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Quantitative evaluation of besifloxacin ophthalmic suspension by HPLC, application to bioassay method and cytotoxicity studies

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ABSTRACT

Besifloxacin (BSF) is a synthetic chiral fluoroquinolone developed for the topical treatment of ophthalmic infections. The present study reports the development and validation of a microbiological assay, applying the cylinder-plate method, for determination of BSF in ophthalmic suspension. To assess this methodology, the development and validation of the method was performed for the quantification of BSF by high performance liquid chromatography (HPLC). The HPLC method showed specificity, linearity in the range of 20–80 μg mL^{-1} (r=0.9998), precision, accuracy and robustness. The microbiological method is based on the inhibitory effect of BSF upon the strain of *Staphylococcus epidermidis* ATCC 12228 used as a test microorganism. The bioassay validation method yielded excellent results and included linearity, precision, accuracy, robustness and selectivity. The assay results were treated statistically by analysis of variance (ANOVA) and were found to be linear (r=0.9974) in the range of 0.5–2.0 μg mL^{-1}, precise (inter-assay: RSD=0.84), accurate (101.4%), specific and robust. 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 methods. These results confirm that the proposed microbiological method can be used as routine analysis for the quantitative determination of BSF in an ophthalmic suspension. A preliminary stability study during the HPLC validation was performed and demonstrated that BSF is unstable under UV conditions. The photodegradation kinetics of BSF in water showed a first-order reaction for the drug product (ophthalmic suspension) and a second-order reaction for the reference standard (RS) under UVA light. UVA degraded samples of BSF were also studied in order to determine the preliminary in vitro cytotoxicity against mononuclear cells. The results indicated that BSF does not alter the cell membrane and has been considered non-toxic to human mononuclear cells in the experimental conditions tested.

1. Introduction

Increased research and development of synthetic drugs in the last decades are reflected in the large number of drugs available. Fluoroquinolones are a group of chemotherapeutic compounds of synthetic origin, characterized by a pharmacological and therapeutic effect that is increasingly promising at each generation [1,2]. BSF (Fig. 1) is a synthetic chiral fluoroquinolone, marketed in an enantiomeric pure form and developed for the topical treatment of ophthalmic infections. It was approved by FDA in May 2009 and it is the only ophthalmic fluoroquinolone that has not been studied primarily for systemic use [3,4].

It became part of the therapeutic arsenal in Brazil in 2011, but it has been sold in the USA by Bausch & Lomb under the tradename of Besivance® ophthalmic suspension 0.6%, formulated with DuraSite® technology that allows the active ingredient to remain longer on the surface of the eye [5].

The literature has reported the determination of BSF in biological fluids [6–10], but no published scientific papers refer to the determination of BSF in raw material and its pharmaceutical form, ophthalmic suspension.

The importance of developing and validating analytical methods for this drug is justified by its therapeutic potential, the absence of such specific information in the literature, as well as the knowledge that the poor quality of anti-infective products is related to the emergence of resistant strains, resulting from the administration of subtherapeutic doses. Therefore this study is relevant for therapeutic application in the international market.

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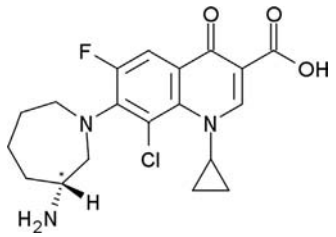

Fig. 1. Chemical structure of BSF.

In order to overcome this lack of information about validated methodologies, the present study reports the development and validation of two analytical methods to determine BSF in ophthalmic suspension: HPLC and microbiological assay by agar diffusion using the cylinder-plate methods. The HPLC method developed and validated was chosen as a comparison method for the determination of BSF in ophthalmic suspension.

A preliminary stability study was performed during HPLC validation and shows that BSF is unstable under light conditions. This led to the study of the degradation kinetics of BSF under UVA light. The UVA degraded samples of BSF were also studied in order to determine the preliminary in vitro cytotoxicity against mononuclear cells.

2. Materials and methods

2.1. Chemicals

Besifloxacin hydrochloride RS (99.6%) was acquired from Sequoia Research Products. Besivance® (Bausch & Lomb Incorporated) besifloxacin ophthalmic suspension 0.6% was obtained by the courtesy of Bausch & Lomb Incorporated and purchased in the local market.

The excipient ingredients contained in suspension form (polycarbophil, mannitol, polaxamer 407, sodium chloride, edetate disodium dehydrate, sodium hydroxide and water for injection) were of pharmaceutical grade and were obtained from different suppliers. All chemicals used were of analytical grade and all solvents were of HPLC grade. Acetonitrile was purchased from Tedia® (Fairfield, USA). Phosphoric acid and triethylamine were purchased from Merck (Darmstadt, Germany).

Sodium chloride, sodium hydroxide, Grove–Randall's 1 culture medium and Grove–Randall's 11 culture medium were obtained from Merck® (Darmstadt, Germany). Purified water was obtained using a Milli-Q Plus® (Millipore, Bedford, USA).

2.2. Apparatus

A photostability UV chamber (1.0 m × 0.17 m × 0.17 m) was used with mirrors in its interior, equipped with a UVA lamp (Light Express®, 352 nm, 30 W) and UV cuvettes (Ultra Vette®, São Paulo, Brazil) for photodegradation studies.

