Talanta 83 (2011) 1774–1779

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Short communication

Gemifloxacin mesylate (GFM) stability evaluation applying a validated bioassay method and *in vitro* cytotoxic study

Clésio S. Paim[a,*], Fernanda Führ[a], Aline B. Barth[a], Carlos E.I. Gonçalves[b], Nance Nardi[b], Martin Steppe[a], Elfrides E.S. Schapoval[a]

[a] *Programa de Pós-Graduação em Ciências Farmacêuticas, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul (UFRGS), Avenida Ipiranga 2752/402, CEP 90610-000, Porto Alegre, RS, Brazil*
[b] *Departamento de Genética, Instituto de Biociências, Universidade Federal do Rio Grande do Sul (UFRGS), Avenida Bento Gonçalves 9500, Prédio 43323, Bloco III, CEP 91501-970, Porto Alegre, RS, Brazil*

ARTICLE INFO

Article history:
Received 23 September 2010
Received in revised form
24 November 2010
Accepted 26 November 2010
Available online 4 December 2010

Keywords:
Gemifloxacin
Microbiological assay
Fluoroquinolones
Staphylococcus epidermidis
In vitro cytotoxicity assay

ABSTRACT

The validation of a microbiological assay applying the cylinder–plate method to determine the quinolone gemifloxacin mesylate (GFM) content is described. Using a strain of *Staphylococcus epidermidis* ATCC 12228 as the test organism, the GFM content in tablets at concentrations ranging from 0.5 to 4.5 μg mL^{-1} could be determined. A standard curve was obtained by plotting three values derived from the diameters of the growth inhibition zone. A prospective validation showed that the method developed is linear (r = 0.9966), precise (repeatability and intermediate precision), accurate (100.63%), specific and robust. GFM solutions (from the drug product) exposed to direct UVA radiation (352 nm), alkaline hydrolysis, acid hydrolysis, thermal stress, hydrogen peroxide causing oxidation, and a synthetic impurity were used to evaluate the specificity of the bioassay. The bioassay and the previously validated high performance liquid chromatographic (HPLC) method were compared using Student's t test, which indicated that there was no statistically significant difference between these two validated methods. These studies demonstrate the validity of the proposed bioassay, which allows reliable quantification of GFM in tablets and can be used as a useful alternative methodology for GFM analysis in stability studies and routine quality control. The GFM reference standard (RS), photodegraded GFM RS, and synthetic impurity samples were also studied in order to determine the preliminary *in vitro* cytotoxicity to peripheral blood mononuclear cells. The results indicated that the GFM RS and photodegraded GFM RS were potentially more cytotoxic than the synthetic impurity under the conditions of analysis applied.

1. Introduction

Fluoroquinolone gemifloxacin mesylate (GFM, Fig. 1a) is a synthetic, broad-spectrum antibacterial agent. It is unique in its dual targeting capacity – achieving adequate plasma concentrations to inhibit both topoisomerase IV and gyrase – distinguishing it from other fluoroquinolones, which inhibit one but not both targets. GFM has excellent *in vitro* activity against both Gram-positive and Gram-negative organisms, including potent antibacterial activity against respiratory tract infection pathogens, particularly *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis*. It also has excellent activity against atypical organisms, such as *Legionella pneumophila*, *Mycoplasma pneumoniae*, and *Chlamydia pneumoniae* [1–3].

The literature has reported microbiological assays to determine fluoroquinolones in pharmaceutical formulations, such as ofloxacin [4], sparfloxacin [5], gatifloxacin [6], moxifloxacin [7], enrofloxacin [8], as well as studies describing the determination of GFM in biological fluids by HPLC–MS [9,10], HPLC–ESI–MS/MS [11] and in tablets by spectrophotometry [12,13]. However, a microbiological assay to determine GFM in tablets has not been reported yet. Moreover, it allows the potency of GFM to be assessed, which is very important for the analysis of antibiotics. The development of alternative analytical methodologies, such as a simple, operationally inexpensive microbiological assay using agar diffusion for antibiotics, represents a great advantage for quality control laboratories not equipped with specialized, sophisticated instruments [14]. Furthermore, the bioassays' low cost, simple procedures allowed them to become an alternative methodology for drug potency assessment in pharmaceutical formulations.

The aim of this study was to develop and validate a simple, specific, accurate and reproducible microbiological assay by agar diffusion using the cylinder–plate method to quantify GFM in

* Corresponding author. Tel.: +55 51 33085214; fax: +55 51 33085378.
E-mail address: csoldatelli30@hotmail.com (C.S. Paim).

