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Quantification of Ofloxacin in Human Aqueous Humour of the Eye by LC-MS-MS

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Abstract

A reliable, repeatable and a sensitive analytical method was developed and validated for the quantification of ofloxacin (a polar molecule) in human Aqueous Humour (AH) of the eye. The chromatography analysis was performed in tandem mass spectrometry (LC- ESI-MS/MS). Using Synergy Hydro PR column polar endcapping (mainly via H-bonding) interacts with polar compounds resulting in high retention. The method was validated over a linear concentration range of 0.1-100.0 ng/mL with the R^2 value being higher than 0.999. The Lower Limit of Quantification (LLOQ) for ofloxacin was quantified at 0.05 ng/mL with sufficient specificity, accuracy, and precision. Both the precision (Coefficient of Variation (CV); 4.28%) and accuracy (Relative Error (RE); 4.46%) were within acceptable criteria of<15%. The chromatographic run time was short within 7 minutes, while the recovery was found ≥100%. The analytical method was successfully validated for the stability of ofloxacin in human AH at various storage conditions (room temperature, fridge, freezer, and freeze-thaw). Furthermore, the developed method was applied to quantify the level of ofloxacin in twenty-one human AH samples as collected intraoperatively from the anterior chamber of patients undergoing cataract surgery following topical application. The mean concentration of ofloxacin was measured at 644.33 ± 1.16 ng/mL.

Keywords: Ofloxacin; Ophthalmology; Human aqueous humour; LC–MS/MS; Method validation

Abbreviations: AH, aqueous humour; CSs, calibration standards; CV, coefficient of variation; EMA, European Medicines Agency; ESI- MS/MS, electrospray ionization tandem mass spectrometry; FDA, Food and Drug Administration; HPLC, high-performance liquid chromatography; IS, internal standard; LC-MS/MS, liquid chromatography-tandem mass spectroscopy; LLOQ, lower limit of quantification; LLOD, lower limit of detection; MIC, minimum inhibitory concentration; MRM, multiple reaction mode; WS, working solutions; QC, quality control; RE, relative error; PP, protein precipitation.

Introduction

Ofloxacin (±)-9-fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H-pyrido [1,2,3-de] [1,4] benzoxazine-6-carboxylic acid) is a fluoroquinolone antibiotic, which structurally belongs to the nalidixic acid family, and is a racemate of two enantiomers, (R)-(+)-ofloxacin and (S)-(-)-ofloxacin (levofloxacin). The molecule is characterized by its tricyclic structure and a methyl group at the C-3 position in the oxazine ring, resulting in an asymmetric center at this position [1,2] (Figure 1).

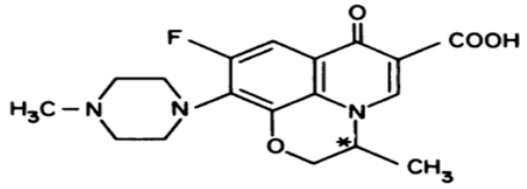

Figure 1: Structure of Ofloxacin Asterisk identifies an asymmetric carbon in ofloxacin, the S-enantiomer of which (methyl group projecting above the page) is DR-3355 (789).

Therapeutically ofloxacin is being used in its racemate form and, since 1998, additionally as the enantiopure S-(-) isomer [3]. It acts by inhibiting topoisomerase II (DNA gyrase) and topoisomerase IV, both required for bacterial DNA replication, and exhibits a wide spectrum of antibacterial activity against both gram-positive and gram-negative bacteria, particularly the *Enterobacteriaceae*, methicillin-sensitive *Staphylococcus epidermidis*, and *Staphylococcus aureus* at minimum inhibitory concentrations below to 0.5 mg/L [1, 4]. However, resistance of *S. aureus* against ofloxacin is constantly increasing. Higher concentrations of the antibiotic are required to inhibit *Streptococcus pneumoniae* and *Pseudomonas aeruginosa*. Although is poorly active against anaerobes, some species are susceptible or moderately susceptible (e.g. *Peptostreptococcus* species, *Bacteroides melaninogenicus*, *Clostridium perfringens*, *Veillonella* species, *Mobiluncus* species). *Ureaplasma urealyticum* and *Mycoplasma* species show moderate susceptibility, while *Chlamydia trachomatis* is susceptible [5-7]. Its low toxicity, high stability, high potency, high intrinsic solubility, low allergenicity, low minimal inhibitory concentration, long half-life,