A dry air oven (Biomatic®, Porto Alegre, Brazil) was utilized for thermal stability studies.

An electronic caliper (Mitutoyo®, Tokyo, Japan) was used to measure the diameters of growth zone inhibition.

The HPLC system consisted of an Agilent® liquid chromatograph (Santa Clara, CA, USA) with a model Q 1311A quaternary pump, ALS-G1329 auto sampler, TCC-G1316A column oven, G1315B photodiode-array detector and ChemStation® manager system software, using an Agilent® Eclipse Plus C18 column (150 mm × 4.6 mm i.d., 5 μm, Santa Clara, CA, USA). For the cytotoxicity assay a Ficoll-Paque gradient centrifuge (Sigma®), Hank's medium, centrifuge, hemocytometer ABX-Micros 60®, commercial kit (Doles® reagents, Goiânia, Brazil) and an Envision plate reader (Perkin Elmer®) were used.

2.3. BSF RS solutions

An accurately weighed amount of BSF RS equivalent to 10.0 mg of BSF base was transferred quantitatively to a 200 mL volumetric flask and dissolved in water to obtain a final concentration of 50.0 μg mL^{-1}. Aliquots of this solution were diluted in water to achieve concentrations of 0.5, 1.0 and 2.0 μg mL^{-1} (S1, S2 and S3, respectively). These solutions were kept protected from the light and used in a microbiological assay. In the HPLC method (APPLICATION), the concentration of 500.0 μg mL^{-1} of BSF dissolved in water was used. Aliquots of this solution were removed and diluted in mobile phase for analysis on HPLC.

A stock solution of 500.0 μg mL^{-1} was also used for stability tests and kinetics studies.

A solution of 2.0 mg mL^{-1} was used for the cytotoxicity assay.

2.4. Sample solution preparation

An aliquot equivalent to 5 mg of BSF base was removed from the sample bottle and transferred to a 100 mL volumetric flask with the aid of 50 mL of water, kept in an ultrasonic bath for 25 min, and the volume was completed with the same solvent. Aliquots of this solution were further diluted in water to achieve concentrations of 0.5, 1.0 and 2.0 μg mL^{-1} (T1, T2 and T3, respectively). These solutions were protected from light and used in the bioassay.

To perform the analysis by HPLC method, a concentration of 500.0 μg mL^{-1} of BSF sample/drug product dissolved in water was used. From this solution aliquots were removed and diluted in a mobile phase for HPLC analysis.

The solution of 500.0 μg mL^{-1} was also used for stability tests and kinetics studies.

A solution of 2.0 mg mL^{-1} was used for a cytotoxicity assay.

2.5. HPLC method

This method was developed and validated in order to monitor and compare the results obtained with the microbiological assay for determination of BSF in an ophthalmic suspension.

The BSF concentration analysis was performed on an Agilent® liquid chromatograph with a C18 column (Agilent® Eclipse Plus 5 μm, 150 mm × 4.6 mm). The mobile phase comprised a mixture of 0.5% triethylamine solution (pH adjusted to 3.0 with 10% phosphoric acid) and acetonitrile (74:26, v/v) at a flow rate of 1.0 mL min^{-1}. The injection volume was 20 μL for both reference substance and drug product solutions. The temperature was set at 25 °C in the column oven. BSF was determined by UV detection at 295 nm using a photodiode-array.

2.6. Microbiological assay

2.6.1. 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 was standardized according to the procedure described in the Brazilian [11] and United States [12] Pharmacopeia. Prior to use, the microorganism was 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. Using a spectrophotometer (Analyser®, São Paulo, Brazil) with wavelength set at 580 nm and a 10 mm absorption cell, the broth containing the microorganism was diluted in 0.9% NaCl sterile solution to give a suspension with

25 ± ± 2% turbidity (transmittance) with the same sterile solution as the blank. From this standardized suspension, aliquots of 2.0 mL were added to each 100 mL of Grove–Randall's 11 culture medium, kept in a water bath at 48 °C, and used as an inoculated layer in the plate.

2.6.2. Agar diffusion bioassay

The bioassay described followed the 3 × 3 parallel line assay design according to the Brazilian and European Pharmacopoeia [11,13].

The base layer agar was composed of 20 mL Grove–Randall's 11 culture medium that was poured into a 100 × 20 mm Petri dish. After layer solidification, portions of 5 mL of inoculated Grove–Randall's 11 medium were poured onto the base layer. In each plate, six stainless steel cylinders of uniform size (8 × 6 × 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 concentrations of the sample solutions (T1, T2, and T3). Six plates were used for each assay. The plates were incubated at 35 ± ± 1 °C aerobically for 18 h. The growth inhibition zone diameters (mm) were carefully measured with a digital caliper (Mitutoyo®, Tokyo, Japan). All experiments were performed in a biological safety cabinet and the infected material was decontaminated before being discarded.

2.6.3. Calculations

The BSF potency was statistically calculated by a parallel-line model for the 3 × 3 assay design [14]. Analysis of variance (ANOVA) was used for the statistical validation of the bioassays, evaluating regression, parallelism and linearity for each assay.