0039-9140/$ – see front matter.
doi:10.1016/j.talanta.2010.11.069

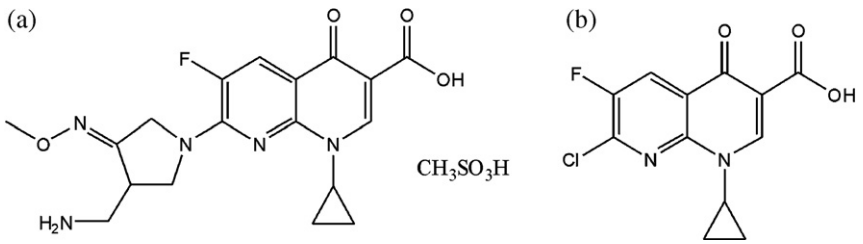

Fig. 1. Chemical structures of GFM (a) and its synthetic impurity: 1-cyclopropyl-6-fluoro-7-chloride-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylic acid (b).

tablets as an alternative to the physicochemical methods described in the literature. Moreover, a high performance liquid chromatographic (HPLC) method, developed and validated in our laboratory, was chosen as a comparison method to determine GFM in degraded samples.

GFM RS, photodegraded GFM RS and synthetic impurity samples were also evaluated to determine the preliminary *in vitro* cytotoxicity when exposed to mononuclear cells. In agreement with ICH [15], it is important to evaluate the biological safety of an individual impurity or a given impurity profile, including degradation products.

2. Materials and methods

2.1. Chemicals

GFM RS (99.0%) was acquired from Toronto Research Chemicals, Inc. (Ontario, Canada). The synthetic impurity, 1-cyclopropyl-6-fluoro-7-chloride-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylic acid (Fig. 1b), was acquired from AK Scientific Inc. (Mountain View, CA, United States). Factive® (Aché, Brazil) coated tablets containing 320 mg of gemifloxacin were purchased from the local market. The excipient ingredients contained in the dosage form (microcrystalline cellulose, crospovidone, titanium dioxide, magnesium stearate, hydroxypropyl methylcellulose, polyethylene glycol, and povidone) were all of pharmaceutical grade and acquired from different suppliers. All chemicals used were of analytical grade and all solvents were of HPLC grade. Methanol was purchased from Tedia (Fairfield, USA). Sodium chloride, sodium hydroxide, dibasic potassium phosphate, monobasic potassium phosphate and medium number 1 [16,17] were obtained from Merck (Darmstadt, Germany). RPMI 1640 medium and Hank's balanced salt solution were purchased from Sigma® (Missouri, USA). Purified water was obtained using a Milli-Q Plus® (Millipore, Bedford, USA).

2.2. Apparatus

Photodegradation studies were carried out in a photostability UV chamber (1.0 m × 0.17 m × 0.17 m) with mirrors in its interior equipped with a UVA lamp (Light Express®, 352 nm, 30 W), and UV cuvettes (Ultra Vette®, São Paulo, Brazil) were employed as containers for the samples. For thermal stability studies, a dry air oven (Biomatic®, Porto Alegre, Brazil) was used. An electronic caliper (Mitutoyo®, Tokyo, Japan) was utilized to measure the diameters in the zone of growth inhibition (mm).

An Agilent® liquid chromatograph (Santa Clara, CA, United States) equipped with a model Q 1311A quaternary pump, ALS-G1329A auto sampler, TCC-G1316A column oven, G1315B photodiode-array detector and ChemStation manager system software was used to control the equipment, to calculate data and to analyze the responses from the HPLC system. The chromatographic separation was performed on an Agilent Eclipse® XDB RP-18 column (150 mm × 4.6 mm i.d., 5 μm, Santa Clara, CA, United States).

For the cytotoxic assay, a Ficoll-Paque® gradient centrifuge (Amersham Pharmacia Biotech, Uppsala, Sweden) and a FACSCalibur® cytometer equipped with 488 nm argon laser (Becton Dickinson, San Diego, CA, USA) were used.

2.3. GFM RS solutions

An accurately weighed amount of GFM RS equivalent to 10.0 mg of gemifloxacin base was transferred to a 100 mL volumetric flask and dissolved in methanol to obtain a final concentration of 100.0 μg mL^{-1}. Aliquots of this solution were diluted in buffer number 1, pH 6.0 [16,17], to achieve concentrations of 0.5, 1.5, and 4.5 μg mL^{-1} (S1, S2, and S3, respectively). These solutions were kept protected from light.

