

Development and Validation of a New Ultra-fast HPLC Method for Quantification of Levofloxacin in Rabbit Aqueous Humour: Application to a Pharmacokinetic Study

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Abstract

A specific, sensitive, reliable, low-cost, isocratic, ultra-fast HPLC-DAD for Levofloxacin quantification in rabbit aqueous humor was developed, validated and applied to a pharmacokinetic study, conducted on commercial formulation of Levofloxacin. Separations were obtained on a XB-C18 column (100 Å, 100 mm × 4.60 mm, 2.6 µm, Phenomenex) with an isocratic mobile phase consisting of 18% acetonitrile and 82% triethylamine 0.5% in water (pH adjusted to 2.5 with H₃PO₄) at a flow rate of 0.5 ml/min. Detection of levofloxacin was done at 292 nm, and the column temperature was 40°C. The total analysis run-time was 5 min per sample, and no interfering peaks from aqueous humor were detected. Method was found to be selective, linear (R²=0.99984), accurate (intra-day recovery, 98.24%-100.04%) and precise (RSD, ≤ 5.50%). Pharmacokinetic parameters in rabbit aqueous humors were calculated by PKSolver add in program and are in agreement with literature data. The outlined method can be efficiently applied for monitoring levofloxacin in aqueous humor in pharmacokinetics studies.

Keywords: Levofloxacin; Rabbit; Aqueous humor; Pharmacokinetic; HPLC; Diode array detector

Introduction

Levofloxacin (LFX) is a third-generation fluoroquinolone with activity against gram-positive and gram-negative bacteria. LFX inhibits DNA gyrase, type II topoisomerase, and topoisomerase IV, enzymes essential to separate replicated DNA and block cell division [1].

LFX topical ophthalmic solution is indicated for the treatment of corneal ulcers and bacterial ocular infections caused by susceptible strains [2-6]. LFX (Figure 1) is an amphoteric molecule (pK_{a1} and pK_{a2} values of 6.22 and 7.81 respectively) with a log P of 0.59 at the isoelectric pH of 7.10 [7,8].

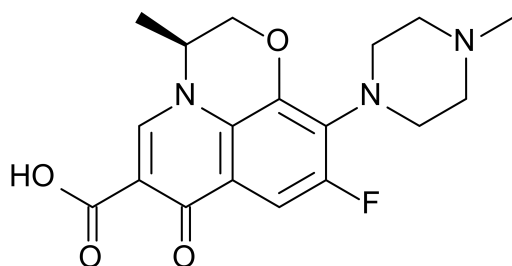


Figure 1: Molecular structure of LFX.

Recently there is a renewed interest in LFX since it is used in different postoperative eye surgery protocols, especially in cataract

surgery. Currently, in Europe is on-going an international clinical study comparing eye drops of LFX and dexamethasone (7 days) followed by dexamethasone (7 days) vs. tobramycin and dexamethasone (14 days) for the prevention and treatment of inflammation and infection associated with cataract surgery (LevoDesa_04-2017, Leader-7) [9]. However, the major disadvantage of LFX eye drops is related to the frequent instillations because the drug is rapidly removed from the ocular surface. Oftaquix[®], an eye drop formulation based on 0.5% LFX marketed in Europe, needs to be instilled up to 8 times in the first two days of bacterial eye infection treatment. This frequent administration regimen could decrease the patient compliance. To overcome this problem many research projects to increase the bioavailability of topical LFX drops have been developed, and others are currently ongoing [10-16].

As a consequence, the increased use of LFX in ophthalmic and in pharmaceutical technology research fields requires the development of readily applicable, fast and low-cost analytical method for pharmacokinetics studies in pre-clinical and clinical monitoring.