2.7. Method validation (HPLC method and microbiological assay) (ICH Q2, USP)

The methodologies were validated according to USP 34 [12] and the International Conference on Harmonization (ICH) guidelines [15] by determining the specificity, linearity, precision, accuracy, robustness, and stability of the reference standard and sample solutions.

2.7.1. Specificity

HPLC method – Forced degradation studies were performed for BSF RS and the drug product (500 μg mL^{-1}) to show the specificity of the method. The interference of excipients was also evaluated and all the solutions used in the assay were protected from the light. Blank solutions were used during the analysis. Fresh BSF RS and drug product solutions were prepared and compared to quantitative results of the forced degradation studies. The stress conditions were the following:

Acid hydrolysis: Sample solutions were prepared and maintained in 1.0 M HCl for 1 h and stored at room temperature. After this time, aliquots of these solutions (500 μg mL^{-1}) were removed, neutralized with 1.0 M NaOH and diluted in water to achieve a theoretical concentration of 50 μg mL^{-1}.

Basic hydrolysis: Sample solutions were prepared and maintained in 1.0 M NaOH for 1 h and stored at room temperature. After this time, aliquots of these solutions (500 μg mL^{-1}) were removed, neutralized with 1.0 M HCl and diluted in water to achieve a theoretical concentration of 50 μg mL^{-1}.

Oxidative degradation: Sample solutions were prepared in a 30% H_2O_2 solution and stored at room temperature for 1 h. After this time, aliquots of these solutions (500 μg mL^{-1}) were removed and diluted in water to achieve a theoretical concentration of 50 μg mL^{-1}.

Thermal degradation: Aqueous sample solutions were maintained at 60 °C in a dry air oven for 24 h. After this time, aliquots of these solutions (500 μg mL^{-1}) were removed and diluted in water to achieve a theoretical concentration of 50 μg mL^{-1}.

Photodegradation: Each solution was prepared in water and exposed to UVA radiation (352 nm) and UVC radiation (254 nm) for 1 h. The stress degradation study was performed by exposing the solutions in quartz cells in the photodegradation chamber. Subsequently, these solutions were diluted in a mobile phase to achieve a concentration of 50 μg mL^{-1}. Control samples were protected from light with aluminum foil and were also placed in the light chamber and exposed concurrently

Microbiological assay – Specificity was studied by analyzing the sample in the presence of the excipients from the formulation (placebo) and degradation products. The ability to determine BSF RS in the presence of excipients and/or degradation products was evaluated by comparing the results obtained from the placebo suspension and degraded samples analyzed by bioassay and the HPLC method. In order to find out whether some components of the pharmaceutical form could interfere with the assay, the sample solutions were assayed and compared to freshly prepared BSF RS solutions at the same theoretical concentrations.

The stressed condition and excipients solutions were prepared as below:

Photodegradation: Aqueous drug product solutions (500 μg mL^{-1}) were exposed to UVA radiation (352 nm) for 120 min. A stress degradation study was conducted by exposing the solution in quartz cells in the photodegradation chamber. Subsequently, these solutions were removed and diluted in water to achieve the theoretical concentrations of 0.5, 1.0 and 2.0 μg mL^{-1}. Samples protected from light with aluminum foil were also exposed in the light chamber and used as control samples.

Excipients: The amount of formulation excipients contained in an ophthalmic suspension of besifloxacin was accurately weighed and dissolved in water.

2.7.2. Linearity

HPLC method – Three calibration curves were prepared with six concentrations (20.0, 30.0, 40.0, 50.0, 60.0, 70.0 and 80.0 μg mL^{-1}) of BSF RS. For each concentration solutions were prepared and injected in triplicate. The peak areas of the chromatograms were plotted against the respective concentration of the drug to obtain the analytical curves. The regression line was calculated by using the least squares method and the curves were validated though analysis of variance.

Microbiological assay – In order to assess the validity of the assay, three doses of the reference substance were used in six independent assays. Linearity was evaluated by linear regression and deviation analysis, which was calculated by the least-squares method.

2.7.3. Precision

HPLC method – Precision was determined using the parameters of repeatability (intra-day) and intermediate precision (inter-day), analyzing six BSF sample solutions prepared at 50 μg mL^{-1} in triplicate on three different days. The results were expressed as relative standard deviation (RSD) of the analytical measurements.

Microbiological assay – The precision of the method was determined by repeatability and intermediate precision and was expressed as relative standard deviation (RSD). Repeatability was examined by assaying three samples of BSF on the same day (intra-day) and under the same experimental conditions that were compared against the BSF reference standard. The intermediate precision of the method was determined on 3 days (inter-day).

2.7.4. Accuracy

HPLC method – Accuracy was calculated as a percentage by adding known amounts of BSF RS to sample solutions (40 μg mL^{-1}) to yield final concentrations of 50.0, 60.0 and 70.0 μg mL^{-1}, corresponding to 25%, 50% and 75% of the analytical concentrations.

Microbiological assay – To determine the accuracy of the proposed method, the test was performed over three concentration levels adding 10% of BSF RS to each concentration of the samples (0.55, 1.1 and 2.2 μg mL^{-1}).