2.4. Preparation of the sample solution

To prepare the sample solution, 20 Factive® coated tablets were weighed and finely powdered. A quantity equivalent to 10.0 mg of gemifloxacin base was transferred into a 100 mL volumetric flask with 60 mL methanol, kept in ultrasonic bath for 30 min, and the volume was completed with the same diluent. Aliquots of this solution were diluted in buffer number 1, pH 6.0 [16,17], to achieve concentrations of 0.5, 1.5, and 4.5 μg mL^{-1} (T1, T2, and T3, respectively), which were used in the bioassay. These solutions were kept protected from light.

2.5. Microorganism and inoculum standardization

The strain of *Staphylococcus epidermidis* ATCC 12228 obtained from INCQS (Rio de Janeiro, Brazil) was cultivated after reconstitution and maintained in medium number 1. The microorganism standardization was prepared according to the procedure described in the Brazilian Pharmacopoeia [16] and USP 32 [17] for microbiological assays with antibiotics. Prior to use, the microorganisms were transferred and inoculated in a test tube containing the same medium, which was maintained in a dry air oven for 24 h at 37 ± 2 °C. The growth was suspended in 0.9% NaCl sterile solution. Using a spectrophotometer (Analyser®, São Paulo, Brazil) set at 580 nm and a 10 mm absorption cell, the solution containing the microorganisms was diluted to achieve a suspension turbidity of 25 ± 2% (transmittance) using a 0.9% NaCl sterile solution as a blank. From this inoculated saline solution, 2.0 mL portions were added to each 100 mL of medium number 1, kept in water bath at 48 °C and employed as the inoculated layer in the plate.

2.6. Agar diffusion bioassay

The bioassay described here followed the 3 × 3 parallel line assay design (three doses of the standard and three doses of the sample in each plate), with eight plates for each assay, according to the Brazilian and European Pharmacopoeias [16,18]. The base layer agar was composed by 20 mL of medium number 1 that was poured into a 100 mm × 20 mm Petri dish. After that layer solidified, 5.0 mL

portions of the inoculated medium 1 were poured onto the base layer. In each plate, 6 stainless steel cylinders of the same size (8 mm × 6 mm × 10 mm) were placed on the surface of the inoculated medium. Three alternated cylinders were filled with 200 μL of the reference solutions (S1, S2, and S3), and the other three cylinders were filled with the sample solutions (T1, T2, and T3). The plates were incubated at 35 ± 1 °C aerobically for 18 h. The diameters (mm) of the growth inhibition zone were carefully measured with an electronic caliper (Mitutoyo®). All experiments were performed in a biological safety cabinet and the infected material was decontaminated before being discarded.

2.7. Calculations

The GFM potency was statistically calculated by the parallel-line model for the 3 × 3 assay design. An analysis of variance (ANOVA) was used for the statistical validation of the bioassays, evaluating the regression, parallelism and linearity of each assay [16,19].

2.8. Validation of the method

The methodology was validated according to the USP 32 [17] and the International Conference on Harmonization (ICH) [20] by determining the following operational characteristics: linearity, precision, accuracy, specificity, robustness, stability of the reference standard and sample solutions.

2.8.1. Linearity

Three doses of the reference substance were used in nine independent assays. Linearity was evaluated by linear regression and deviation analysis, which were calculated by the least-squares method.

2.8.2. Precision

The precision of the method was determined by repeatability and intermediate precision and was expressed as the relative standard deviation (RSD). Repeatability was examined by assaying three GFM samples on the same day (intraday) and under the same experimental conditions, and intermediate precision was evaluated by analysis on 3 days (interday).

2.8.3. Accuracy

Accuracy was determined by recovering known amounts of GFM RS added to the sample solutions. An accurately weighed amount of tablets equivalent to 10.0 mg of gemifloxacin base was transferred to a 100 mL volumetric flask and dissolved in methanol (100.0 μg mL^{-1}). 1.0, 3.0 and 4.5 mL aliquots of this solution were transferred into volumetric flasks containing 1.0, 3.0 and 4.5 mL of GFM RS (10.0 μg mL^{-1}). Buffer number 1 pH 6.0 was added to achieve concentrations of 0.55, 1.65 and 4.95 μg mL^{-1} that correspond to 110.0% of the nominal concentrations (as described in Section 2.4). The cylinders were filled up with the solutions described above and recovery rates of the added GFM RS were calculated.