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and lipophilic properties for better intraocular uptake than previous antibacterial agents have rendered ofloxacin one of the most commonly used topical fluoroquinolones for the treatment of ocular infections and is commonly applied, pre- or postoperatively, in numerous eye surgeries [5,6,8]. Ofloxacin's therapeutic value after topical application depends on whether its concentration at the site of action is higher than the Minimum Inhibitory Concentration (MIC) for the infective pathogens. Hence, evaluation of its intraocular penetration is essential to ensure the effectiveness of the followed regimen [9].

Human Aqueous Humour (AH) is an important transparent intraocular fluid (98% water), that fills both the anterior and the posterior chambers of the eye and it is formed by the ciliary body, a structure supporting the lens. In human, the total volume of AH is approximately 250 µL. All plasma proteins are present in human AH although in very low concentration (from 0.05 g/L to 0.15 g/L) to preserve its optical transparency. Furthermore, it consists of a high concentration of ascorbic acid, glucose, glutathione, and electrolytes (calcium, sodium, phosphate, potassium, bicarbonate, magnesium, chloride) [10]. Anatomical structures surrounding human AH render its collection an invasive procedure, which is usually possible intraoperatively using specific techniques to avoid harming the eye [11].

In this study, a validated chromatographic methodology based on High-performance Liquid Chromatography (HPLC) linked to electrospray ionization tandem mass spectrometry (ESI-MS/MS) was developed. The developed method was used to measure ofloxacin's levels in human AH samples aspirated intraoperatively from the anterior chamber of patients undergoing cataract surgery following topical application. Accurate *in vivo* quantification of ofloxacin in human AH of the eye facilitates evidence-based therapeutical approach in ophthalmology.

Materials and Methods

Chemicals and reagents

Ofloxacin, acetonitrile, formic acid, and methanol (HPLC grade) were all obtained from Sigma-Aldrich, Germany. Ciprofloxacin (≥ 98%) was used as an Internal Standard (IS) obtained from (Merck®, Germany). A Milli Q-Integral system (Merck-Millipore, Watford, UK) was used to purify water (18.2 MΩ).

Instrumentations

Ofloxacin analysis was performed on a Waters HPLC system (Alliance HT 2795) equipped with a temperature-controlled auto-sampler and a degasser. Chromatographic separation was achieved in a Synergi Hydro-RP column 100 × 2 mm, 4 µm, Proguard 2 mm to 8 mm (Phenomenex, Washington, USA), using a mobile phase consisting of (solvent A) 0.1% formic acid in aqueous and (solvent B) acetonitrile. The column temperature was preserved at 40°C throughout all the experiments, while the sample temperature in the autosampler tray was maintained at 15°C.

A linear gradient condition was applied, initiating at 10% of solvent B maintained for 0.5 min gradually, the elution was augmented to 90% of solvent B for the following 1 min, and then immediately decreased to 10% of solvent B until 1.5 minutes, and these conditions were maintained till the end of the run (7 minutes). For sample analysis 50 µL was injected, arising the immediate commence of the gradient.

For the detection of ofloxacin, Micromass Quattro Micro tandem MS system (Waters, Milford, MA, USA), was used.

MS system was set in the positive ion mode, operating the electrospray ion source, while its settings were ameliorated in the following manner: desolvation temperature, 400°C; source temperature, 100°C; desolvation gas flow, 500 L/h; and collision gas (argon) flow, 50 L/h.

