Journal of Chromatography B, 867 (2008) 105–110

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Quantitative determination of besifloxacin, a novel fluoroquinolone antimicrobial agent, in human tears by liquid chromatography–tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 3 January 2008
Accepted 26 March 2008
Available online 1 April 2008

Keywords:
Besifloxacin
Fluoroquinolone
Antimicrobial agent
Human tears
LC/MS/MS
Pharmacokinetics

ABSTRACT

A rapid and sensitive method was developed using high-performance liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) for the quantification of besifloxacin in human tears using sparfloxacin as the internal standard (IS). Besifloxacin was extracted from human tear samples using an ammonium formate buffer at pH 3.25. The method was validated over a concentration range of 2–2000 ng/mL, with a total run time of less than 4 min. The overall intra- and inter-day precision for this method was less than 6%. The method was used to measure besifloxacin concentrations in tear samples collected after topical ocular administration to humans; besifloxacin concentrations were 610 ± 540 μg/g (15 min) and 1.60 ± 2.28 μg/g (24 h).

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1. Introduction

The fluoroquinolones are a class of synthetic antibacterial agents which are known for their broad spectrum of antibacterial activity [1,2]. Since their introduction in the late 1980s, fluoroquinolones have become very important and frequently used antibacterial agents in medical and surgical specialties, such as ophthalmology. Many fluoroquinolones have been marketed and used in ophthalmology, including ciprofloxacin, ofloxacin, norfloxacin, levofloxacin, and more recently gatifloxacin and moxifloxacin [2–5]. Despite the relatively large number of treatment options for ophthalmic fluoroquinolones, the search continues for new fluoroquinolone agents, primarily driven by the desire to improve potency against a wide spectrum of microbial pathogens and to mitigate the increasing threat of bacterial resistance to antibiotics [6].

Besifloxacin (7-[(3R)-3-aminohexahydro-1H-azepin-1-y1]-8-chloro-1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-3-quinolinecarboxylic acid; Fig. 1) is a novel, chiral synthetic fluoroquinolone being developed by Bausch & Lomb for the topical treatment of ophthalmic infections. Structurally, besifloxacin has an N-1 cyclopropyl group, which provides broad-spectrum activity against aerobic bacteria [7]. The main mechanism of action of besifloxacin is the inhibition of both DNA gyrase and topoisomerase-IV enzymes that are involved in coiling and uncoiling of bacterial DNA. This activity is enhanced by a C-8 chloride substituent in besifloxacin, which also exhibits improved activity against gram-positive bacteria relative to older fluoroquinolones while still retaining potent efficacy against anaerobic bacteria [7].

Multiple approaches using high-performance liquid chromatography coupled with UV or fluorometric detection have been used in the past to quantitate various fluoroquinolones in human body fluids [8–12]; however, these detectors do not provide the same degree of versatility, speed, specificity, and sensitivity in bioanalysis as the high-performance liquid chromatography-electrospray tandem mass spectrometer (LC/MS/MS). Since the quantification of besifloxacin in tissues to support topical ocular pharmacokinetic studies requires a highly sensitive and specific bioanalytical assay, the LC/MS/MS was chosen for the quantitation of besifloxacin. The present paper describes an LC/MS/MS method for the quantification of besifloxacin in human tears using artificial tears as a surrogate matrix.