2.8.4. Specificity

The ability of the proposed method to determine GFM in the presence of degradation products was assessed by comparing the results obtained from the degraded samples analyzed by bioassay and by the HPLC method (developed and validated in the previous studies). Under all conditions, the sample solutions were assayed and compared to freshly prepared GFM RS solutions at the same theoretical concentrations. Specificity was also checked using the synthetic impurity 1-cyclopropyl-6-fluoro-7-chloride-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylic acid (CAS 100361-18-0) and the excipients of the formulation to determine whether these substances could interfere with the assay. The preparations of the stressed GFM, excipients and synthetic impurity solutions were the following.

2.8.4.1. Acid hydrolysis. Sample solutions were prepared and maintained in 0.1 M HCl for 1 h and stored at room temperature. After that, aliquots of these solutions (100.0 μg mL^{-1}) were removed, neutralized with 0.1 M NaOH and diluted in buffer number 1 pH 6.0 to achieve theoretical concentrations of 0.5, 1.5 and 4.5 μg mL^{-1}.

2.8.4.2. Basic hydrolysis. Sample solutions were prepared and maintained in 0.01 M NaOH for 3 h and stored at room temperature. After that, aliquots of these solutions (100.0 μg mL^{-1}) were removed, neutralized with 0.01 M HCl and diluted in buffer number 1 pH 6.0 to achieve theoretical concentrations of 0.5, 1.5 and 4.5 μg mL^{-1}.

2.8.4.3. Oxidative degradation. Sample solutions were prepared in a 30% H_2O_2 solution and stored at room temperature for 20 h. After that, aliquots of these solutions (100.0 μg mL^{-1}) were removed and diluted in buffer number 1 pH 6.0 to achieve theoretical concentrations of 0.5, 1.5 and 4.5 μg mL^{-1}.

2.8.4.4. Thermal degradation. Methanolic sample solutions were maintained at 60 °C in a dry air oven for 64 h. After that, aliquots of these solutions (100.0 μg mL^{-1}) were removed and diluted in buffer number 1 pH 6.0 to achieve theoretical concentrations of 0.5, 1.5 and 4.5 μg mL^{-1}.

2.8.4.5. Photodegradation. Methanolic sample solutions (100.0 μg mL^{-1}) were exposed to UVA radiation (352 nm) for 4 min. The stress degradation study was performed exposing the solution in quartz cells in the photodegradation chamber, where the samples were positioned horizontally to provide maximum area of exposure to the light source. After that, these solutions were removed and diluted in buffer number 1 pH 6.0 to achieve theoretical concentrations of 0.5, 1.5 and 4.5 μg mL^{-1}. Control samples protected from light with aluminum foil were also placed and exposed concurrently in the light chamber.

2.8.4.6. Synthetic impurity. 10.0 mg of 1-cyclopropyl-6-fluoro-7-chloride-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylic acid was accurately weighed and dissolved in a 100 mL volumetric flask with methanol to generate a concentration of 100 μg mL^{-1}. Aliquots of this solution were diluted in buffer number 1 pH 6.0 to provide concentrations of 0.5, 1.5, and 4.5 μg mL^{-1}.

2.8.4.7. Excipients. The amount of formulation excipients contained in 10.0 mg GFM tablets was accurately weighed and dissolved in a 100 mL volumetric flask with methanol. The same aliquots used to prepare the sample solutions were withdrawn and diluted in buffer number 1 pH 6.0.

2.8.5. Robustness

Determined by analyzing the sample solution under the following conditions: ±0.2% variation in the inoculum concentration and ±0.2 unit variation in the pH value.

2.8.6. Stability of GFM RS and sample solutions

Determined using the HPLC method previously developed and validated in our laboratory. The solutions were analyzed for 18 h under the chromatographic conditions described in Section 2.8.8.

2.8.7. Cytotoxicity assay

GFM RS and the synthetic impurity solutions were prepared in aqueous solution (1.0 mg mL^{-1}) and diluted in RPMI 1640 medium

immediately before use to the concentrations of 10.0, 30.0, and 90.0 μg mL^{-1}. The photodegraded solution was prepared by exposing GFM RS in aqueous solution (1.0 mg mL^{-1}) to UVA radiation (352 nm) for 30 min. After the period of exposure, the solution was diluted under the same conditions described above.

