Talanta 119 (2014) 367–374

Contents lists available at ScienceDirect

Talanta

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

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

Márcia C.N. Costa [a,*], Amanda T. Barden [a], Juliana M.M. Andrade [b], Tércio P. Oppe [a], Elfrides E.S. Schapoval [a]

[a] Programa de Pós-Graduação em Ciências Farmacêuticas, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul., Av. Ipiranga 2752, Lab. 402, F: 55-51-3308-5214 Porto Alegre/RS, CEP 90610-000, Brazil

[b] Programa de Pós-Graduação em Ciências Farmacêuticas, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul. Av. Ipiranga 2752, Lab. 505-H, F: 55-51-3308–5258 Porto Alegre/RS, CEP 90610-000, Brazil

ARTICLE INFO

Article history:
Received 18 May 2013
Received in revised form
8 October 2013
Accepted 17 October 2013
Available online 20 November 2013

Keywords:
Besifloxacin
Method validation
HPLC
Bioassay
Cytotoxicity

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.

* Corresponding author: Tel.: +55 51 3308 5214; fax: +55 51 3308 5378.
E-mail address: marciacncosta@yahoo.com.br (M.C.N. Costa).

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http://dx.doi.org/10.1016/j.talanta.2013.10.051

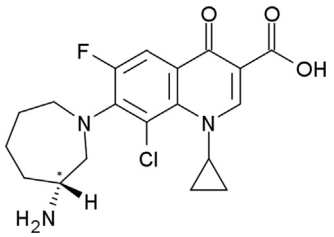

Fig. 1. Chemical structure of BSF.

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