The capillary voltage was set at 3.5 kV and the multiplier at 650 V, while the cone voltage values and collision energy for both Ofloxacin and IS were set at 33V and 16eV, correspondingly. Moreover, both Ofloxacin and IS detection was performed operating the multiple reaction mode (MRM) scan, the selected transitions m/z were →362.14>318.11 and 332.12>288.15, correspondingly (Figures 2-4). Lastly, data processing was carried out by MassLynx v.4.0 software.

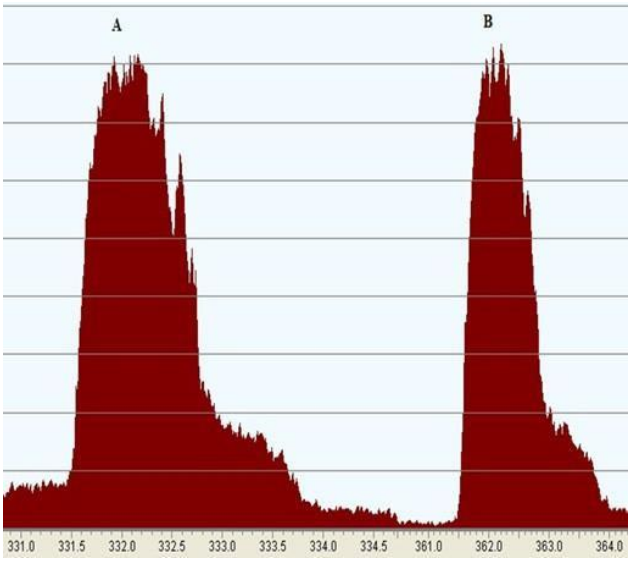

Figure 2: Representative chromatograms of Ms scan transitions of (A) ciprofloxacin at 332.12 [M+H]+1 and (B) ofloxacin at 362.14 $[M+H]^{+1}$.

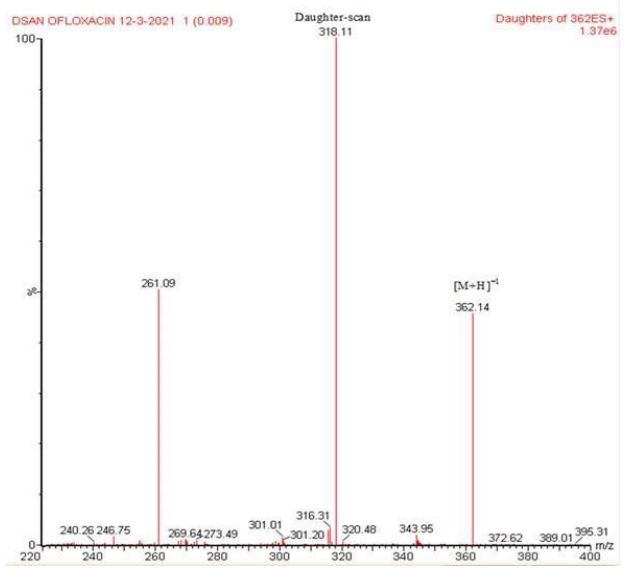

Figure 3: Representative chromatogram of Multiple-Reaction Monitoring (MRM) transitions of ofloxacin (362.14 $[M+H]^{+1}$).