Human mononuclear cells were separated from the peripheral blood of three healthy donors. Heparinized venous blood was diluted 4:3 with Hank's balanced salt solution. The mononuclear cells were isolated by centrifugation on a Ficoll-Paque® gradient centrifuge and washed twice in Hank's solution. Viable cells were counted by trypan blue exclusion in a Neubauer chamber. The mononuclear cells were washed and resuspended in RPMI to a concentration of 10^6 viable cells in 1.0 mL. These cell suspensions were dispensed in 96-well plates (100 μL in each well), and the samples were immediately added (100 μL in each well) to those cells. In each well the final analyzed concentration of each sample was 10.0, 30.0 and 90.0 μg mL^{-1}, in triplicate. Controls with mononuclear cells were included. Cells were cultivated in a humidified 5% CO_2 incubator at 37 °C for 24 and 72 h. Cell viability was determined by flow cytometry after addition of propidium iodide. Analyses were conducted on a FACSCalibur® cytometer equipped with 488 nm argon laser using the CellQuest® Software. The WinMDI 2.8 software was used to obtain the final results.

2.8.8. HPLC method

The GFM concentration analysis was performed on an Agilent® liquid chromatograph with a RP-18 column. The mobile phase comprised a mixture of 0.3% triethylamine solution (pH adjusted to 3.0 with 10% phosphoric acid) and acetonitrile (80:20, v/v) at a flow rate of 1.0 mL min^{-1} and isocratic elution. The injection volume was 20 μL for both reference substance and drug product solutions and the run time was 8 min. The temperature was set at 25 °C in the column oven. GFM was determined by UV detection at 272 nm using photodiode-array.

2.8.9. Comparison of methods

In order to compare the bioassay developed against a second well-characterized procedure (the previously validated HPLC method), the precision results of both methods were statistically analyzed using the Student's statistical *t* test, which indicates whether there is a significant difference between two methods at a 5% significance level.

3. Results and discussion

3.1. Analytical method

The potency of an antibiotic may be demonstrated under suitable conditions by comparing the growth inhibition of sensitive microorganisms produced by known concentrations of the antibiotic to be examined and a RS [17]. In this experimental study a 3 × 3 design, using three dose levels for standard and sample solutions, was used following the procedure described in the Brazilian and European Pharmacopoeias [16,18]. The calculation procedure normally assumes a direct relationship between the observed diameter of the inhibition zone and the logarithm of the applied dose.

Biological methods are advantageous because the measured parameters and the therapeutic properties of the drug are the same. Impurities and related substances do not interfere, maintaining the precision of the analytical method [21]. Therefore, microbial or biological assays remain, in general, the standard for dispelling doubts about the potential loss of activity [17].

The experimental conditions of the proposed method were tested and adjusted to accurately determine the performance of the assay. The *in vitro* activity of GFM was tested against *S. epidermidis* ATCC 12228 and *Micrococcus luteus* ATCC 9341. The strain

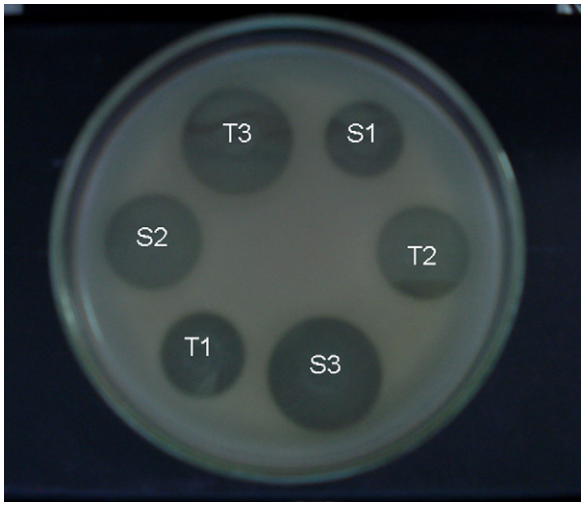

Fig. 2. Agar diffusion assay (cylinder–plate method) using a strain of *S. epidermidis* ATCC 12228. GFM reference substance concentrations: 0.5 (S1), 1.5 (S2), and 4.5 (S3) μg mL^{-1}. Sample concentrations: 0.5 (T1), 1.5 (T2), and 4.5 (T3) μg mL^{-1}.

of *S. epidermidis* ATCC 12228 was shown to be an appropriate test microorganism because of its sensitivity to GFM and ability to form sharply defined inhibition growth zones, allowing accurate measurements (Fig. 2). For handling microorganisms, all safety procedures (wearing masks, gloves and goggles) were adopted. All assays were performed in a laminar air flow cabinet, and the infected material was decontaminated before being discarded.

The stability analysis of GFM RS and sample solutions, both quantified using the HPLC method, demonstrated that the solutions were stable during the period of analysis (18 h) as the GFM area remained constant and the chromatograms did not show degradation peaks.

