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Analytical Chemistry Insights

A UPLC–MS Method for the Determination of Ofloxacin Concentrations in Aqueous Humor

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ABSTRACT: A rapid, simple, and specific method based on ultra performance liquid chromatography (UPLC) with mass spectrometry detection has been developed for quantitative analysis of ofloxacin in human aqueous humor using tobramycin as internal standard (IS). Chromatographic separation was achieved on a Waters Acquity UPLC BEH C18 Shield column (150 × 2.1 mm, 1.7 µm) eluted with 95:5 water: acetonitrile (v/v) containing 0.1% formic acid and a flow rate of 0.3 mL/minute. The total analysis time was three minutes with ofloxacin eluting at 1.67 ± 0.03 minutes. The linearity of the method ranged from 0.1 to 8 µg/mL with $r^2 = 0.998$. The method was validated according to FDA guidelines with respect to linearity, accuracy, precision, specificity, and stability. The limits of detection and quantification were 0.03 and 0.10 µg/mL, respectively. The developed method was successfully applied to the analysis of samples that have been obtained from patients.

KEYWORDS: UPLC, ofloxacin, mass spectrometry, aqueous humor

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Introduction

Fluoroquinolones structurally belong to the nalidixic acid family. They are broad spectrum synthetic antimicrobial agents that inhibit topoisomerase II (DNA gyrase) and topoisomerase IV[1,2] enzymes required for bacterial DNA replication. Ofloxacin is a second generation fluoroquinolone with enhanced antimicrobial activity against aerobic Gram-negative and positive bacteria.[3] It is a racemic mixture, which consists of 50% levofloxacin and 50% of its "mirror image" or enantiomer, dextrofloxacin. Ofloxacin is one of the most commonly used topical fluoroquinolones because of its high potency, high stability, low toxicity, low allergenicity, high intrinsic solubility, low minimal inhibitory concentration, and long half life.[4] There are several routes of administration of ofloxacin: oral, intravenous, and eye drops. Ofloxacin is used for the treatment of ocular infections and is commonly applied in several ophthalmic surgeries, pre- or postoperatively.

Many methods describing the determination of ofloxacin concentration in biofluids have been reported; HPLC with fluorescence or UV detection is the technique most commonly used.[5,6] LC-MS/MS has been used for the sensitive determination of ofloxacin in human plasma;[7] the authors achieved sensitivity of 78 ng/mL. Ofloxacin concentration has been determined in aqueous humor by LC-UV or fluorescence detection;[4,8–10] but with no mass detector. However, the high selectivity and sensitivity of mass spectrometry coupled with the rapid analysis offered by ultra performance liquid chromatography (UPLC) has not been exploited for analysis and quantification of ofloxacin in aqueous humor.

The objective of this study was to develop and validate a UPLC–ESI-MS analytical method for the quantification of ofloxacin in aqueous humor. Validation was conducted based on FDA international guidelines for bio-analytical assay validation.[11]

Experimental

Materials and reagents. High purity ofloxacin and tobramycin (Fig. 1) were purchased from Sigma-Aldrich, Germany. UPLC-grade solvents; Acetonitrile, methanol, and formic acid were purchased from Acros Organics, USA. Ultrapure water was supplied by a Milli-Q, Malva purification system.

Apparatus. The chromatographic equipment consisted of a Waters Acquity UPLC-ESI-MS system with a Waters 2966 Photodiode Array detector (PAD) coupled to a Waters Micromass ZQ mass spectrometer, a binary solvent delivery pump, an autosampler with cooling device, and a thermostatic column compartment. The column was an Acquity UPLC BEH Shield C18 (1.7 μm, 2.1 × 150 mm). The software packages for data acquisition and handling were the Waters Empower and the Waters Mass Lynx 4.1.

Chromatographic conditions. The mobile phase consisted of a mixture of A: 0.1% aqueous formic acid and B: 0.1% formic acid in acetonitrile, delivered at a constant flow rate of 0.30 mL/minute. Separation was achieved by isocratic elution under the following conditions: 95% solvent A and 5% solvent B for three minutes at a flow rate of 0.30 mL/minute. Column temperature was kept at 30°C, whereas sample temperature was 10°C. The monitoring wavelength was 230 nm, in which the analyte shows the optimum absorbance. All mobile phases were filtered through a 0.22-μm Millipore filter (durapore membrane filter). The injection volume was 10 μL. After each injection, the sample manager underwent a needle wash process with methanol.