Accurate aliquots of 0.10, 0.20, and 0.40 mL of the reference standard solution (5.0 μg mL^{-1}) were transferred into 10 mL volumetric flasks together with aliquots of 0.10, 0.20 and 0.40 mL of sample solutions (50 μg mL^{-1}) and diluted with water to final concentrations of 0.55, 1.1 and 2.2 μg mL^{-1}, respectively (corresponding to 110.0% of the nominal concentrations). The cylinders were filled with the solutions described above and recovery rates of the added BSF RS were calculated.

2.7.5. Robustness

HPLC method – The factors investigated in the robustness evaluation were pH of the aqueous phase (3.0 ± 0.1), percentage of acetonitrile ($26\% \pm 1$), flow (1.0 mL $min^{-1} \pm 0.1$) and percentage of triethylamine ($0.5\% \pm 0.1$). The results obtained under these conditions were compared with the original conditions.

Microbiological assay – Determined by analyzing the sample solution under $\pm 0.2\%$ variation in the inoculum concentration.

2.8. BSF photodegradation kinetics

Aqueous sample solutions (500 μg mL^{-1}) were exposed to UVA radiation (352 nm) according to the photodegradation studies described in Section 2.7.1. At pre-established times (60, 120, 180, 240 and 300 min), 1.0 mL aliquots of the solution were withdrawn and diluted in water to achieve the concentration of 50 μg mL^{-1}. Aliquots of this solution were diluted in water to achieve concentrations of 0.5, 1.0 and 2.0 μg mL^{-1} for the bioassay according to Sections 2.5–2.7.

2.8.1. Kinetic calculation

The reaction order for the kinetics of BSF degradation was determined representing the residual drug concentration versus time (zero order reaction), log concentration versus time (first order reaction) and the inverse of a function of concentration versus time (second order). Linear regression coefficients (r) were obtained, and the coefficient closer to unity indicates the order of reaction. Kinetic parameters such as apparent order degradation rate constant (k) and t_{90} (time at which 90% of the concentration of the drug is unchanged) were obtained.

2.9. Cytotoxicity assay

Cytotoxicity or cell death by necrosis can be determined by membrane integrity. The intracellular enzyme lactate dehydrogenase (LDH) is rapidly released into the medium when the cell membrane is damaged. A commercial kit (DOLES Reagentes, Goiânia, Brazil) was used to determine LDH release.

Human mononuclear cells of five healthy donors were separated from the peripheral blood. Heparinized venous blood was diluted 1:1 (v/v) using 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 in a Neubauer chamber. The mononuclear cells were washed and suspended in Hank's solution to a concentration of 10^7 cells for 1.0 mL.

Pipetting the reagents into a 96-well plate was done in the following order: ferric alum, substrate and sample solution. The sample solutions were composed of solutions containing BSF SR and drug production (with and without degradation under UVA light). The photodegraded solutions were prepared by exposing the aqueous solution besifloxacin (2.0 mg mL^{-1}) to UVA radiation during 5 h. After the exposure period, the solution was diluted with Hank's to a final concentration of 10, 100 and 200 μg mL^{-1}, in triplicate. After the samples were added, the plates were maintained at 37 °C. Simultaneously, a blank test (baseline) was performed, in triplicate, with ferric alum and substrate. A positive control test was also carried out with Triton X-100 (100% cell death). A blank solution was also made with Hank's solution and water. Thereafter, an NAD solution was added to each well-plate and after the time indicated by the kit, the stabilizing solution was pipetted. After this procedure, ultraviolet absorbance was measured at 492 nm using an Envision plate reader (Perkin Elmer). The blank measurement was subtracted from the measurements obtained for each sample.

2.10. Comparison of the methods

All results in this study were compared with those obtained by the validated high-performance liquid chromatography (HPLC) method that was previously described.

3. Results and discussion

3.1. HPLC method

This method was developed and validated in order to monitor and compare the results obtained with the microbiological assay for determination of BSF in an ophthalmic suspension. For this reason, the results will be shown briefly.

The chromatographic conditions were adjusted in order to provide a good performance of the assay. During method optimization, different columns, organic solvents and the aqueous phase were tested. After testing various combinations of the mobile phase, the best condition with a retention time of 4.9 min for BSF was obtained using an Agilent C-18 column (150 mm × 4.6 mm i.d., particle size 5 μm) maintained at room temperature (25 °C) and the mobile phase was composed of water with 0.5% triethylamine adjusted to pH 3.0 with phosphoric acid and acetonitrile (74:26, v/v). The addition of triethylamine improved peak symmetry.

3.2. Bioassay method

Determining antimicrobial potency is important for controlling the quality of pharmaceutical preparations, and it is necessary to develop economic and practical procedures that can be validated and applied to the determination of these drugs [16]. The potency of antibiotics is generally determined by comparing the dose which inhibits the growth of a susceptible microorganism with the dose of preparation of the reference antibiotic that produces similar inhibition under the same working conditions. A reduction in microbial activity can reveal changes not demonstrable by chemical methods [12].

