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Research article

Simultaneous quantification of gatifloxacin, moxifloxacin, and besifloxacin concentrations in cornea and aqueous humor by LC-QTOF/MS after topical ocular dosing

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ABSTRACT

The fourth-generation fluoroquinolones are widely used as ophthalmic antimicrobials. This study aimed to validate a new analytical technique for simultaneous quantification of gatifloxacin, moxifloxacin, and besifloxacin concentrations in the cornea and aqueous humor by liquid chromatography (LC) coupled to quadrupole time-of-flight mass spectrometry (QTOF/MS) at 10 min and 1 h after instillation of topical ophthalmic antimicrobial suspensions. It was used twenty-two male dogs without ocular lesions verified by ophthalmic and histologic examinations. Methanol:water (4:1) was used for the extraction procedure for cornea and acetonitrile:water (4:1) was used for aqueous humor. The chromatographic separations were carried out on a C18 column with a linear gradient of water and methanol, both containing 0.1% formic acid. The total chromatographic run time was 4 min. Mass spectrometry analyses were performed on a Xevo™ G2-S QTof tandem mass spectrometer, operated in a positive ion electrospray ionization (ESI+) mode. The retention times were approximately 1.42 min for gatifloxacin, 1.87 min for moxifloxacin, and 3.01 min for besifloxacin. No interference peak was detected for the three tested antimicrobials in samples obtained from both cornea and aqueous humor, ensuring that the peak response was exclusive to the analyte of interest. The limit of detection for the three antimicrobials was 0.11 μg/mL and the limit of quantification was 0.42 μg/mL for both cornea and aqueous humor samples. At both time points post instillation of the three antimicrobials, moxifloxacin had the highest corneal concentration and besifloxacin demonstrated the highest concentration in the aqueous humor.

1. Introduction

Fluoroquinolones are antimicrobial drugs used worldwide for the treatment of infections in humans and animals. The pharmacological mechanism of action of fluoroquinolones involves binding to topoisomerase IV and gyrase in the presence of DNA, causing changes in the conformation of the formed protein. Quinolones are bacteriostatic agents because they prevent bacteria replication (Cheng, Hao, Dai, Liu, & Yuan, 2013; Aldred, Kerns, & Osheroff, 2014; Tse-Dinh, 2015). The fourth-generation quinolones are products of chemical modifications resulting in an extension of the antimicrobial spectrum of former generations to broad-spectrum activity covering gram-negative and gram-positive bacteria. These compounds are widely used for the prophylaxis and treatment of ocular infection (Mather, Karenchak, Romanowski, & Kowalski, 2002; Scoper, 2008).

Fourth-generation fluoroquinolone ophthalmic solutions include gatifloxacin, moxifloxacin (McGee, Holt, Kastner, & Rice, 2005), and besifloxacin (Deschênes & Blondeau, 2015; Gu et al., 2016a). The ophthalmic suspension of besifloxacin, a more recent fluoroquinolone developed exclusively for ophthalmic use, contains a mucoadhesive polymer (Deschênes & Blondeau, 2015; Gu et al., 2016a; Chung et al., 2013) that may contribute towards its ability to maintain concentrations in both cornea and aqueous humor, perhaps by reducing elimination due to lacrimation.

For effective therapeutic action, these drugs must be present in certain concentrations at the target tissue. Intraocular penetration of the drug is hampered by defense mechanisms protecting the eyes – the two most important being the corneal barrier affecting drug permeability and lacrimal drainage of the drug (Samuelson, 2013; Nautscher, Bauer, Steffl, & Amselgruber, 2015). Drugs with high intraocular penetration and those that maintain adequate intraocular concentrations are considered the most effective topical ocular antimicrobials (Chung et al., 2013; Maggs, 2008).

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Reported methods for measuring fluoroquinolones in ocular samples are high-performance liquid chromatography with fluorescence detection (FLD) (Basci, Hanioglu-Kargi, Soysal, Bozkurt, & Kayaalp, 1997; Davis et al., 2010; Matsuura, Suto, Akura, & Inoue, 2013) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Gu et al., 2016b; Proksch et al., 2009). Methods for measurement in plasma also include high-performance liquid chromatography with ultraviolet detection (HPLC-UV) (Cavazos-Rocha, Carmona-Alvarado, Vera-Cabrera, Waksman-de-Torres, & Salazar-Cavazos, 2014). To the best of our knowledge, liquid chromatography (LC) coupled to quadrupole time-of-flight mass spectrometry (QTOF/MS) has not been reported for determination of fluoroquinolones in ocular samples.

The aim of this study was to develop and validate an analytical method for the detection and quantification of gatifloxacin, moxifloxacin, and besifloxacin concentrations in cornea and aqueous humor by a LC-QTOF/MS employing QuanTOF technology. Another aim was to determine the concentrations of these antimicrobials in the cornea and aqueous humor of dogs, 10 min and 1 h after instillation of these topical ophthalmic suspensions.

2. Materials and methods

2.1. Reagents and standard solutions

Gatifloxacin sesquihydrate and moxifloxacin hydrochloride were obtained from Fluka - Sigma-Aldrich (St. Louis, MO, USA), and besifloxacin hydrochloride was acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). LC-MS-grade methanol was purchased from Baker (Xalostoc, México), and formic acid was purchased from Tedia (Ohio, USA).