2. Experimental

2.1. Materials and reagents

Besifloxacin HCl (99.4% pure) was obtained from Bausch & Lomb, whereas the internal standard (IS) sparfloxacin was purchased from Sigma–Aldrich (St. Louis, MO). In all instances, besifloxacin concentrations were corrected for the salt content, and are presented

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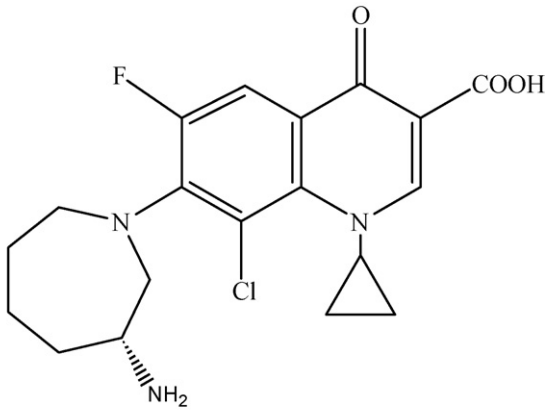
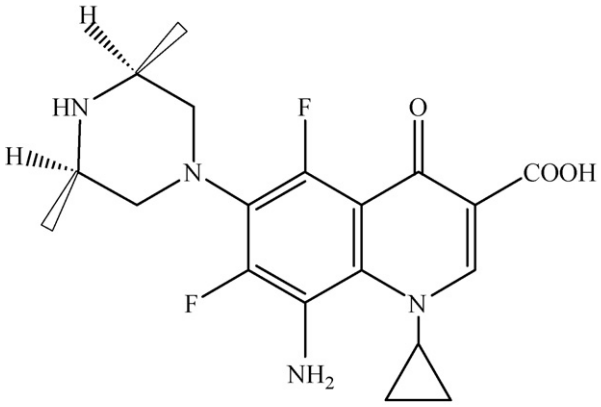
doi:10.1016/j.jchromb.2008.03.025



Besifloxacin ($C_{19}H_{21}ClFN_3O_3$, MW 393.8)



Sparfloxacin ($C_{19}H_{22}F_2N_4O_3$, MW 392.4)

Fig. 1. Chemical structure of besifloxacin and sparfloxacin (internal standard).

herein as free base equivalents. Chemical structures of besifloxacin and sparfloxacin are shown in Fig. 1. HPLC-grade acetonitrile, methanol, and isopropyl alcohol were obtained from Burdick & Jackson (Morristown, NJ). Ammonium formate, formic acid and sodium hydroxide (10 M) were obtained from Sigma–Aldrich (St. Louis, MO). Deionized water was USP grade. An artificial tear solution was used as a surrogate matrix and was prepared based on a published method with minor modifications [13]. The artificial tear solution contained the major types of proteins, glycoproteins, and lipids present in real human tears. While the use of a surrogate matrix is not common, it is occasionally necessary when authentic blank matrix is not readily available from commercial sources, such as in this case [14,15]. Schirmer Tear Test strips were supplied from Eagle Vision (Memphis, TN). All other reagents were of analytical grade and were obtained from standard commercial suppliers.

2.2. Chromatographic and mass spectrometric conditions

2.2.1. Chromatographic conditions

The HPLC system consisted of Shimadzu pumps (Shimadzu Co., Columbia, MD), an on-line vacuum degasser, a column temperature controller (Shimadzu Co.) and a CTC HTS PAL autosampler (LEAP Technologies, Carrboro, NC) with 100-μL loop and 100-μL syringe/syringe adapter, running under Cycle Composer software with Macro Editor. The analytical column was a Pursuit® Diphenyl (30 mm × 2.0 mm, 5 μm, Varian, Palo Alto, CA) with a guard column of the same stationary phase with a column temperature of 30 °C. The analytes were eluted using a mobile phase composed of 5-mM ammonium formate in water, pH 3.25, and a combination of acetonitrile:methanol (20:80 v/v). The injection volume was 30 μL with a flow rate of 0.80 mL/min and the run time was 4 min.