The quantification of antimicrobial agents by chemical methods, such as HPLC and UV spectrophotometry, although accurate, does not provide a true indication of biological activity. The test microbiological agar diffusion-plate cylinder allows drug potency to be quantified by measuring the area of microorganism growth inhibition, caused by the diffusion of the drug through the agar [16].

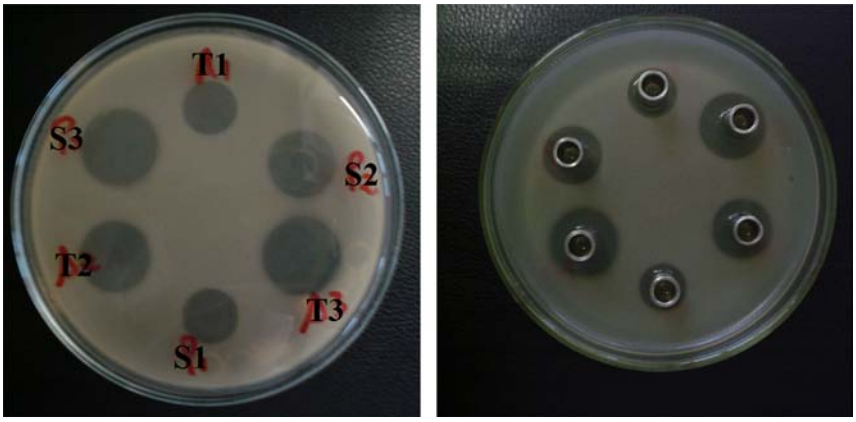

Fig. 2. Photos of inhibition zones obtained by microbiological method for determination of besifloxacin (front and back of the card).

Table 1
Parameters tested during the development of microbiological assay for BSF ophthalmic suspension determining.

Parameter	Condition
Culture media	Grove–Randall number 1 Grove–Randall number 11
Microorganisms	*Kochuria rhizophila* ATCC 9341 *Staphylococcus aureus* ATCC 6538P *Staphylococcus epidermidis* ATCC 12228
Diluent solution	Water Phosphate buffer pH 6.0 Phosphate buffer pH 7.0
Standard curve ($\mu g\ mL^{-1}$)	0.5; 1.0; 2.0 0.5; 1.5; 4.5
Inoculum (%)	1 2

Table 2
Final condition parameters established for microbiological assay of BSF.

Parameter	Condition
Standard curve ($\mu g\ mL^{-1}$)	0.5, 1.0 and 2.0
Diluent solution	Water
Microorganism	*Staphylococcus epidermidis* ATCC 12228
Inoculum (%)	2.0
Incubation temperature (°C)	35 ± 2
Incubation time (h)	18
Culture media	Grove–Randall number 11

No official microbiological cylinder-plate assays are described in the official codes to determine BSF in pharmaceutical formulations, which demonstrates the relevance of the work developed here.

In this work we chose to use a 3×3 design in which three concentration levels of the sample and reference solutions were tested (Fig. 2), maintaining a geometric progression of the values used, following the procedure described in the Brazilian (2010) and European Pharmacopoeia (2005). It is possible to directly relate the diameter of the zone of inhibition as a function of the logarithm of the dose used. It is assumed that the response curve (reference and sample) should display the same inclination [14].

The experimental conditions were adjusted to accurately determine the assay performance. Some parameters (Table 1) were tested earlier to establish the final conditions described in Table 2.

All assays were performed in a laminar air flow cabinet, and the infected material was decontaminated before being discarded. During microorganism handling, all safety procedures (use of mask, gloves and goggles) were followed.

3.3. Method validation (HPLC and bioassay methods)

3.3.1. Specificity

HPLC method – The specificity analysis revealed that the HPLC method did not show any interference from the formulation excipients, since there were no other peaks eluting at the same time as BSF. Photodiode array detection also supported the specificity of the method and provided evidence of peak purity for BSF. The BSF drug product solutions were submitted to different stress conditions to induce drug degradation. These sample solutions showed stability against thermal and basic degradation conditions without formation of degradation product peaks or reduction in the BSF content (Fig. 3A and E). There was a reduction in the content of BSF under photodegradation, acid and oxidative conditions, but no additional peak majority of degradation products was formed (Fig. 3B–D and F). The chromatographic peak purity tool, applied to BSF peaks, demonstrated that they remained pure in all cases, confirming the absence of other substance coeluting at the same retention time.

Bioassay method – To demonstrate the specificity, a placebo solution was evaluated and showed no zones of inhibition when assessed against a freshly prepared solution of BSF. A sample solution was subjected to forced photolytic degradation (UVA) and analyzed. The results showed a degradation of about 19% when the sample solution was exposed to UV radiation for 120 min, similar to those found by the HPLC method (22%). It was observed that the degradation product formed did not interfere with the determination of the microbiological method for BSF.

The method showed specificity, because the excipients from the formulation and the possible degradation product formed did not interfere with the determination of BSF.

3.3.2. Linearity

HPLC method – The method was linear ($r=0.9998$) at the drug concentration range of 20–80 $\mu g\ mL^{-1}$. The representative linear equation was $y=34.766x+4.4175$, where x is the concentration and y is the area. According to ANOVA there is a linear regression ($p<0.05$) and there is no deviation from linearity ($p>0.05$). Student's t-test was performed to verify the significance of the experimental intercept in the linear regression equation. According to the results, there were no significant differences from the theoretical zero value for $p>0.05$.