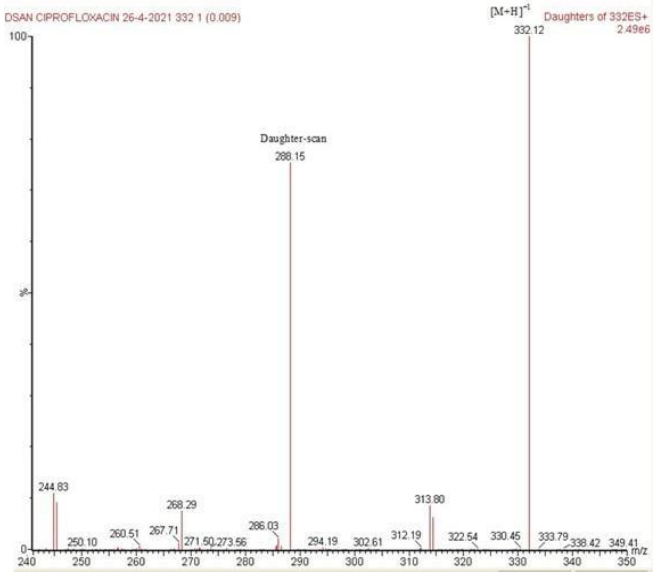

Figure 4: Representative chromatogram of Multiple-Reaction Monitoring (MRM) transitions of ciprofloxacin (332.12, $[M+H]^{+1}$).

Stock solution preparation

Stock solution of ofloxacin and IS (1 mg/mL) were separately prepared in the solvent mixture of acetonitrile-water (50:50, v/v). Moreover, Working Solutions (WS) and Quality Control samples (QC) of ofloxacin were prepared by consecutive dilution of the corresponding stock solution with the solvent mixture.

Calibration samples and quality control samples preparation

Calibration Standards (CSs) of ofloxacin ranging from 0.1-100 ng/mL were prepared by adding 10 µL of each known WS (ranging from 0.01-10 ug/mL) of ofloxacin, with 10 µL of IS solution to 30 µL of drug-free human AH. The quality control samples (WS of 0.04, 4 and 8 ug/mL) were prepared in the same way to the calibration standard at three concentration levels: low, mid and high (0.4, 40 and 80 ng/mL) for ofloxacin. To ensure complete mixing the samples were vortexed and therefore, all the stocks, working solutions, and QC solutions were stored at -20°C. Moreover, the repeatability of the method was verified using five replicates of QC samples were extracted with the calibration standards on the day of the analysis.

Sample preparation and extraction procedure

For samples preparation process protein precipitation extraction method was used, where 300 µL of acetonitrile was added to 50 µL (30 µL of AH+10µL from WS of ofloxacin +10 µL from WS of IS) of each spiked calibration standard or QC sample. Followed by vortexing the solutions for 1 min and then by centrifuging at 10000 g, for 5 minutes. The supernatant of the solution was transferred to a new Eppendorf for drying on a Centrivap Cold Trap concentrator (Labconco, USA). Reconstitution of dried extracts was performed in solvent A (1000 µL). Subsequently, Regenerated Cellulose (RC) 0.22 µm filters (Phenomenex, Torrance, US) were used for filtering purposes. 50 µL of the sample was introduced into the system of chromatography LC-MS/MS.

Deep frozen patient samples were thawed at room temperature, vortexed and then 30 µL of each sample was spiked with 10 µL of IS. The same extraction procedure as described above was used for the preparation of the patient samples.

Method validation

The bioanalytical method validation was based on the guidelines of the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA) [12,13] for (a) linearity, (b) precision, (c) accuracy, (d) matrix effect, and (e) stability.

Stability studies of ofloxacin in human AH were evaluated with spiked samples at concentration levels of low, medium, high and at LLOQ at the following conditions: (a) after remaining for a week at room temperature (b) after remaining for a week in the refrigerator (4-8°C), (c) after three freeze–thaw cycles (freeze at -20°C and thaw at room temperature), and (d) after remaining for 6 months at -20°C.