2.2.2. Mass spectrometric conditions

Besifloxacin and the IS were detected using an API 4000 SCIEX triple-quadrupole tandem mass spectrometer (Applied Biosystems/MDS SCIEX, Concord, Ontario, Canada). The mass spectrometer was operated in the TurboIonSpray™ mode with positive ion detection. The TurboIonSpray™ temperature was maintained at 600 °C, with an ion spray voltage of 4000 V applied to the electrode. Nitrogen was used as the sheath and nebulizing gas. The detection and quantification of analytes was performed using the multiple reaction monitoring (MRM) mode using ion precursor → product ion combinations of 394.23 → 356.00 m/z for besifloxacin and 393.22 → 349.10 m/z for the IS. The Q1 and Q3 mass resolutions were set as unit and low resolution, respectively. All raw data were processed with PE SCIEX Analyst Software (Version 1.4.1) from Applied Biosystems.

2.3. Preparation of stock solutions

Stock solutions of besifloxacin and IS were prepared by dissolving each compound in methanol to obtain a final nominal concentration of 4.0 mg/mL and 0.100 mg/mL, respectively, corrected for compound purity. The stock solutions were diluted with water/methanol (50:50 v/v) to make working solutions which were further diluted to prepare the calibration standards and quality

control samples. All solutions, standards and QC samples were stored at −20 °C until used.

2.4. Preparation of standards and quality controls (QCs)

Calibration standards were prepared by spiking Schirmer strips containing artificial tear solution with known concentrations of besifloxacin. The concentrations of besifloxacin used for the calibration curves were: 2.00, 5.00, 25.0, 100, 500, 1200, 1700, and 2000 ng/mL. QC samples (2.00, 6.00, 1000, and 1500 ng/mL) were prepared in a similar manner. All standards and QCs were prepared in individual Sarstedt polypropylene vials and stored at approximately −20 °C protected from light. Artificial tear fluid was used in place of human tears for the calibration curves and QCs in this validation due to limitations on the commercial availability of human tears. Equivalence between these two matrices was demonstrated by comparing QCs made from human tears acquired from human volunteers to a calibration curve prepared from the surrogate tears in one of the validation runs.

2.5. Assay extraction procedure

Quality control samples, calibration standards, human or artificial tears, and study samples were allowed to thaw and come to room temperature before processing. To each vial, 1.00 mL of 5-mM ammonium formate buffer (pH 3.25) was added and vortexed for approximately 1 min followed by shaking on a horizontal shaker for approximately 30 min. After shaking, a 200-μL aliquot of the buffer eluent was added to a 96-well plate, along with 50.0 μL of the IS working solution (10.0 ng/mL) for all samples except for the blanks. Blank samples were prepared by adding 50.0 μL of 80:20 (v/v) water: acetonitrile to the 96-well plate to make up for the lack of internal standard. The prepared plates were vortex-mixed and 20 μL was injected onto the LC/MS/MS.

2.6. Validation studies

A full validation was perfomed on the LC/MS/MS method described above for the quantitation of besifloxacin in human tear samples based on the Guidance for Industry, Bioanalytical Method Validation issued by the US Food and Drug Administration [16]. Method validation experiments were completed within a 5-day period and the parameters for validation included accuracy (%bias), precision (%RSD), selectivity, lower limit of quantitation (LLOQ), and stability.

2.6.1. Recovery

The recovery of besifloxacin during the extraction procedure was estimated by comparing the peak area for besifloxacin or IS following direct injection of aqueous QC samples of HQC (high-level quality control; 15,000 ng/mL), MQC (mid-level quality control, 1000 ng/mL) and LQC (low-level quality control; 6 ng/mL) against the peak areas obtained for QCs spiked in Schirmer strips containing the artificial tear solution and extracted as described above.

2.6.2. Calibration curves

The calibration curves were evaluated by performing quadratic regression with $1/x^2$ weighting using all acceptable calibration standard curve points. The quadratic regression model was used to satisfy the wide dynamic range explored in this method (2–2000 ng/mL). While other regression models were evaluated, this model provided the best fit to the calibration data and ultimately afforded acceptable accuracy and precision, as assessed by the results from QC sample analysis. The coefficient of determination (r^2) was used to evaluate the goodness-of-fit of the calibration curves along with a visual inspection of the residuals plots. The performance of the calibration model was confirmed by back-calculating the measured concentrations of the calibration standards.