Bioassay method – 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 17.51 mm (RSD=0.97) for the lower dose (0.5 $\mu g\ mL^{-1}$), 21.33 mm (RSD=0.88) for the

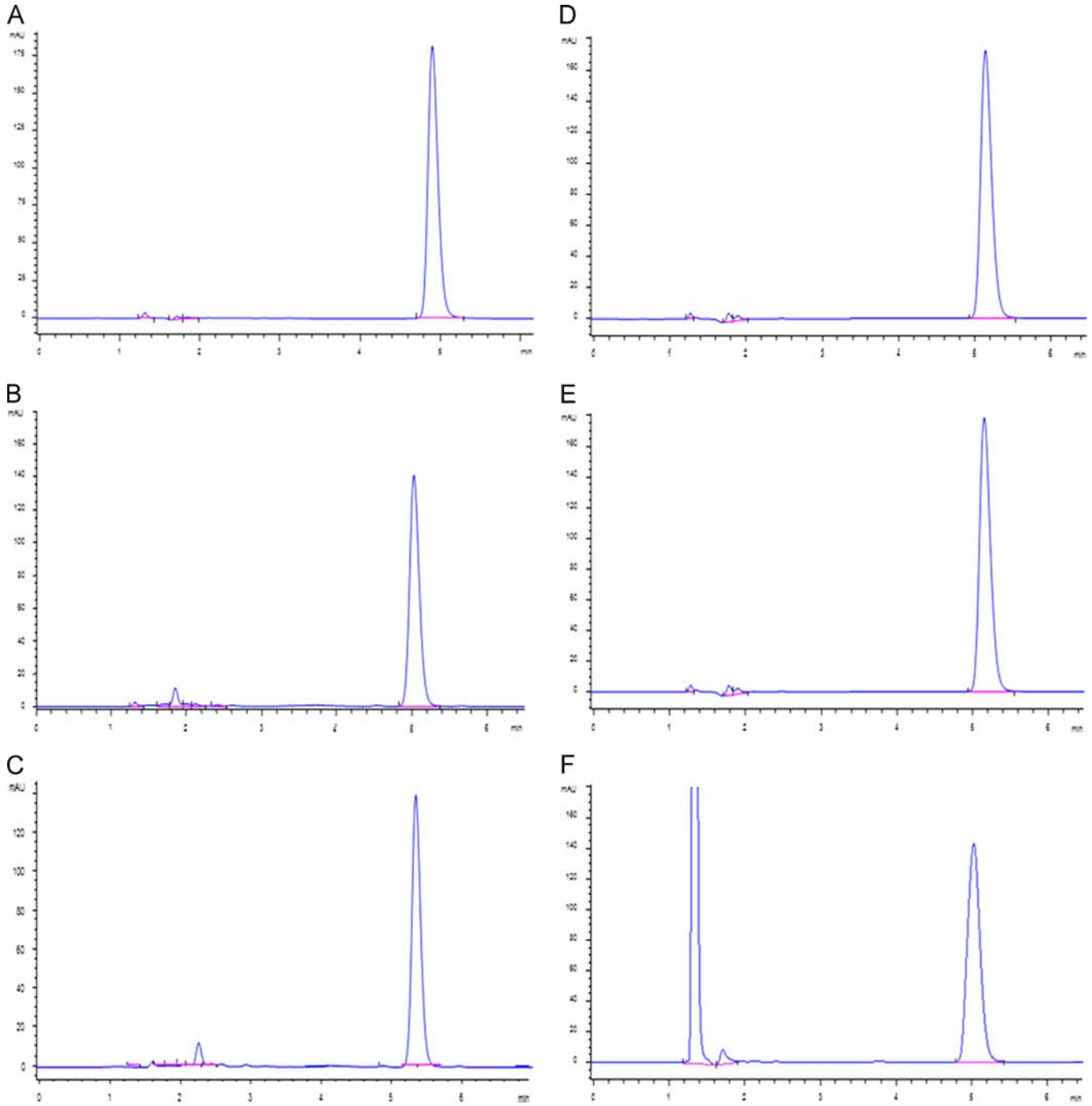

Fig. 3. Representative HPLC chromatograms obtained from the sample solution of besifloxacin submitted to forced degradation: (A) 60 °C during 24 h; (B) UVC light for 1 h; (C) UVA light for 1 h; (D) 1.0 M HCl for 1 h; (E) 1.0 M NaOH for 1 h; (F) 30% H_2O_2 for 1 h.

medium dose (1.0 μg mL^{-1}), and 24.30 mm (RSD=0.55) for the higher dose (2.0 μg mL^{-1}). The calibration curves found for BSF were constructed by plotting the log concentration (μg mL^{-1}) versus the zone diameter (mm) and showed good linearity in the concentration range of 0.5 and 2.0 μg mL^{-1}. The representative linear equation was $y=11.278x+9.7687$, where x is the log concentration and y is the zone diameter. The correlation coefficient ($r=0.9974$) found was considered significant for the method (Fig. 4).