3.2. Linearity

The calculation procedure usually assumes a direct relationship between the observed zone diameter and the logarithm of the applied dose. The corresponding mean zone diameters for reference solutions were: 19.29 mm (RSD = 4.74%) for the lower dose (0.5 μg mL^{-1}), 21.33 mm (RSD = 3.32%) for the medium dose (1.5 μg mL^{-1}), and 24.36 mm (RSD = 1.85%) for the higher dose (4.5 μg mL^{-1}). The calibration curves for GFM were constructed by plotting the log concentration (μg mL^{-1}) versus the zone diameter (mm) and showed good linearity between the 0.5 and 4.5 μg mL^{-1} range. The representative linear equation for GFM was $y = 7.409x + 19.689$, where x is the log concentration and y is the diameter of the inhibition zone. The correlation coefficient ($r = 0.9966$) was highly significant for the method (Fig. 3).

Antibiotic assays must be designed in such a way that they allow the mathematical model's validity to be examined based on a potency equation. According to the Brazilian and European Phar-

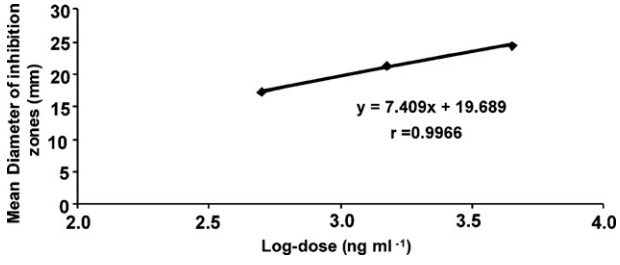

Fig. 3. GFM calibration curve obtained by the microbiological cylinder–plate assay.

Table 1
Intra-assay and inter-assay precision data for the GFM bioassay in the pharmaceutical formulation.

Sample	Day	Potency found[a] (%)	RSD (%)
1		98.37	
2	1	99.26	1.02
3		100.39	
4		97.96	
5	2	98.04	0.30
6		98.50	
7		97.85	
8	3	97.70	0.48
9		98.58	
	Inter-assay	98.52	0.86

[a] Mean of eight determinations.

Table 2
Robustness results and Student's statistical *t* test for the bioassay of GFM pharmaceutical formulation.

Parameters	Potency found[a] (%)	Stat *t*	*t*-Theoretical ($\alpha = 0.05$)
Inoculum concentration (1.8%)	97.49	0.14	2.26
	99.45		
Inoculum concentration (2.2%)	98.12	0.55	
	98.22		
Buffer solution pH (5.8)	100.45	0.50	
	97.42		
Buffer solution pH (6.2)	96.54	1.96	
	97.87		

[a] Mean of eight determinations.

macopoeias [16,18], if a parallel-line model is chosen, the two log dose–response lines of the preparations to be examined as well as the reference preparation must be parallel and must be linear over the range of doses used in the calculation. These conditions were verified by validity tests for a given probability by means of ANOVA, no deviation from parallelism and linearity was found ($p > 0.05$), and the regression was highly significant ($p < 0.05$).

3.3. Precision and accuracy

The precision and accuracy of the assay were also demonstrated. The experimental values obtained for the GFM determined in samples are presented in Table 1. The precision of the assay was determined by repeatability (intra-assay) and intermediate precision (inter-assay), and the results were expressed as the RSD of the measurement series. The repeatability (intraday precision) was studied by the determination of samples in three assays, at the same concentration and under the same experimental conditions. The results obtained showed RSD values lower than 2.0%, indicating good intra-assay precision. Inter-assay variability was calculated from assays on 3 different days and had an RSD of 0.86%.

Accuracy was evaluated by simultaneous determination of the analyte in solutions prepared via the standard addition method. The recovery rates (99.98, 99.36 and 102.65%) were close to the actual sample values.

3.4. Specificity

Alsante et al. [22] suggested a target degradation of 5–20% for establishing the stability-indicating nature of an assay method, since intermediate degradation products should not interfere in any stage of drug analysis. Hence, the same degradation conditions used to validate the stability-indicating HPLC method, previously validated in our laboratory, were employed in the microbiological assay.

During the specificity analysis using the formulation excipients and diluents, no inhibition zone was formed under the study conditions, revealing the absence of interference from these substances. Moreover, growth inhibition zone diameters did not increase when excipients were added to the GFM RS.

Degradation under acidic conditions (0.1 M HCl for 1 h) led the GFM sample solution to be unstable during the validation of the HPLC method. Under those conditions, the drug concentration decreased by 6.52%. Such instability was also detected during the microbiological assay, when drug activity decreased about 9.99% in 0.1 M HCl for 1 h.