Patient samples

Twenty-one patients undergoing cataract surgery, comprised of 12 male and 9 female, with a mean age of 72.5 years ± 7.95 (SD) (aged from 57 to 85), were assigned to receive one drop of ofloxacin 0.3% (Exocin®, Allergan Pharmaceuticals) four times at intervals of 15 min. The eye drops were applied in the middle of the inferior lower fornix. Patients who missed any of the 4 doses were excluded from the study. AH was collected after 1 hour of the last administration, intraoperatively, at the beginning of cataract surgery using a syringe attached to a 27-gauge cannula through a partial-thickness corneal cataract incision. Approximately 100 µL of AH was withdrawn, and were frozen at -70°C. Exclusion criteria were chronic topical ocular treatment, presence of exfoliation material within the anterior segment of the eye, other ocular pathology than cataract, renal or hepatic failure, diabetes mellitus, systemic hypertension, allergy to antibiotics and other local or systematic antibiotic treatment. The study has been approved by the Ethics Committee of the University of Patras for human research. Written informed consent was obtained from all study subjects.

Results

Chromatographic conditions: Optimization

Several parameters were checked to achieve optimization of chromatographic conditions (column type, flow rate, mobile phase, and temperature) with the aim to acquire good peak shape, high resolution, and a short running time. The finest separation results were established by the Phenomenex Synergi Hydro-RP column (in comparison to the Kinetex C8, the Germini-NX C18, and the Jupiter Proteo 90 A column) and operated at temperature of 40°C. A mobile phase was delivered at flow rate of 0.3 mL/min and composed of 0.1% formic acid in aqueous (solvent A) and acetonitrile (solvent B) was found to be appropriate for the separation of the ofloxacin. The highest ofloxacin recovery ≥ 100% from human AH was achieved using protein precipitation method with 0.1% formic acid in acetonitrile. Furthermore, ciprofloxacin was found to be the finest selection for the quantification of ofloxacin in human AH, as it provides good chromatographic conditions and similar extraction recovery. It is chemically similar to ofloxacin (both second-generation fluoroquinolones) and is not expected to be naturally present in human AH. Both contain same functional groups, boiling points, activity, similar molecular masses (331.347 vs. 361.373 g/mol), similar retention time and derivatization. Furthermore, ciprofloxacin is stable and does not interfere with the human AH components. Moreover, the retention time of ofloxacin and ciprofloxacin was 3.27 and 3.35 minutes correspondingly (Figure 5), while the mean Standard Deviation (SD) of all measurements was ≤ 1.16.