According to the Brazilian (2010) and European Pharmacopoeia (2005), if a parallel-line model is chosen, the two log dose–response lines of the preparation to be examined as well as the reference preparation must be parallel and they must be linear

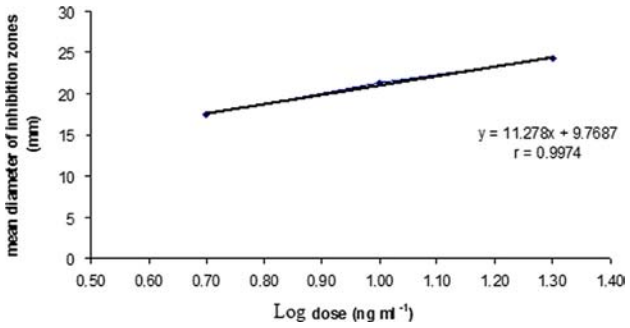

Fig. 4. BSF calibration curve obtained by the microbiological cylinder-plate assay.

over the range of doses used in calculation. These conditions were verified by a validity test for a given probability, usually $p=0.05$. The assays were validated by analysis of variance, as described in these official guidelines. There was no deviation from parallelism and linearity in the results presented here ($p > 0.05$).

3.3.3. Precision and accuracy

HPLC method – The precision of the method was determined by repeatability (intra-day) and intermediate precision (inter-day) and it was expressed as RSD (%). The low values of relative standard deviations (RSD) for repeatability (0.74%, 1.73% and 0.88%) and intermediate precision (1.41%) showed adequate precision of the analytical method.

The accuracy was evaluated by simultaneously determining the analyte in solutions prepared using the standard addition method. The mean results were found to be 101.0%, indicating satisfactory accuracy.

Bioassay method – The microbiological assay also demonstrated precision and accuracy. The precision of the assay was determined by repeatability (intra-assay) and intermediate precision (inter-assay) in which values are expressed as relative standard deviation (RSD) of the series of measurements performed on the same day and on alternate days. Repeatability was demonstrated by the low RSD found in the samples on the same day (0.53 to 1.15). The intermediate precision was determined by analyzing samples on three different days, where an RSD of 0.84% was obtained (Table 3).

Accuracy was evaluated by simultaneously determining the analyte in prepared solutions using the standard addition method. Accuracy expresses the agreement between the accepted value and the value found [15]. The mean values were found to be 101.4%.

3.3.4. Robustness

HPLC method – The method robustness was investigated through little changes in the pH of the aqueous phase, percentage of acetonitrile, flow and percentage of triethylamine in order to verify the influence of these modifications in the chromatographic parameters (system suitability), such as area, theoretical plates, retention time and retention factor.

No significant changes were found in the chromatographic pattern when the modifications were made, under experimental conditions, thus the method was shown to be robust.

Bioassay method – To evaluate the robustness of the method the concentration of the inoculated medium in the determination of BSF (1.8 and 2.2%) varied. The values found in determining BSF parameter related to robustness by modifying the concentration of inoculum (103.9%) were very close to those obtained during the validation tests (104.7%), demonstrating the robustness of the method.

Table 3
Intra-assay and inter-assay precision data for the BSF bioassay in the pharmaceutical formulation.

Sample	Day	Potency found[a] (%)	RSD (%)
1		104.34	
2	1	106.67	1.15
3		104.92	
4		104.78	
5	2	104.98	0.53
6		103.93	
7		103.81	
8	3	105.14	0.69
9		103.98	
	Inter-assay	104.73	0.84

[a] Mean of six determinations.

3.4. Photodegradation kinetics

Considering preliminary stability and photoreactivity studies, the degradation kinetics of BSF under a UVA light condition was performed (because it is an ophthalmic suspension without restriction to daytime use).

All results obtained by microbiological assay were monitored by previously validated HPLC. The effect of light exposure on the residual BSF RS and on the pharmaceutical formulation/ocular suspension is shown in Tables 4 and 5, respectively.

The reaction order for the degradation kinetics of BSF was determined representing the residual drug concentration versus time (zero order reaction), log concentration versus time (first order reaction), and the inverse of a function of concentration versus time (second order). The linear regression coefficients (r) were obtained, and the coefficient closer to unity indicates the order of reaction. BSF RS was found to follow a second-order reaction (A) while the ophthalmic suspension (B) followed a first-order reaction (Fig. 5).

With this information, through mathematical calculations, the first reaction rate constant (k) and lifetime (t_{90}%) were determined. For the BSF ophthalmic suspension (first-order reaction) the constant reaction corresponds to 0.0962 h^{-1}. In other words, it deteriorates at a rate of approximately 9.62% per hour. For BSF RS which follows a second-order reaction, the reaction constant was 0.0025 h^{-1}.

The lifetime (t_{90}%) for BSF RS showed a value of 0.00499 min or 0.299 s, while for the ophthalmic suspension the value was 1.095 h.

3.5. 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

Table 4
BSF RS levels obtained after exposure to UVA light evaluated by HPLC and microbiological method.

