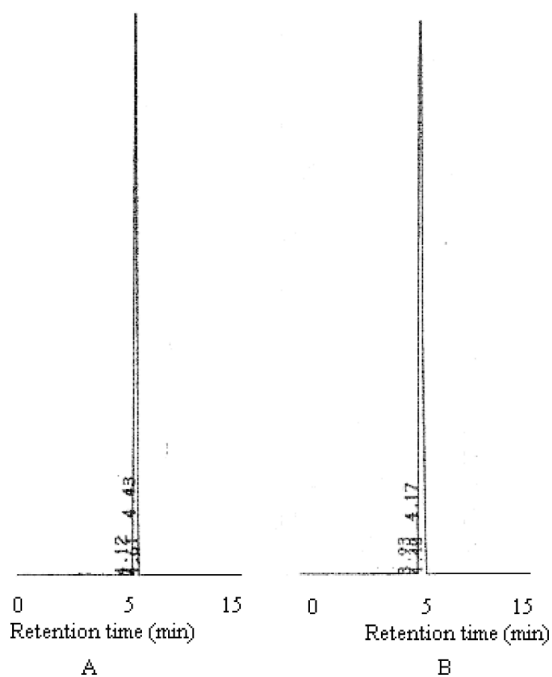


Figure 2. HPLC chromatograms of gatifloxacin reference substance (A) and gatifloxacin tablets (B). Chromatographic conditions: column C_{18} (250 mm x 4.6 mm i.d., 5 μ m particle size), mobile phase: 5% acetic acid-methanol-acetonitrile (70:15:15, v/v/v), flow rate: 1.0 mL/min, detection wavelength: 287 nm, retention time: 5.0 min

cin were constructed by plotting concentration versus peak area and showed good linearity over the concentration range from 4 to 14 μ g/mL ($r^2 = 0.9994$). The method limits of detection and quantification were 0.37 and 1.13 μ g/mL, respectively (Table 1).

Table 1. Validation parameters of gatifloxacin in tablets

Validation parameters	UV-spectrophotometric method	Bioassay	HPLC
Analytical curve	$y = 0.077x + 0.0092$	$y = 5.71 \ln x + 16.29$	$y = 46720.05x - 6353.47$
equation ^a	(4-14) ^a	(4-16) ^a	(4-14) ^a
R ²	0.9998	0.9993	0.9994
LOD ^b	0.21	0.19	0.37
LOQ ^c	0.64 (0.005) ^d	0.57 (0.143) ^d	1.13 (5298.86) ^d

^arange of concentration; ^bs/n = 3; ^cs/n = 10; ^dstandard error of mean, n = 3

The validity of the assay was verified by means of the ANOVA. According to ANOVA (Table 2) there is linear regression ($F_{\text{calculated}} > F_{\text{critical}}$; $P = 0.05$) and there is no deviation from linearity ($F_{\text{calculated}} < F_{\text{critical}}$; $P = 0.05$). The precision of the method was determined by repeatability (intra-day) and intermediate precision (inter-day) and was expressed as R.S.D. (%) of a series of measurement. The experimental values obtained for the determination of gatifloxacin in samples are present in Table 3. The result obtained shows R.S.D. of 0.98 to 1.48% indicating good intra-day precision. Inter-day variability was calculated from assays on 3 days and shows a mean R.S.D. of 1.56%. The accuracy of the method was determined and the mean recovery was found to be 100.17% (Table 4) indicating an agreement between the true value and the value found.

UV method

The proposed UV method allows a rapid and economical quantitation of gatifloxacin in powder for injection without any time-consuming sample preparation. Moreover, the spectrophotometric methods involve simple instrumentation compared with other instrumental techniques. The absorption spectrum of gatifloxacin in aqueous solution is shown in Figure 3. The λ_{max} was found to be 287 nm. This wavelength was used for all measurements. For more accurate analysis, Ringbom curve was constructed and the linear range was observed. The range of calibration curves were constructed from 4 to 14 μ g/mL. The results showed good linearity over this concentration range with a correlation of 0.9998 (Table 1). The method limits of detection and quantification were 0.21 and 0.64 μ g/mL, respectively. According to ANOVA (Table 2) there is linear regression