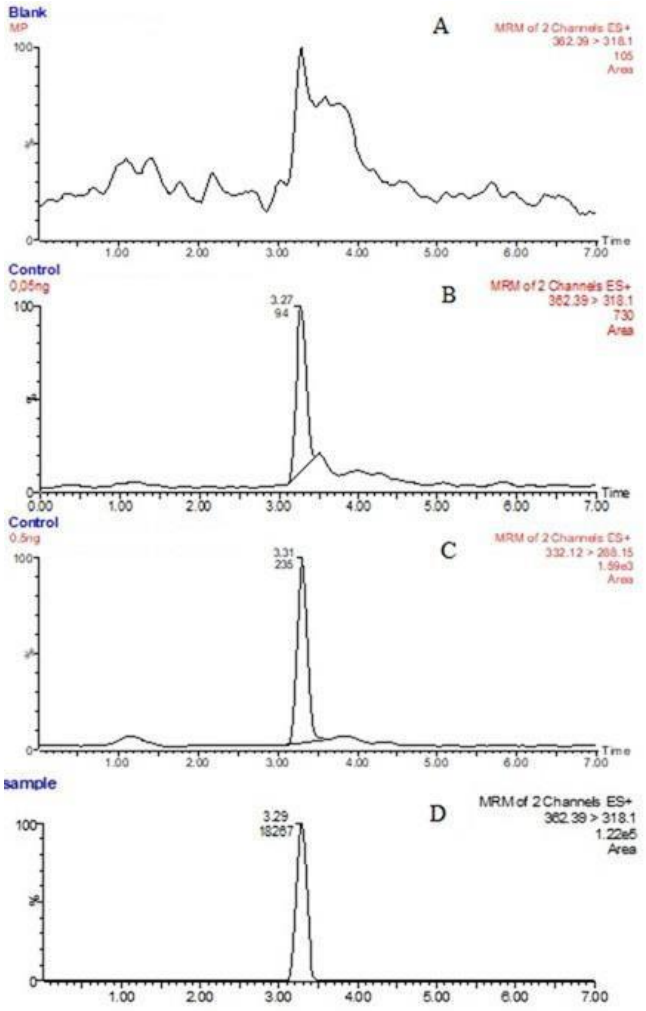

Figure 5: Representative chromatograms of: A) drug-free human AH, B) human AH spiked with 0.05 ng/mL of ofloxacin at LLOQ, C) human AH spiked with 0.5 ng/mL of ciprofloxacin as internal standard at LLOQ and (D) ofloxacin in human AH of indicative patient sample.

Validation of the method

Calibration curves and sensitivity: Calibration curves of ofloxacin were assessed based on the Internal Standard (IS) method by plotting peak area ratio of cited drug versus to the IS. The constructed calibration curves were found to be linear over the range of 0.1-100 ng/mL as shown (Table 1). The regression equation of ofloxacin was also computed and the correlation coefficient was found ≥ 0.9997.

Concentration (ng/mL)	Line equation	r2	LLOD (ng/mL)	LLOQ (ng/mL)
0.1-100.0	Y=995.83X-2054.4	0.9985	0.017	0.05

Table 1: Linear regression equation data of ofloxacin spiked in human AH (n=5)

Sensitivity of the developed method was measured using the lower limit of quantification (Lowest Standard Level (LLOQ) compared to its peak area of blank human AH sample in Figure 5. Taking into consideration, the peak area of blank samples ought to be less than 20% of the mean peak area of LLOQ (0.05 ng/mL) of ofloxacin. The CV and RE (%) were determined as ≤ 4.28% and ≤ 4.46%, correspondingly as shown in Table 2. These results further verify the ability of current analytical method to quantify the LLOQ of ofloxacin with high accuracy and precision.

Level	Nominal concentrations (ng/mL)	Intra-day			Inter-day		
		Accuracy RE (%)	Precision CV (%)	Recovery (%)	Accuracy RE (%)	Precision CV (%)	Recovery (%)
LLOQ	0.05	4.46	1.27	99.51	2.84	2.19	97.23
Low	0.4	2.72	4.28	97.25	3.08	1.23	96.92
Mid	40	3.22	3.07	100.8	2.14	1.31	97.86
High	80	1.18	1.91	101.91	1.69	3.65	98.31

Table 2: Intra- and inter-day accuracy RE (%), precision CV (%) and % recovery of ofloxacin in human AH (n=5).

Detection limit (LLOQ and LLOD): The detection limits (LLOQ and LLOD) were determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be detected with acceptable accuracy and precision. This was performed by the definition of the signal-to-noise ratio with known low concentrations of analyte comparing with those of blank samples. The LLOQ and LLOD of the ofloxacin were found to be 0.05 ng/mL and 0.017 ng/mL respectively, after five replicates. A typical signal-to-noise ratio of 10:1 was found for a quantification limit that can be reliably detected [12].

Precision and accuracy: Intra- and inter- day accuracy and precision were evaluated using spiked samples at four different concentration levels of LLOQ, low, medium and high (0.4, 40 and 80 ng/mL) with five replicates for every concentration level. The precision (signified as CV) was determined to be ≤ 4.28% and the accuracy (signified as RE) was determined to be ≤ 4.46%, as presented in Table 2. The constant values of RE and CV were found to be within 10%, and the recovery are lower than 15% [13].

Selectivity and carryover: To evaluate selectivity six human blank AH samples, each derived from a unique source, were analyzed so as to test the interference throughout analysis. The chromatograms of the drug free human AH did not present any interfering peaks in the retention time of the analyte as it is illustrated (Figure 5).