Mass spectrometric conditions. A single-quadrupole Quattro micro mass spectrometer (ACQUITY SQ Detector) equipped with an electrospray ionization (ESI) interface was used for analytical detection. ESI-MS was operated in positive mode under the following operating parameters: capillary voltage 3.5 kV, cone voltage 30 V, source temperature 140°C, desolvation temperature 250°C, desolvation gas (nitrogen) 500 L/hour, and cone gas (nitrogen) 50 L/hour. All data were acquired and processed using Masslynx 4.1 software (Waters Corp., MA, Milford, USA).

Collection of aqueous humor samples from patients and preparation. The aqueous humor samples (approximately 50 μL) were aspirated from the anterior chamber of the eye by puncturing the anterior chamber using a 30 G needle connected to a tuberculin syringe. Aqueous humor samples were collected and frozen within one hour at −70°C, protected from light, until analysis.

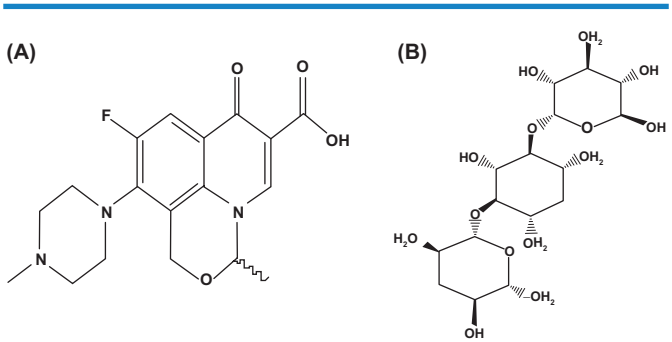

Figure 1. Structure of ofloxacin (**A**) and tobramycin (**B**).

Frozen aqueous humor samples were thawed at room temperature and subjected to protein precipitation as follows: Humor aliquots of 50 and 10 μL of the appropriate internal standard (IS) working solution were added to a 1.5 mL Eppendorf tube and the mixture was vortexed for three minutes. Then, 300 μL of a methanol: acetonitrile (1:2, v/v) mixture was added and the mixture was vortexed for three minutes followed by centrifugation at 10,000 rpm for 8 minutes at 15°C. The supernatant was transferred to a clean Eppendorf tube and evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 100 μL of the mobile phase, vortexed for one minute, centrifuged again under the same conditions, transferred into plastic autosampler vials with preslit septa, and 10 μL was injected into the UPLC–ESI-MS system.

Preparation of standard solutions and quality control (QC) samples. Primary stock solution of ofloxacin and IS was prepared by dissolving appropriate amount of ofloxacin and IS in diluent (water). The calibration curve was constructed using water due to the limited availability of blank aqueous humor.[12,13] These solutions were further diluted to obtain working standard solutions (0.1–8 μg mL^{-1}) in duplicate. The QC samples were prepared with blank aqueous humor. The LOQ, low, medium, and high concentrations of QC samples were 0.1, 0.4, 3, and 7 μg mL^{-1}.

Clinical application. The validated UPLC–ESI-MS method that has been developed was applied to quantify the aqueous humor concentration of ofloxacin after topical administration. Fifty-seven diabetic study subjects, aged 60–80, programmed for cataract surgery, were randomly selected to form three groups. Group A consisted of 19 patients who were assigned to receive four drops of topical ofloxacin 0.3% (Exocin®) in two hour intervals, one day before surgery. Group B (19 patients) were administrated four drops of ofloxacin (Exocin®) one hour before surgery, while Group C (19 patients) received eight drops topical ofloxacin, four drops the day before the surgery in two hour intervals and four drops one hour before cataract surgery, instilled at intervals of 15 minutes. In Group B and Group C aqueous humor samples were collected after 45 minutes of the last eye drop administration. The study has been approved by the Ethics Committee of the University of Patras for human research. Written informed consent was obtained from all the study subjects.