In the last years several chromatographic methods have been developed to determine LFX in tissue, in plasma and in pharmaceutical dosage forms [17-24]. However, there are relatively few HPLC methods available for the analysis of LFX in aqueous humor (AH) and many of these methods require fluorescence detection [25,26]. Fluorescence detector is not common in many laboratories since its use is restricted to fluorescent analytes or requires complicate and time-consuming derivatization procedures with fluorescent probes to analyze not-fluorescent molecules. In addition, other HPLC methods for LFX quantification in AH are based on HPLC-MS and UPLC techniques [27,28] that are not widely available in all laboratories because of the high cost of the equipment.

With this background in mind, we herein proposed a specific, sensitive, reliable, low-cost, isocratic, ultra-fast HPLC-UV/DAD for LFX quantification in rabbit AH. The outlined method was validated and applied to an AH pharmacokinetic study conducted on a commercial formulation of LFX.

Materials and Methods

Chemicals

Water and acetonitrile (HPLC grade) were from Merck (Milan, Italy). LFX hemihydrate standard was kind gifts by Alfa Intes (Napoli, Italy). All other chemicals were reagent grade.

Animal

Male New Zealand albino rabbits (Envigo, Udine, Italy) weighing 1.8-2.2 kg, free of any signs of ocular inflammation or other abnormality, were used. Animal procedures conformed to the ARVO (Association for Research in Vision and Ophthalmology) resolution on the use of animals in research. The animals were maintained in restraining boxes during the experiments.

Levofloxacin formulations

Commercial LFX 0.5% eye drop formulation (Oftaquix[®], Santen Oy, Tampere, Finland) was obtained by a local pharmacy.

HPLC analysis of levofloxacin

HPLC separations were conducted on an Agilent 1260 Infinity II chromatographic system (Agilent Technologies) equipped with a high-performance liquid chromatography (HPLC) ChemStation OpenLab software (M8307AA), a quaternary pump G7111B, a diode array detector (DAD) G7115A and a thermostated column compartment G1316A.

Separations were obtained on a XB-C18 column (100 Å, 100 mm × 4.60 mm, 2.6 µm, Phenomenex) with an isocratic binary mobile phase consisting of 18% acetonitrile and 82% triethylamine 0.5% in water (pH adjusted to 2.5 with H₃PO₄). The flow rate was 0.5 ml/min, the column temperature was 40°C and the detection wavelength was 292 nm. Each analysis was run in triplicate. UV spectra were recorded in the range 200-400 nm, and chromatograms were acquired at 292 and 254 nm. Identification of LFX was performed by HPLC-DAD analysis by comparing the retention time and the UV spectra of the sample with that of authentic reference sample.

Sample preparation

A 100 µL aliquot of rabbit AH was pipetted into a 1 ml Eppendorf tube and a 100 µL aliquot of mobile phase (see above) was added in order to precipitate proteins. The sample was vortex-mixed vigorously for 60s and centrifuged at 10,000 g for 5 min. The supernatant was aspirated with a HPLC syringe, samples were filtered (0.45-µm Spartan filters, Schleicher and Schuell, Dassel, FRG) and injected onto the HPLC system.

The possible absorption of the filter was excluded comparing by HPLC analysis the same sample subjected to a double treatment: centrifuged plus filtered *vs.* centrifuged only. No significant differences in the LFX levels were detected (data not shown).

Calibration

A stock solution of LFX (100.0 µg/ml) was obtained by dissolving an appropriate amount of LFX standard in water. Working standard solutions of LFX were daily prepared by adequate dilution with eluent phase of calculated amount of the stock solution. In order to prepare the AH calibration standards, measured aliquots (100-150 µL) of AH were spiked in an Eppendorf tube, with increasing concentrations of working standard solutions, to obtain ten LFX calibrators (range 3.1-3137.5 ng/ml). Calibration standards were processed as reported in the above mentioned sample preparation procedure and analyzed by HPLC.

Validation

The developed ultra-fast HPLC method was validated according to International Conference on Harmonization Guidelines [29] with regard to linearity, limit of detection (LOD) and quantitation (LOQ), precision (intra-day and inter-day) and accuracy.