2.6.3. Accuracy and precision

The accuracy of the assay (%bias) was calculated by subtracting the theoretical concentration from the mean observed concentration for each QC or standard level, and dividing by their respective nominal values. Precision was measured by the relative standard deviation (%RSD) and was determined by dividing the standard deviation by the mean concentration for each QC or standard level.

2.7. Stability

The stability of besifloxacin was evaluated under the following conditions: room temperature, freeze/thaw cycles, extracted samples at room temperature, autosampler stability, and long-term frozen storage conditions. Also, the dilution integrity was verified by an over-range QC sample assayed in at least six replicates at one or more dilution factors.

2.8. Application of the method

The human tear samples analyzed in this study were obtained from 64 healthy subjects following topical ocular administration of 0.6% besifloxacin HCl in both eyes. A single tear sample was collected from each subject at predetermined times through 24 h after dosing. Each tear sample was collected using a Schirmer Tear Test strip which was weighed before and after tear collection to determine the weight of the tear sample. On average, approximately 10–20 mg of tears was collected from each subject. Besifloxacin concentrations are reported as μg besifloxacin/g of tears. Schirmer Tear Test strips were stored at −20 °C until the samples were analyzed by LC/MS/MS.

3. Results and discussion

3.1. Chromatography

Representative chromatograms obtained from extracted blank human control tear samples as well as a 1000-ng/mL calibration standard are shown in Fig. 2 for besifloxacin and sparfloxacin (IS). Under the chromatographic conditions employed, the average ± SD retention time for besifloxacin and IS was 1.8 ± 0.3 min and 0.7 ± 0.3 min, respectively.

3.2. Selectivity/specificity/carryover

Six lots of human tears were analyzed for the evaluation of selectivity and specificity. These samples did not show any significant interfering peaks at the retention times of either besifloxacin or the IS (Fig. 2).

Carryover was evaluated in all validation runs. An injection of the high calibration standard was directly followed by injection of a control blank (without IS). No peaks greater than 20% of the LLOQ for besifloxacin or greater than 5% of the average IS response were observed at the retention time of interest in the drug or the IS channel for the blank, indicating that any carryover was at an acceptable low level.

3.3. Between-subject variability

Variability between subjects was investigated by preparing six QC pools (one replicate of each) in the range of the calibration curve (1000 ng/mL) from individual matrix donors. The precision and accuracy were 1.1 and 12%, respectively. Since the measured