Time (min)	BSF RS (%)	
	Microbiological assay*	HPLC
0	100.0	100.0
15	99.3	99.5
30	96.9	95.0
60	80.2	87.0
90	76.6	77.0
120	71.6	68.0
150	68.8	63.0

* Mean of six replicates.

Table 5
BSF levels obtained in ophthalmic suspension after exposure to UVA light evaluated by HPLC and microbiological method.

Time (min)	BSF ophthalmic suspension (%)	
	Microbiological assay[a]	HPLC
0	100.0	100.0
60	91.5	91.0
120	81.4	78.0
180	74.7	66.0
240	69.1	60.0
300	60.7	53.0

[a] Mean of three determinations.

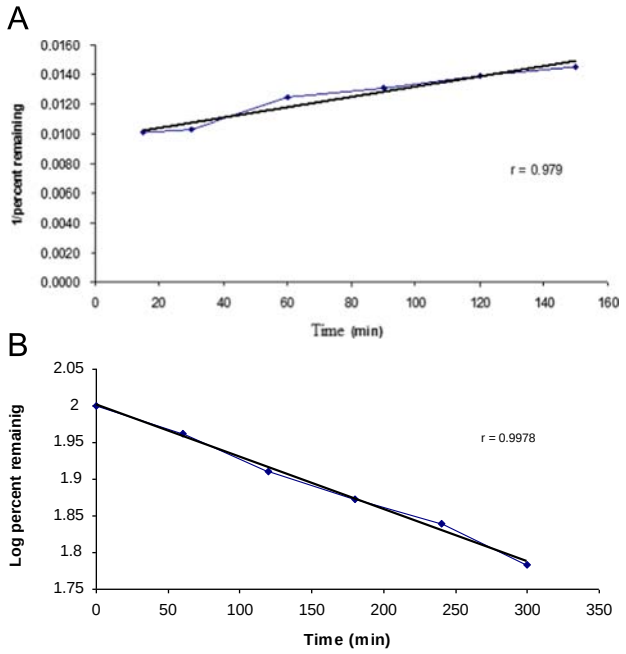

Fig. 5. Second-order plots for the degradation of BSF RS (A) under UVA light and first-order plots of ophthalmic suspension (B) by microbiological assay.

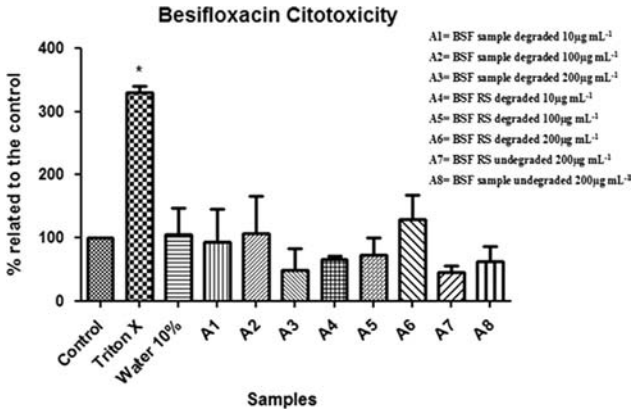

Fig. 6. Results of lactate dehydrogenase (LDH) assay for cellular viability. Values of 100% indicate total cellular viability (control data). Triton X (1%) exhibited cellular death, being used as a positive control. * indicates significant differences between means ($p < 0.05$).

from unstable samples. Non-significant differences ($p > 0.05$) were obtained, indicating that the degraded samples and intact molecule did not show an increase in cytotoxicity after exposure to the assayed cells for 5 h (Fig. 6).

3.6. Comparison of the methods

The results obtained with the cylinder plate assay were comparable with those obtained by HPLC. For the microbiological assay method, the mean potency found (RSD%) was 104.67% ± 0.85% and for the HPLC method, 102.83% ± 1.29%. These results were statistically analyzed using Student's t test and indicated that there was no significant difference between the methods at $\alpha = 0.05$ ($t_{calculated}$ 1.46 < $t_{theoretical}$ 2.12).

4. Conclusions

An HPLC method was developed and validated for quantitative determination of BSF in ophthalmic suspension. Validation experiments provide a proof that the HPLC method is specific, linear in the proposed working range, as well as accurate, precise and robust.

The proposed microbiological cylinder plate assay for determination of BSF in ophthalmic suspension is linear, precise, accurate, specific and robust. Moreover, there is no statistical difference between the microbiological assay and the HPLC method for drug quantification; therefore, these can be interchangeable. The validation proved that the microbiological assay is an excellent alternative method for analyzing the pharmaceutical dosage form in the BSF ophthalmic suspension, being a useful tool to supplement or replace physico-chemical methods in quality control.

The degradation of BSF RS and the ophthalmic suspension during the photochemical process are found to follow second-order and first-order reaction kinetics, respectively. The kinetic parameters of the degradation rate constant, and $t_{90\%}$ could be predicted.

The cytotoxicity test showed that at all concentrations tested (10–200 μg mL^{-1}), regardless of whether there is degradation, BSF did not alter the cell membrane and, thus, it is considered non-toxic to human mononuclear cells under the experimental conditions tested.

These studies proved to be useful tools to control the quality of the BSF ophthalmic suspension, encouraging its use in routine analysis.

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