During the initial forced degradation experiments analyzed by HPLC, fast degradation was observed in a 0.1 M NaOH solution at room temperature. As a result, the drug was exposed to a 0.01 M NaOH solution for 3 h to achieve a lower degradation rate. The same conditions were applied to the microbiological study, and results showed 5.06% drug degradation in the pharmaceutical preparation, similar to the results by the HPLC method (6.84%).

Under photolytic conditions (UVA radiation), fast GFM degradation was found in a methanolic solution. Results showed 13.49% degradation when the drug solution was exposed to UVA radiation for 4 min and analyzed by microbiological assay. Similar results were obtained by the HPLC method (14.27%).

The GFM sample solution submitted to thermal degradation conditions (60 °C for 64 h) showed degradation of 10.14%. Similar results were obtained with the HPLC method (9.89%).

The oxidative stress conditions could not be evaluated due to the bactericide activity of the 30% hydrogen peroxide solution used as the oxidative agent.

Results also showed that the GFM synthetic impurity had no activity against *S. epidermidis* in the applied conditions, as grown inhibition zone diameters did not change when the synthetic impurity was added to the GFM sample solution.

Considering all the stress conditions used to develop and validate both methods, it is possible to conclude that the drug is unstable under photolytic, thermal, acid and alkaline conditions. Moreover, this study suggests that the degradation products formed and the GFM synthetic impurity, at the concentrations applied, did not show activity against *S. epidermidis*, demonstrating the selectivity of the microbiological assay used to determine GFM in coated tablets.

3.5. Robustness

Table 2 shows the robustness results of the analytical method. The values obtained during the assays were statistically compared against the precision results of the bioassay method ($\alpha = 0.05$). The quantification of GFM in coated tablets using the bioassay method can be considered robust, because none of the factors under study had a significant effect on the determination of drug potency.

3.6. Cytotoxicity assay

A cytotoxicity assay with mononuclear cells was performed to evaluate the effect of the degraded structures in relation to the intact molecule, to foresee possible undesirable effects resulting from unstable samples. Non-significant differences ($p > 0.05$) were obtained, indicating that the degraded samples did not show an increase in cytotoxicity when exposed to the assayed cell culture after 24 h of exposure. In contrast, after 72 h of incubation, the GFM RS and photodegraded GFM RS showed cytotoxic effects with a reduction in cell viability (Table 3). This effect was not observed for the synthetic impurity in the concentrations under study.

Table 3
Results of cell viability obtained by *in vitro* cytotoxic assay with mononuclear cells for GFM RS, photodegraded GFM RS and synthetic impurity.

Concentration ($\mu g\,mL^{-1}$)	GFM RS	Photodegraded GFM RS	Synthetic impurity
Cell viability (%)[a] ± SD (incubation time 72 h)			
10	99.88 ± 0.21	100.00 ± 0.0	98.81 ± 2.06
30	85.52 ± 6.57	87.80 ± 5.32	98.44 ± 2.69
90	52.87 ± 6.44	59.11 ± 4.69	96.77 ± 5.15

[a] Mean of three analyses.

The results of the statistical analysis of cell viability showed that there is no significant difference between the cytotoxicity of GFM RS and that of the photodegraded GFM at $90\,\mu g\,mL^{-1}$ after 72 h of incubation (ANOVA, $\alpha = 0.05$). Such results corroborate other studies that investigated the potential cytotoxicity of quinolones [23,24]. The synthetic impurity showed little cytotoxicity under the study conditions.

3.7. Comparison of methods

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 the results obtained from the antibiotic bioassay with those from chemical methods have turned out to be disappointing. Therefore, bioassays continue to play an essential role in the manufacturing and quality control of antibiotic medicines and still demand considerable skills and expertise to assure success [25]. For method comparison purposes, GFM samples in coated tablets were determined by the microbiological assay and HPLC methods. For the microbiological assay method, the mean potency found (±RSD%) was 98.52% ± 0.86% and for the HPLC method, 99.57 ± 1.57%. These results were statistically analyzed using Student's *t* test and indicated that there was no significant difference between the methods at $\alpha = 0.05$ ($t_{calculated}$ 1.87 < $t_{theoretical}$ 2.06). Therefore, the methods developed and validated provided similar results for GFM quantitation in tablets. Moreover, potency results were within the pharmacopeial potency limits of 90–110% applied to antimicrobial pharmaceutical preparations [17].