Results and Discussion

Mass condition and chromatographic condition optimization. MS analytical parameters were carefully optimized for the determination of ofloxacin in aqueous humor and of tobramycin, the IS in the specific chromatographic method. The mass spectrometer was tuned in positive ionization mode because these pharmaceutical compounds produce much stronger signal in positive ion mode than in negative ion mode,

owing to the presence of nitrogen atoms in their structure. In the precursor ion full-scan spectra, the most abundant ions were protonated molecules $[M + H]^+$ m/z 362.16 $[M + H\text{-}CO_2]^+$ m/z 318.11 and $[M + H\text{-}CO_2\text{-}C_3H_7N]^+$ m/z 261.13 for ofloxacin and $[M + H]^+$ m/z 468.17 [M + H-(amino-alpha-D-glucopyranose)]$^+$ m/z 324.16 for tobramycin. The variety of mass conditions so as to gain the maximum abundance of the analyte's ions was examined. All the mass spectrometric parameters in positive ion mode, such as capillary and cone voltage, ESI source and desolvation temperature, and flow rate of desolvation and cone gas were further adjusted in order to obtain the optimal intensity of protonated molecules $[M + H]^+$ of the analyzed compounds.

Various isocratic as well as gradient mobile phases in various volume ratios of the elution system were evaluated for chromatographic behavior, sample throughput, and the ionization responses of ofloxacin and the IS. Two different mixtures for mobile phase were tested. The first one consisted of water (0.1% formic acid) as solution A and methanol (0.1% formic acid) as solution B. In the second one, methanol was replaced by acetonitrile. The ratio for isocratic elution was at first 70% A: 30% B and then the solution B was reduced by 5% each time. Similarly, the starting ratio for the gradient elution was 70% A: 30% B.

The isocratic elution system with conditions of 95% water (0.1% formic acid): 5% acetonitrile (0.1% formic) indicated the best response and peak shape (Fig. 2). The overall chromatographic run time was completed within three minutes at a flow rate of 0.3 mL/minute.

Method validation.

Selectivity. Selectivity was studied by comparing chromatograms of six different drug-free samples of aqueous humor from six subjects with those of corresponding calibration standard humor samples spiked with ofloxacin and IS. All blank aqueous humor samples that were analyzed under the same conditions, were found to be free of interference peaks from aqueous humor ingredients at the t_R of the analyte and the IS, demonstrating the selectivity of the assay.