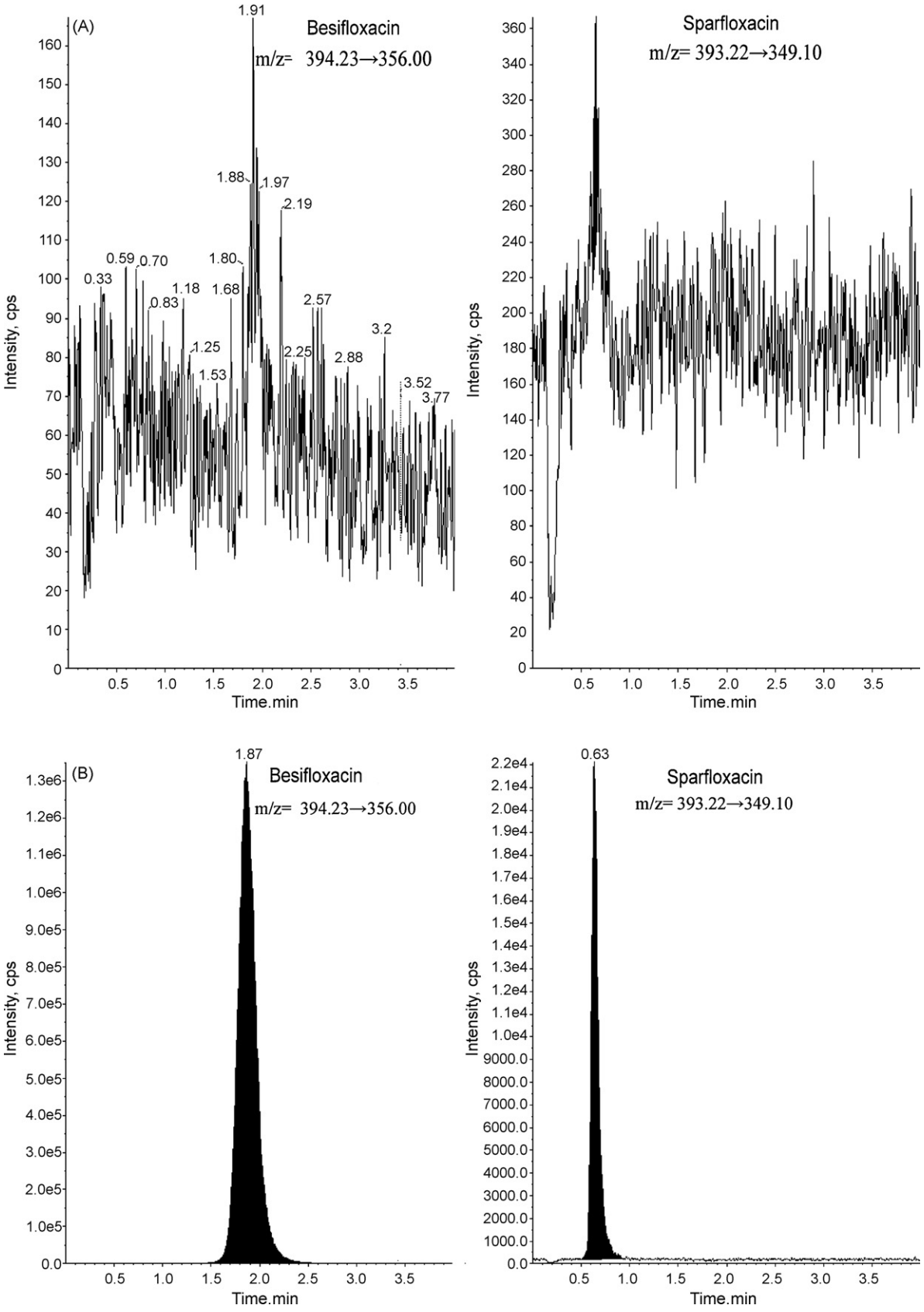


Fig. 2. Selected reaction monitoring chromatograms of besifloxacin and the internal standard (sparfloxacin) in control blank (A) and from a medium calibration standard (1000 ng/mL) (B) in extracted human tears.

Table 1
Summary of extraction recovery of besifloxacin and sparfloxacin

QC samples	Theoretical concentration (ng/mL)	Besifloxacin %recovery	Sparfloxacin %recovery	*n*
LQC	6.00	92.6	96.3	6
MQC	1000	86.2	98.4	6
HQC	1500	102.3	103.5	6
Overall mean		93.7	99.4	

Table 2
Summary of besifloxacin calibration standards

Theoretical concentration (ng/mL)	Measured concentration mean (ng/mL)	SD	Precision[a] %RSD	Accuracy[b] %bias	*n*[c]
2.00	2.00	0.117	5.8	0.0	10
5.00	4.99	0.0973	1.9	−0.2	10
25.0	24.5	0.629	2.6	−2.0	10
100	100	2.63	2.6	0.0	10
500	506	16.8	3.3	1.2	10
1200	1220	14.1	1.2	1.7	10
1700	1700	14.5	0.8	0.0	10
2000	1970	36.9	1.9	−1.5	10

[a] (SD/mean) × 100.
[b] [(Measured mean conc. − Theoretical conc.)/Theoretical conc.)] × 100.
[c] Two replicates from five separate runs.

accuracy was less than 15%, this suggests that the analog IS was properly controlling any matrix effects that might be present. Due to the limited commercial supply of blank human tear samples, an ion suppression experiment was not performed.