4. Conclusions

For routine quality control of medicines, it is essential to employ well-characterized, fully validated analytical methods to obtain reliable results that can be satisfactorily interpreted. The results obtained in this study show that the proposed microbiological method for the determination of GFM in a pharmaceutical dosage form is accurate, specific, robust, and possesses excellent linearity and precision characteristics. Moreover, there is no statistical difference between the microbiological assay and the HPLC method (developed and validated in a previous study) for drug quantification; therefore, these can be interchangeable. The bioassay is less expensive and very appropriate when an HPLC system is not available for antibiotic potency determination. Therefore, the proposed bioassay is a useful methodology for quality control and stability studies of GFM in pharmaceutical products. The *in vitro* cytotoxicity assay with mononuclear cells demonstrated that both the photodegraded GFM RS and the GFM RS have similar cytotoxicity under the study conditions.

Acknowledgement

The authors wish to thank CAPES (Brazil) and CNPq (Brazil) for their financial support.

References

[1] FDA, Food and Drug Administration, Information prescribing, http://www.fda.gov/medwatch/safety/2007/May_PI/Factive_PI.pdf.
[2] D.M. Johnson, R.N. Jones, M.E. Erwin, Diagn. Microbiol. Infect. Dis. 33 (1999) 87.
[3] R. Grossman, J. Rotschafer, J. Tan, Am. J. Med. 118 (2005) 29S.
[4] L.S. Ev, E.E.S. Schapoval, J. Pharm. Biomed. Anal. 27 (2002) 91.
[5] H.R.N. Marona, E.E.S. Schapoval, Informacion Tecnologica 9 (1998) 251.
[6] H.R.N. Salgado, C.C.G.O. Lopes, M.B.B. Lucchesi, J. Pharm. Biom. Anal. 40 (2006) 443.
[7] F.L.B. Guerra, C.S. Paim, M. Steppe, E.E.S. Schapoval, J. AOAC Int. 88 (2005) 1086.
[8] M.J. Souza, C.F. Bittencourt, P.S. Souza, Int. J. Pharm. 271 (2004) 287.
[9] A. Alen, E. Bygate, M. Vousden, S. Oliver, M. Johnson, C. Ward, A. Cheon, Y. Choo, I. Kim, Antimicrob. Agents Chemother. 45 (2001) 540.
[10] E. Doyle, S. Fowles, D. McDonnell, R. McCarthy, S. White, J. Chromatogr. B 746 (2000) 191.
[11] V.R. Robledo, W.F. Smyth, Anal. Chim. Acta 623 (2008) 221.
[12] M.V. Krishna, D.G. Sankar, E-J. Chem. 5 (2008) 493.
[13] M.V. Krishna, D.G. Sankar, E-J. Chem. 5 (2008) 515.
[14] M.J. Souza, R.R. Kulmann, L.M. Silva, D.R. Nogueira, E.S. Zimmermann, C.A. Schmidt, J. AOAC Int. 89 (2006) 1367.
[15] I.C.H., Harmonised Tripartite Guideline: Impurities in New Drug Products – Q3B(R2), Commission of the European Communities, Geneva, 2006, pp. 1–11.
[16] Farmacopéia Brasileira, 4th ed., Atheneu, São Paulo, 1988. Parte I, v.5.2. 17–v.5.2.17-11.
[17] The United States Pharmacopoeia, 32nd ed. (USP 32), United States Pharmacopoeia Convention, Rockville, MD, 2009.
[18] European Pharmacopoeia, 5th ed. European Directorate for the Quality of Medicines, Strasbourg Cedex, France, 2005, pp. 1695–1697.
[19] W. Hewitt, Microbiological Assay for Pharmaceutical Analysis: a Rational Approach, Interpharm, Boca Raton, 2003.
[20] International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceutical for Human Use: Validation of Analytical Procedures, Text and Methodology — Q2(R1), 2005.
[21] N.A. Hodjes, in: M.E. Aulton (Ed.), Pharmaceutics: The Science of Dosage Form Design, Churchill Livingstone, London, 2001, pp. 623–643.
[22] K.M. Alsante, A. Ando, R. Brown, R. Ensing, T.D. Hatajik, W. Kong, Y. Tsuda, Adv. Drug Deliv. Rev. 59 (2007) 29.
[23] A. Foroumadi, M. Oboudiat, S. Emami, A. Karimollah, L. Saghaee, M.H. Moshafid, A. Shafieea, Bioorg. Med. Chem. 14 (2006) 3421.
[24] A. Pardakhty, A. Foroumadi, M. Hashemi, S. Rajabalian, M. Heidari, Toxicol. In Vitro 21 (2007) 1031.
[25] R.M. Baird, N.A. Hodges, Handbook of Microbiological Quality Control, Taylor & Francis, London, 2000.