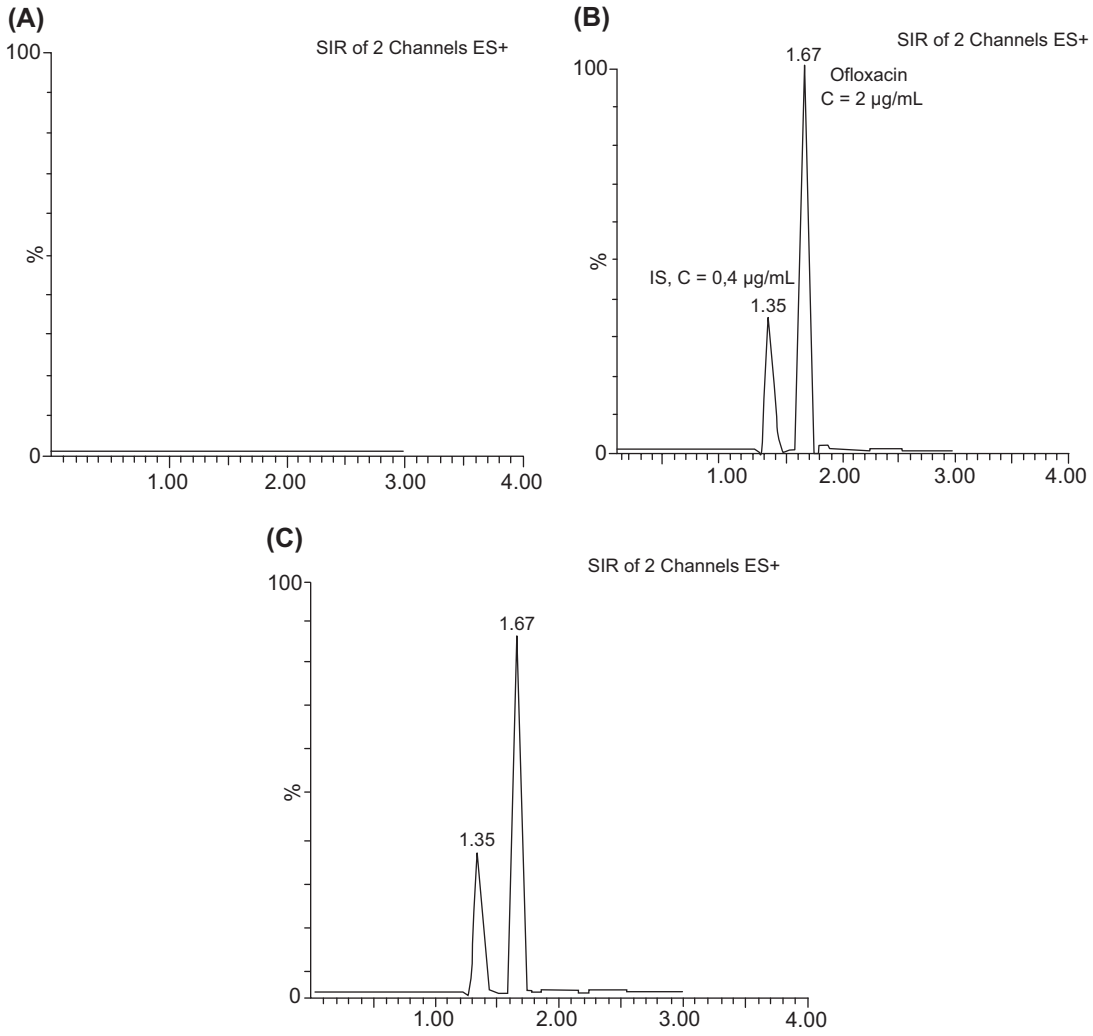

Figure 2. Representative UPLC–ESI-MS chromatograms of ofloxacin and the IS. (**A**) Blank aqueous humor, (**B**) aqueous humor spiked with ofloxacin (2 µg mL^{-1}) and IS (0,4 µg mL^{-1}) (**C**) aqueous humor sample collected after four drops administration of topical ofloxacin (SIR: 362.84 and 468.46 ofloxacin and IS respectively). *Y*-axis shows the % relative intensity and *X*-axis the elution time. The t_R for ofloxacin and IS are 1.67 and 1.35 minutes, respectively.

Table 1. Calibration equation parameters for the determination of ofloxacin in aqueous humor by the developed UPLC-ESI-MS methodology.

CALIBRATION EQUATION	Y = 0.9742*X + 0.3566
Slope	0.9742
Intercept	0.3566
Correlation coefficient (r)	0.999
LOD (μg/mL)	0.03
LOQ (μg/mL)	0.1
Range (μg/mL)	0.1–8

Calibration model and LLOQ. Calibration curve construction was based on the analysis of the calibration standards ($n = 6$) at eight concentration levels ranging from 0.1 to 8 μg mL^{-1} (0.1, 0.5, 0.8, 1, 2, 4, 6 and 8 μg mL^{-1}) and plotting the peak area ratios of ofloxacin to IS against the nominal calibration standard concentration. Two different samples were prepared at each concentration and each sample was analyzed four times. Four reference curves in four different days were constructed and equations of the straight lines ($y = ax + b$), the slope (a, slope), the ordinate on the start (b, Y-intercept), the coefficient of determination r^2, and standard deviation of the intercept on the top (SD, Y-intercept) were calculated. The results are presented in Table 1. The correlation coefficient (r) of the calibration curve was 0.9988, indicating good correlation whereas the equation of the fitted model is shown in Table 1.

Accuracy and precision. The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value/concentration of the analyte. Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. Accuracy of the developed method is determined by conducting recovery experiments. The precision of an analytical method expresses the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. For the determination of intra-day precision and accuracy, samples were spiked with the analyte and the IS at four different levels. Those were analyzed with six replicates for each level, whereas analysis on three different days provided data for the determination of inter-day precision and accuracy. According to the FDA guidance, the precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV. The accuracy and precision data are presented in Table 2.

Recovery-matrix effect (ME). The % relative recovery (R%) of ofloxacin was tested and evaluated in comparison with five replicates of QC samples at the three QC concentration levels (0.1, 0.4 and 7 μg mL^{-1}) for ofloxacin and at 0.4 μg mL^{-1} for the tobramycin with those of post-extraction spiked humor samples. The recovery data are presented in Table 3.

In order to evaluate the ME on the ionization of the analyte, the potential suppression or enhancement due to humor components, five aliquots of blank aqueous humor were processed according to the sample pretreatment procedure and then reconstituted with suitable volumes of ofloxacin working solution at the four concentration levels and tobramycin, as above. The corresponding peak areas of the samples, (A), were compared with those of equivalent concentrations of the analyte and IS working standard solutions in the mobile phase (B). The extraction recoveries of ofloxacin from QC samples were 96.2, 95.4, and 92.9% at low, medium, and high concentrations, 0.1, 0.4 and 7.0 μg mL^{-1}, respectively, whereas the recovery was 93.3% for tobramycin as IS. In terms of ME, all the ratios defined above, were within the acceptable limits (85–115%). No significant ME for ofloxacin was observed, indicating that ion suppression and enhancement from aqueous humor were negligible for this method. The results are tabulated in Table 3.