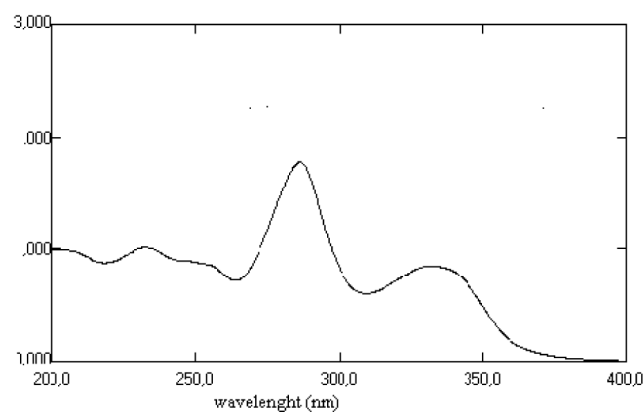


Figure 3. Absorption spectrum of gatifloxacin (287 nm)

Table 2. Statistical results of linear regression analysis in the determination of gatifloxacin by proposed spectrophotometric method, bioassay and HPLC

Statistical parameters	UV-spectrophotometric method	Bioassay	HPLC
Regression analysis			
Slope (S.E. ^a)	0.077 (5.26x10 ⁻⁴)	2.4814 (0.066)	46720.05 (550.45)
95% confidence limits of slope	0.076-0.079	1.63-3.32	45191.98-48248.15
Intercept (S.E. ^a)	0.0092(0.005)	16.297 (0.143)	-6353.47 (5298.86)
95% confidence limits of intercept	-0.005-0.023	14.47-18.12	-21063.68-8356.06
Analysis of variance			
Linear regression ^b	16600.14 (4.75)	6679.94 (4.24)	14260.64 (4.75)
Linearity deviation ^c	1.87 (3.26)	0.93 (4.24)	1.69 (3.26)

^a Standard error of mean. ^bTheoretical value of F is based on one-way ANOVA test at P = 0.05 level of significance. ^cValues in parentheses are the corresponding critical values for F at P = 0.05

Table 3. Percentual amount of gatifloxacin in tablets by three different methods

Method	Theoretical amount ^b (mg)	Experimental amount ^a (mg) ± RSD (%) intra-day	Purity (%)	R.S.D.(%) Inter-day
Bioassay	400	397.84 ± 0.45	99.46	0.87
		402.88 ± 0.28	100.72	
		404.56 ± 0.44	101.14	
Spectrophotometric	400	392.49 ± 1.26	98.12	1.32
		393.80 ± 0.11	98.45	
		402.17 ± 0.89	100.54	
HPLC (%)	400	396.04 ± 1.48	99.01	1.56
		403.64 ± 0.99	100.91	
		408.52 ± 0.98	102.13	

^a Mean of six replicate analysis. ^b Theoretical amount: 400 mg per tablet. R.S.D. = relative standard deviation

($F_{\text{calculated}} > F_{\text{critical}}$; P= 0.05) and there is no deviation from linearity ($F_{\text{calculated}} < F_{\text{critical}}$; P= 0.05). Table 3 shows the experimental values obtained for the determination of gatifloxacin in samples, indicating a satisfactory intra-day variability (R.S.D. of 0.11-1.26%) and inter-day variability (R.S.D. of 1.32%) A good accuracy of the method was verified with a mean recovery of 98.60% (Table 4).

Bioassay

In this work experimental 3x3 design using three dose levels for each standard and sample were used following the procedure described in Brazilian and European Pharmacopoeias.^{26,27} The calculation procedure normally assumes a direct relationship between the observed zone diameter and logarithm of applied dose. The calibration curve for gatifloxacin was constructed by plotting log of concentrations (µg/mL) versus zone diameter (mm) and showed good linearity on the 4-16 µg/mL range. The coefficient of regression was $r=0.9993$. The experimental values obtained for the determination of gatifloxacin in samples are present in Table 1. According Brazilian, European, and British Pharmacopoeias,²⁶⁻²⁸ if a parallel-line model is chosen, the two log dose-response lines of the preparation to be examined and the reference preparation must be parallel and they must be linear over the range of doses used in the calculation. These conditions must be verified by validity tests for a given probability, usually $P = 0.05$. The assays were validated by means of the analysis of variance, as descri-