Carryover was determined by the injection of a high concentration of ofloxacin (100 ng/mL) followed by the injection of blank samples containing only reconstitution buffer. The response of the blank sample was found less than 20% of the corresponding peak area of the LLOQ, which mean the carryover is considered acceptable.

Recovery: Four QC concentration levels (0.1, 0.4, 40 and 100 ng/mL) (n=5) were recruited in order to estimate the recovery of the method. Recovery was calculated 97.23 ± 3.64%, 96.92 ± 3.08%, 97.86 ± 2.14% and 98.31 ± 1.69% for LLOQ, low, mid and high concentration levels, respectively. IS recovery (n=5) was determined equal to 102.01 ± 5.67%. After the extraction procedures, the recoveries were consistent and precise between analytes and the IS.

Matrix effect: Evaluation of the matrix effect was performed by calculating the ratio of the peak area of analytes spiked in blank human AH after extraction with those of analytes dissolved in mobile phase at the same concentration. The matrix effect was 96.51 ± 4.46%, 97.25 ± 2.72% and 100.80 ± 2.81% for ofloxacin at the LLOQ, low, mid and high concentration levels, respectively, while the matrix effect for the IS was 98.95 ± 5.32%. All samples were analyzed in triplicates and the CV was found to be less than 15% as shown (Table 2).

Stability study: The stability of ofloxacin in human AH was studied using spiked samples at concerning level of 0.4, 40 and 100 ng/mL under various conditions: (a) after a week in the refrigerator (4°C-8°C), (b) after three freeze–thaw cycles (freeze at -20°C), (c) at room temperature, (d) after 6 months at -20°C. Each estimation is based in five measurements (n=5). Precision CV (%) and accuracy RE (%) were lower than 6.86% and 9.51% (<15%), respectively, showing that ofloxacin remains stable in the AH at the various storage conditions (Tables 3 and 4).

Nominal concent-rations (ng/mL)	After a week in the refrigerator (4-8°C)			After 3 cycles freeze/thaw (-20°C)		
	Accuracy RE (%)	Precision CV (%)	Recovery (%)	Accuracy RE (%)	Precision CV (%)	Recovery (%)
0.4	2.59	2.38	97.41	9.51	3.63	90.49
40	1.56	2.91	101	4.88	1.01	99.12
80	1.28	6.86	101.25	2.86	4.83	100.86

Table 3: Stability of ofloxacin spiked in human AH (n=5) after a week in the refrigerator (4°C -8°C) and after three freeze/thaw cycles at -20°C.

Nominal concentrations (ng/mL)	After a week at RT			After 6 months in the freezer (-20°C)		
	Accuracy RE (%)	Precision CV (%)	Recovery (%)	Accuracy RE (%)	Precision CV (%)	Recovery (%)
0.4	5.73	1.08	99.19	6.87	2.25	93.34
40	1.27	3.07	99.88	4.27	4.03	107.27
80	2.01	6.52	102.01	5.81	4.37	102.17

Table 4: Stability of ofloxacin spiked in human AH (n=5) after 6 months in the freezer at −20°C and after a week at room temperature (RT).

Samples patient quantification: Current method has been effectively applied for the quantification of ofloxacin in human AH, as shown (Figure 5 and Table 5). The mean human AH concentration of ofloxacin (± SD) was 644.33 ± 2.43 ng/ml (n=21), the concentration range was from 232.72 to 1409.02 ng/ml, indicating that an approximately six-fold difference between the highest and lowest concentrations was easily detectable.