Stability. Analyte stability in aqueous humor was studied by repeated analysis (five replicates) of QC samples at two concentration levels (low and high) after various storage and handling conditions. Short-term stability was assessed at room temperature for 12 hours, whereas long-term stability was evaluated at −70°C for 20 days. Freeze–thaw stability was evaluated after three freeze–thaw cycles, which consisted of storage at −35°C for a minimum of 18 hours, followed by thawing at room temperature. Finally post-preparative stability was assessed by analyzing pre-treated QC samples stored in the autosampler at 10°C for 20 hours. The results from the stability tests are presented in Table 4, and indicated

Table 2. The accuracy and precision for the ofloxacin analysis in aqueous humor.

LEVEL	ADDED CONCENTRATION (μg mL^{-1})	FOUND CONCENTRATION (μg mL^{-1})	SD	ACCURACY, RE (%)	INTRA-DAY *CV	INTER-DAY *CV
LLOQ	0.1	0.094	0.003	−6.0	3.2	9.1
LQC	0.4	0.457	0.026	14.3	5.7	5.6
MQC	3	3.29	0.13	9.6	4.0	9.5
HQC	7	7.41	0.26	5.9	3.5	8.6

*Intra-day: $n = 6$; inter-day: $n = 6$ series per day within three days.

Table 3. The recovery and ME of ofloxacin.

STATISTICAL VARIABLE	NOMINAL CONCENTRATION (μg mL^{-1})			
	OFLOXACIN			TOMBRAMYCIN
(μg mL^{-1})	0.1	0.4	7.0	0.4
Mean R (%) ± SD	96.2 ± 7.2	95.4 ± 7.9	92.9 ± 5.9	93.3 ± 5.0
Mean ME (%) ± SD	96.8 ± 2.8	95.6 ± 2.1	92.4 ± 4.4	94.9 ± 3.1

Table 4. The stability of ofloxacin in aqueous humor at low and high QC levels ($n = 6$).

STORAGE	NOMINAL CONCENTRATION (μg mL^{-1})	MEAN VALUE (μg mL^{-1})	SD	CV	% NOMINAL
Freeze–thaw stability	0.4	0.42	0.035	8.4	105
	7	7.5	0.43	5.7	107
Long-term stability	0.4	0.43	0.042	9.8	108
	7	7.6	0.50	6.6	109
Short-term stability	0.4	0.39	0.036	9.3	97
	7	6.8	0.41	6.0	97
Post-preparative stability	0.4	0.43	0.033	7.6	108
	7	7.3	0.30	4.1	104

that ofloxacin could be characterized as stable under routine laboratory conditions. The method is therefore proved to be applicable for routine analysis.

Clinical application. The validated UPLC-ESI-MS method was successfully applied to the analysis of clinical samples obtained after topical administration of ofloxacin. The mean aqueous humor concentration of ofloxacin is shown in Table 5.

As it is figured out from Table 5, the mean value in group C is statistical significantly higher than the other two groups ($P < 0.001$, ANOVA test with Bonferroni correction).

The results showed that patients in group A and B who were assigned to receive ofloxacin four times one day or one hour prior to surgery achieved similar aqueous humor antibiotic levels. However, patients in group C who received a repeated ofloxacin regimen (one day and one hour prior to surgery) yielded an aqueous humor antibiotic concentration approximately 1.7 times higher than those in patients of group A and B.

Table 5. The mean concentration of ofloxacin in aqueous humor samples.

PATIENTS ($n = 57$)	OFLOXACIN (μg mL^{-1})		
	MEAN	SD	*P**
Group A	1.099	0.273	
Group B	1.078	0.303	
Group C	1.797	0.314	<0.001

*ANOVA test.

Conclusion

The described UPLC-ESI-MS validated analytical methodology enables the rapid and selective assay of ofloxacin in aqueous humor. This is the first application of UPLC-MS for the determination of ofloxacin in aqueous humor and the method is equally sensitive as LC-fluorescence applied in aqueous humor[9] or LC-MS/MS determination in plasma.[7] Furthermore, the developed method is advantageous in its high speed (analysis time of 3 minutes) and the selectivity. It can be automated and is completely validated according to FDA guidelines with satisfactory data for all the parameters under scrutiny. We also describe the application of the UPLC-ESI-MS method to the analysis of clinical samples. Therefore, the developed UPLC-ESI-MS method allows the reproducible sensitive measurement of ofloxacin with improved throughput in aqueous humor and can be applied to studies of bioavailability of ofloxacin in the anterior chamber of the eye.