The standard stock solution was prepared in methanol:water (4:1) at a concentration of 1000 μg/mL and stored at −20 °C. The working standard solutions were prepared weekly in methanol:water (4:1) to yield final concentrations of 0.42, 0.83, 1.67, 3.33, 8.33, and 16.66 μg/mL and also stored at −20 °C.

2.2. Animals and experimental design

The experimental protocol used in this study was approved by the Ethics Committee in Animal Experimentation at the Universidade Federal de Minas Gerais-UFMG (protocol #266/2014) and by the Ethics Committee of the Center for Zoonosis Control of the Belo Horizonte city, Minas Gerais state, Brazil.

Male dogs, aged one to seven years, from the Center for Zoonosis Control of the Belo Horizonte city, were used. All dogs were euthanized as they were carriers of canine visceral leishmaniasis. The dogs were previously selected by ophthalmologic examination, which included assessment of eye health through inspection using a flashlight, direct ophthalmoscope (Heine Beta 200S, Heine & Co., Herrsching, Germany), and portable slit lamp (Alltion (Wuzhou) Co., Wuzhou, Guangxi, China). Animals showing any clinically diagnosable ocular disorders were not included in the study. After collection of aqueous humor and cornea, the eyes were individually identified, fixed in Davidson's solution (Latendresse, Warbrittion, Jonassen, & Creasy, 2002), embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin. After histological examination, four eyes from two animals were discarded from the study. Thus, a total of 40 eyes from 20 dogs were used in the study.

The ophthalmic suspensions used were gatifloxacin 0.3% (Zymar, Allergan Produtos Farmacêuticos, São Paulo, SP, Brazil), moxifloxacin 0.5% (Vigamox, Alcon Laboratórios do Brasil, São Paulo, SP, Brazil), and besifloxacin 0.6% (Besivance, Bausch & Lomb Incorporated, Tampa, Florida, USA). Each dog was instilled with a different antimicrobial for each eye. Antimicrobial ophthalmic suspensions were instilled at the top side of each dog's eyeball. The volume of instillation was 45 μL for gatifloxacin, 38 μL for moxifloxacin, and 32 μL for besifloxacin. Antimicrobial concentrations were measured at 10 min and 1 h. The numbers of eyes that were instilled with ophthalmic suspension at each time of penetration are shown at Table 1.

After 10 min and 1 h had elapsed, the animals were submitted to an euthanasia procedure using intravenous xylazine administration (6 mg/kg), followed by thiopental (25 mg/kg) and magnesium sulfate injection (in increasing amounts until cardiac arrest was induced). After euthanasia, each eyeball was removed by lateral canthotomy followed by sectioning of periocular tissue towards the optic nerve. The eyeball was removed and conjunctiva excess was detached.

Samples of aqueous humor were collected from the anterior and posterior chamber by using a 29G needle (12.7 mm × 0.33 mm), which was inserted into the cornea near the limbus and parallel to the iris. Approximately 500 μL of aqueous humor was collected. The samples were placed in 1.5-mL microcentrifuge tubes and stored in a freezer Revco® at −80 °C until processing. Each cornea was removed from eyeball and placed in 2.0-mL microcentrifuge tubes and stored at −80 °C until analyses.

2.3. Sample preparation

The methods for sample preparation were based on preliminary tests using blank samples fortified with the antimicrobials, which tested different types of solvents (acetonitrile, methanol, and mixtures with water) and solvent volumes.

Each cornea was weighed and fragmented before placing into a 2-mL plastic tube containing 1.5 mL of methanol:water (4:1, v/v). The mixture was placed into a laboratory ultrasonic bath (40 kHz) for 25 min at 35 °C, vortexed at a high speed for 10 s, and then centrifuged at 1300 × *g* for 10 min at 9 °C. The supernatant was filtered through 0.20-μm PTFE membrane filters (Chromafil O-20/15 MS) and transferred to vials for injection into the LC-TOF-MS system.

An aliquot of 500 μL of aqueous humor was placed into a 2-mL plastic tube containing 1.0 mL of acetonitrile:water (4:1, v/v). The mixture was vortexed at a high speed for 10 s and then centrifuged at 1300 × *g* for 10 min at 9 °C. The supernatant was filtered through 0.20-μm PTFE membrane filters (Chromafil O-20/15 MS) and transferred to vials for injection into the LC-TOF-MS system.

2.4. Chromatographic conditions

The chromatographic separations were carried out using a Waters Acquity UPLC system (Waters, Milford, USA). Injection volume was 5 μL. Separation was carried out on an Acquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm, Waters, Milford, USA) with a linear gradient of water containing 0.1% formic acid (phase A) and methanol containing 0.1% formic acid (phase B). The gradient elution program of A was 75–50% for 3 min, 50–5% for 0.2 min, 5% for 0.2 min, 5–75% for 0.1 min, and 75% for 0.5 min, at a flow rate of 0.3 mL/min maintained at 45 °C. The total chromatographic run time was 4 min.

2.5. Mass spectrometric conditions

Mass spectrometry analysis was performed on a Xevo™ G2-S QTof (quadrupole hybrid with orthogonal acceleration time-of-flight) tandem mass spectrometer with QuanTOF™ technology (Waters, Milford, USA). The data acquisition was performed in positive ion electrospray ionization (ESI+) mode. The desolvation gas was nitrogen and the

Table 1
Number of eyes that were instilled by ophthalmic suspension of gatifloxacin, moxifloxacin, and besifloxacin.

Antimicrobial	10 min	1 h
Gatifloxacin	7	7
Moxifloxacin	6	7
Besifloxacin	7	6