Table 4. Recovery data of standard solutions added to the samples analyzed by using the proposed HPLC, bioassay and UV-spectrometry method

Sample	Added amount (µg/mL)	Found amount (µg/mL)	Recovery ^a (%) ± R.S.D.
Bioassay	0.5	0.48	100.20 ± 0.26
	1.0	0.96	97.60 ± 0.65
	1.5	1.48	100.13 ± 1.0
UV-spectrometry	2.0	2.02	101.65 ± 0.19
	4.0	3.97	100.05 ± 0.43
	6.0	5.91	100.74 ± 0.52
HPLC	0.4	0.39	99.47 ± 0.41
	0.8	0.78	97.50 ± 0.29
	1.6	1.67	104.79 ± 0.45
	2.4	2.37	98.94 ± 0.37

^a Mean of three replicate analysis. R.S.D. = relative standard deviation

bed in these official codes. There are no deviation from parallelism and linearity with results obtained here ($P = 0.05$). Table 3 shows the experimental values obtained for the determination of gatifloxacin in samples, indicating a satisfactory intra-day variability (R.S.D. of 0.28 - 0.45%) and inter-day variability (R.S.D. of 0.87%). A good accuracy of the method was verified with a mean recovery of 99.31% (Table 4). The quantification of antibiotic components by chemical methods such as HPLC and UV spectrophotometry, although precise, cannot provide a true indication of biological activity. Attempts to correlate antibiotic bioassay results with those from chemical methods have proved disappointing. Therefore, bioassays continue to play an essential role in manufacturing and quality control of antibiotic medicines, and still demand considerable skill and expertise to assure success.²⁹

Although the biological assays have a high variability, the analysis of the obtained results could demonstrate that the proposed method is very useful to the determination of this drug in pharmaceutical dosage forms.

Analysis of variance of analytical methods

The data obtained by bioassay, spectrophotometric analysis and HPLC presented in Table 3 were statistically comparable by ANOVA test. The mean percent levels determined by the method proposed did not differ significantly at the $P < 0.05$ (Table 5).

Table 5. Analysis of variance of the different method to quantify gatifloxacin in dosage forms

Source of preparation	d.f	SS	Variance	F
Treatments	2	560641.6	280320.8	1.91 (4.46) ^a
Residual	6	879944.6	146657.4	
Total	8	1440586		

*P, 0.05. ^aTheoretical value of F is based on one-way ANOVA test at P = 0.05 level of significance.

We had developed three methods of analysis of gatifloxacin both raw material and tablets.¹⁵⁻¹⁷ All methods showed good linearity ($r^2 > 0.9993$) in the concentrations of 4–16 µg/mL. Repeatability, intra-day and inter-day variability were evaluated for the precision of the methods (Table 3). This paper compared these methods on the basis of precision, accuracy, repeatability, cost and ease of handling. All proposed methods yielded coefficients of variation less than 2%. These results can indicate a good precision.²⁵

The reported methods for the determination of gatifloxacin in pharmaceutical forms give accurate and precise results. It is evident that HPLC was the most expensive method. This procedure requires specialized equipment and expensive solvents as well. The spectrophotometric analysis also requires specialized equipment but cost of spectrophotometer is less than an HPLC apparatus. The bioassay is clearly the least expensive method studied, and requires no specialized equipment. Ease of handling, as measured by technician time required for performance of a single assay can be another determination factor to select a laboratory routine method. However, bioassay is limited by incubation and counting time resulting 20 h. Spectrophotometry and HPLC both provide results in 2 h.

The data obtained by bioassay, spectrophotometric analysis and HPLC were statistically comparable by ANOVA test. The mean percent levels determined by the method proposed did not differ significantly at the $P < 0.05$. On the other hand, HPLC technique can show interfering peaks and bioassay can quantify antibacterial activity. Our study leads to conclude that the bioassay and HPLC have clearly displayed advantages. The application of each method as a routine analysis should be investigated considering cost, simplicity, equipment, solvents, speed, and application to large or small workloads.

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