Author Contributions

Conceived and designed the experiments: CG, PP. Analyzed the data: ML, OM. Wrote the first draft of the manuscript: PP, CA. Contributed to the writing of the manuscript: PP, CA, OM, ML. Agree with manuscript results and conclusions: PP, CA, OM, ML, CD. Jointly developed the structure and arguments for the paper: ML, CD. Made critical revisions and approved final version: PP, CA, OM, ML, CD.

DISCLOSURES AND ETHICS

As a requirement of publication the authors have provided signed confirmation of their compliance with ethical and legal obligations including but not limited to compli-

ance with ICMJE authorship and competing interests guidelines, that the article is neither under consideration for publication nor published elsewhere, of their compliance with legal and ethical guidelines concerning human and animal research participants (if applicable), and that permission has been obtained for reproduction of any copyrighted material. This article was subject to blind, independent, expert peer review. The reviewers reported no competing interests.

REFERENCES

1. Lesher GY, Froelich EJ, Gruett MD, Bailey JH, Brundage RP. 1,8-Naphthyridine derivatives: a new class of chemotherapeutic agents. *J Med Pharm Chem*. 1962;91:1063–1065.
2. Gellert M, Mizuuchi K, O'Dea MH, Nash HA. DNA gyrase: an enzyme that introduces superhelical turns into DNA. *Proc Natl Acad Sci U S A*. 1976;73(11):3872–3876.
3. Andriole CL, Andriole VT. Are all quinolones created equal? *Mediguide Infect Dis*. 2001;21:1–5.
4. Giamarellou H, Kanellas D, Kavouklis E, Petrikkos G, Gabriel L. Comparative pharmacokinetics of ciprofloxacin, ofloxacin and pefloxacin in human aqueous humour. *Eur J Clin Microbiol Infect Dis*. 1993;12(4):293–297.
5. Macek J, Ptácek P. Determination of ofloxacin in human plasma using high-performance liquid chromatography and fluorescence detection. *J Chromatogr B Biomed Appl*. 1995;673(2):316–319.
6. Wongsinsup C, Taesotikul W, Kaewvichit S, Sangsrijan S, Sangsrijan S. Simple extraction and determination of ofloxacin in human plasma by high-performance liquid chromatography with fluorescence detector. *CMU J Nat Sci*. 2009;8(2):165–174.
7. Meredith SA, Smith PJ, Norman J, Wiesner L. An LC-MS/MS method for the determination of ofloxacin in 20 µl human plasma. *J Pharm Biomed Anal*. 2012;58:177–181.
8. Yalvac IS, Basci NE, Bozkurt A, Duman S. Penetration of topically applied ciprofloxacin and ofloxacin into the aqueous humor and vitreous. *J Cataract Refract Surg*. 2003;29(3):487–491.
9. Basci NE, Hanioglu-Kargi S, Soysal H, Bozkurt A, Kayaalp SO. Determination of ofloxacin in human aqueous humour by high-performance liquid chromatography with fluorescence detection. *J Pharm Biomed Anal*. 1997;15(5):663–666.
10. Beck R, van Keyserlingk J, Fischer U, Guthoff R, Drewelow B. Penetration of ciprofloxacin, norfloxacin and ofloxacin into the aqueous humor using different topical application modes. *Graefes Arch Clin Exp Ophthalmol*. 1999;237(2):89–92.
11. Guidance for Industry. *Bioanalytical Method Validation*. Rockville, USA: Department of Health and Human Services, Food and Drug Administration; 2001.
12. Keski-Rahkonen P, Pärssinen O, Leppänen E, Mauriala T, Lehtonen M, Auriola S. Determination of tamsulosin in human aqueous humor and serum by liquid chromatography-electrospray ionization tandem mass spectrometry. *J Pharm Biomed Anal*. 2007;43(2):606–612.
13. Schwartz M, Kline W, Matuszewski B. Determination of a cyclic hexapeptide (L-743 872), a novel pneumocandin antifungal agent in human plasma and urine by high-performance liquid chromatography with fluorescence detection. *Anal Chim Acta*. 1997;352:299–307.