3.4. Extraction recoveries

The absolute recovery of besifloxacin and IS was determined by comparing the measured peak areas of normal quality control samples with those of blank sample extracts reconstituted with standard solutions prepared at the expected final extract concentrations. Concentrations of the six replicates were 6.00, 1000, and 1500 ng/mL. The overall mean recovery of besifloxacin was 93.7% and the overall mean recovery of the IS was 99.4%. Results are shown in Table 1.

3.5. Calibration curves

The MS response for besifloxacin was linear over the concentration range of 2.0–2000 ng/mL, affording an LLOQ of 2.00 ng/mL. Linearity was evaluated by performing quadratic regression with $1/x^2$ weighting using acceptable calibration standard curve points. The mean coefficient of determination (r^2) for besifloxacin determined during the validation ($n = 5$) was 0.999. The results of back-calculated concentrations, accuracy (%bias) and precision (%RSD) of besifloxacin calibration samples are presented in Table 2, and calibration curve parameters are shown in Table 3. Overall, the results indicated that the method was reliable and reproducible within the analytical range.

3.6. Accuracy and precision

Intra- and inter-assay precision was determined from the relative standard deviations (%RSD) of the quality control samples (LLOQ, LQC, MQC, and HQC). The intra-assay precision ranged from 0.9 to 5.8%. The inter-assay precision ranged from 3.0 to 5.0%. Table 3 shows a summary of the results for the intra- and inter-assay precision.

Intra- and inter-assay accuracy was determined by comparing the mean measured concentrations of the quality control samples with their nominal concentrations determined within a single bioanalytical run or in separate runs, respectively. The intra-assay accuracy ranged from 8.0 to 17.5%. The inter-assay accuracy ranged from −3.8 to 2.0%. The intra- and inter-assay accuracy are described in Table 4.

3.7. Stability

The stability of besifloxacin tested under a variety of conditions during the validation is presented in Table 5. Matrix stability was assessed from sets of quality control samples held at room temperature for approximately 24 h in buffer solution and 2 h on Schirmer strips. The samples were assayed against a freshly prepared calibration curve. The precision results for besifloxacin in buffer solution ($n = 6$) ranged from 1.2 to 1.6% and the accuracy ranged from −8.3 to 2.0%. The precision results for the stability of the drug on Schirmer strips for 2 h ranged from 1.8 to 1.9% and the accuracy ranged from −11 to −4.7%. Results from experiments to assess the 24-h stability in buffer and the 2-h stability on Schirmer strips at room

Table 3
Summary of calibration curve parameters for besifloxacin

Curve number	*A*	*B*	*C*	*R*-squared	LLOQ (ng/mL)	ULOQ (ng/mL)
1	6.58E-07	0.0143	0.000218	0.998777	2.00	2000
2	2.55E-07	0.0147	−0.000928	0.999207	2.00	2000
3	3.7E-07	0.0148	0.00109	0.999522	2.00	2000
4	3.7E-07	0.0149	0.000678	0.999247	2.00	2000
5	1.28E-07	0.013	0.00327	0.997472	2.00	2000
Mean	3.56E-07	0.0143	0.000866	0.999		
SD	1.96E-07	0.00078	0.00154	0.000813		
%CV	55.1	5.5	177.8	0.1		
n	5	5	5	5		

Regression footnote(s): Response = $A \times (\text{conc.} \times 2) + B \times \text{conc.} + C$.