Patients	Concentrations (ng/mL)
Patient 1	562.68 ± 0.34
Patient 2	715.32 ± 1.78
Patient 3	1014.23 ± 0.97
Patient 4	290.04 ± 0.22
Patient 5	406.20 ± 0.22
Patient 6	903.81 ± 1.25
Patient 7	485.75 ± 2.09
Patient 8	1409.02 ± 2.34
Patient 9	823.94 ± 0.75
Patient 10	1169.66 ± 0.81
Patient 11	620.21 ± 0.52
Patient 12	563.79 ± 0.81
Patient 13	708.15 ± 0.53
Patient 14	232.72 ± 0.33
Patient 15	322.17 ± 0.35
Patient 16	609.09 ± 4.39
Patient 17	295.33 ± 0.19
Patient 18	611.26 ± 0.34
Patient 19	438.12 ± 0.28
Patient 20	662.43 ± 3.23
Patient 21	687.08 ± 2.72

Table 5: Concentrations of ofloxacin (ng/mL) in human AH patients, quantified with the developed method.

Discussion

Various analytical methods for the quantification of ofloxacin in human AH (chromatographic techniques including the use of LC-MS/MS) have been reported [9,14-19]. The previous developed methods for the quantification of ofloxacin in human AH reach a minimum sensitivity of 10 ng/ml [9]. Furthermore, Hows et al. [18] developed a methodology for the quantification of polar substances (dopamine, nor epinephrine, 5-hydroxytryptame and cocaine) using a Synergy Hydro PR column. In the current study this well-known procedure was optimized for the quantification ofofloxacin analysis. Ofloxacin is a polar compound and the use of the modified Synergi Hydro RP interacts with the column polar endcapping (mainly *via* H-bonding), as it provides improved hydrogen capabilities and hydrophobic interactions, ideal for polar bases retention under 100% aqueous conditions. A dense bonded phase coverage combined with the high (475 m^2/g) 4 µm silica surface area permits substantial interaction between the sample analyte and the bonded phase. Greater hydrophobicity allows resulting of higher percentage organic mobile phase in shorter run and re-equilibration times, and in LC-MS/MS in improved sensitivity. Dense bonding and endcapping make Synergi Hydro-RP compatible with a variety of MS-compatible mobile phase modifiers such as formic acid, ammonium formate, and acetic acid. Running a 100% aqueous mobile phase on a C18 column can provide improved retention of polar compounds [19]. The used of gradient elution in combination with mild ionization in ESI-MS/MS (ion spray voltage was set at 3,5 kV and the source temperature at 100° C) as previously dıscraped (section Instrumentations) gave good result in analysis of ofloxacin.

Moreover, the current work concentrated on a simple extraction method, maintaining equivalent time rates of ofloxacin recovery. A simple step process (Protein Precipitation (PP)) was selected for the extraction of analytes from human AH samples, avoiding the low recoveries offered by liquid-liquid extraction and the expensive solid-phase extraction. Methanol and acetonitrile are typically the most common solvents for PP. However, methanol is less effective for PP than acetonitrile [20]. Therefore, the selection of acetonitrile in 0.1% formic acid to precipitate proteins ensured rendering clean samples and simultaneously achieving the high recovery of ofloxacin (≥100%).

According to FDA and EMA guidelines, the LC-ESI-MS/MS developed methodology was accomplished and validated efficaciously [12,13]. Good recovery (≥ 100%), specificity, accuracy (RE 1.18% - 4.46%), and reproducibility (CV 1.27%-4.28%), simplicity and short run time were achieved. Furthermore, the LLOD and LLOQ of 0.017 and 0.05 ng/mL correspondingly, were achieved. In addition, it provides satisfactory validation results without any noteworthy interference effect grace to the plain PP and separation by chromatography of endogenous substances in human AH from the analyte. The developed analytical method will facilitate the evidence based approach in ophthalmic therapeutics [21].

Conclusion

To summarize, the current analytical methodology was effectively applied to quantify the amount of ofloxacin in human AH after topical application. Thus, our method can be utilized to quantify ofloxacin in future ophthalmology studies. Moreover, the developed methodology may be used in the analysis of other biological fluids such as plasma or urine, due to its favorable analytical characteristics.

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Declaration of Interests

None.

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