Table 4
Intra- and inter-day accuracy and precision of besifloxacin

Accuracy and precision	QC samples	Theoretical concentration (ng/mL)	Measured mean concentration (ng/mL)	SD	Precision[a] %RSD	Accuracy[b] %bias	*n*
Intra-day	LLOQ	2.00	2.35	0.136	5.8	17.5	4
	Low	6.00	6.54	0.058	0.9	9.0	4
	Mid	1000	1140	33.7	3.0	14.0	4
	High	1500	1620	23.8	1.5	8.0	4
Inter-day	LLOQ	2.00	1.97	0.098	5.0	−1.5	18
	Low	6.00	5.77	0.206	3.6	−3.8	26
	Mid	1000	1020	33.8	3.3	2.0	26
	High	1500	1490	44.0	3.0	−0.7	26

[a] (SD/mean) × 100.
[b] [(Measured mean concentration − theoretical concentration)/theoretical concentration)] × 100.

Table 5
Summary of stability of besifloxacin under various storage conditions

Matrix stability at room temperature	At least 24 h for buffer and 2 h for Schirmer strips
Extract stability at room temperature	At least 74 h in substitute matrix and human tears
Freeze/thaw stability	Three cycles in buffer and Schirmer strips at approximately −20 °C
Long-term matrix stability	At least 51 days for buffer and 197 days for Schirmer strips at approximately −20 °C
Stock solution stability at room temperature	At least 6 h for besifloxacin and 2 h for sparfloxacin
Stock solution stability at −20 °C	At least 14 days for besifloxacin and 25 days for sparfloxacin

temperature showed that besifloxacin was stable under these conditions.

The stability of besifloxacin in extracted samples was determined by storing sets of processed quality control samples at room temperature for approximately 74 h prior to injection. The samples were assayed with a freshly prepared calibration curve. The precision for the QCs in the artificial tear matrix ranged from 1.4 to 3.8% and the accuracy ranged from −2.2 to 3% ($n = 6$). The precision for the QCs in human tears ranged from 0.9 to 6.5% and the accuracy ranged from 5.3 to 16% (16% for the LLOQ; $n = 4$).

Stability of besifloxacin and the IS in solution was assessed by comparing the mean peak area ratios of fresh standard solution to those of solutions held at −20 °C for 14 days for besifloxacin and 25 days for IS. Additionally, room temperature stability of besifloxacin and the IS in solution was assessed for a period of 6 h for besifloxacin and 2 h for IS.

Sets of quality control samples were stored at −20 °C for approximately 51 days for buffer and 197 days for Schirmer strips. The samples were then assayed along with a freshly prepared calibration curve and the results indicated that besifloxacin was stable for at least 197 days under these conditions.

3.8. Dilution study

Dilution integrity was determined using a QC sample that was diluted 100-fold with artificial tears into the calibration curve range. This QC had an accuracy and precision of −0.2% and 0.9%, respectively ($n = 6$). These results indicated that dilution of samples up to 100-fold with surrogate blank matrix does not compromise the assay.

3.9. Application of the method

The validated analytical method was used for the quantitation of besifloxacin in human tear samples collected during a clinical study with besifloxacin. Maximal besifloxacin tear levels after topical ocular administration of 0.6% besifloxacin were 610 ± 540 μg/g. The lowest besifloxacin concentrations (1.60 ± 2.28 μg/g) were observed in tear samples collected 24 h after dosing. A detailed description of the ocular pharmacokinetics of besifloxacin will be published elsewhere.

3.10. Conclusion

An LC/MS/MS method has been developed utilizing artificial tears for the quantitative analysis of besifloxacin in human tear samples with a lower quantitation limit of 2 ng/mL and a total chromatographic run time of 4 min. The current method was sensitive, reproducible, precise and accurate and was successfully applied to study the topical ocular pharmacokinetics of besifloxacin in healthy human subjects.

Acknowledgements

The authors wish to acknowledge and thank Raphaële Siou-Mermet and Pr Claude Dubray for overseeing all aspects of the clinical pharmacokinetic study.

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