Ocular pharmacokinetic study

Albino rabbits (n=12) were treated with the formulation under investigation. Each rabbit received a single topical instillation of 50 µL commercial formulation of LFX into the conjunctival sac of both eyes *via* a micropipette. Next, the eyelids are closed gently for 30 seconds. After instillation of the eye drops the AH was collected at different times (15, 30, 45, 60, 120 and 240 min), for each time 2 rabbits were used.

Rabbit were killed by Tanax injection and AH (100 µL) was collected by a syringe with a 26 G needle and stored at -20°C prior to HPLC analysis. Pharmacokinetic parameters to evaluate the AH bioavailability, such as C_{max}, T_{max}, T_{1/2}, K_{el} and AUC, were determined [30].

Results and Discussion

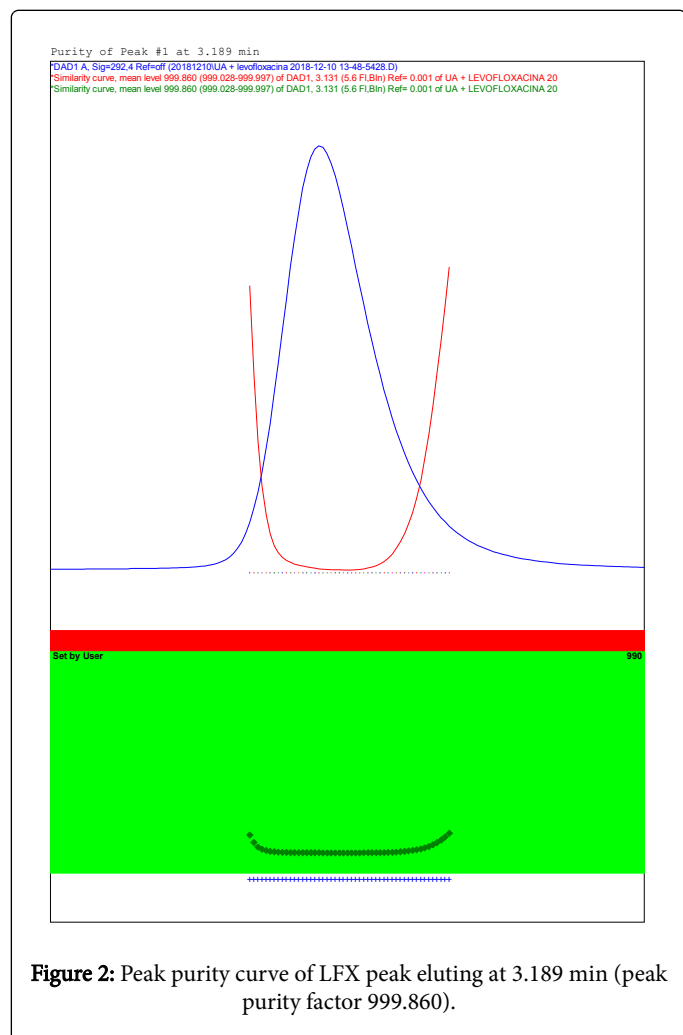
Method development

The effect of the pH and the composition of the mobile phase were evaluated as important factors to improve separations and peak symmetry of LFX. LFX is amphoteric due to the presence of carboxylic and piperazinyl moieties with pKa values of 6.18 and 7.81, respectively [7,8]. In order to suppress the carboxylic ionization, we decided to explore a mobile phase with a pH in the range 2.5-3.5. In addition, to avoid secondary interactions between free silanol groups and the protonated amino groups of LFX piperazinyl moiety, that causing peak tailing and increasing retention time, we decided to use triethylammonium phosphate as additive, in order to shield the silanol groups and, consequently, improve the peak symmetry. The effect of column temperature in the range 40-50°C was also evaluated to obtain the best peak shape. In summary, we find that an optimum combination of triethylammonium phosphate buffered at pH 2.5, a proton-acceptor solvent (i.e. acetonitrile) and a column temperature of 40°C ensure an optimum solvent strength and solvent-type selectivity to obtain good separation with acceptable peak shape (tailing factor=1.52).

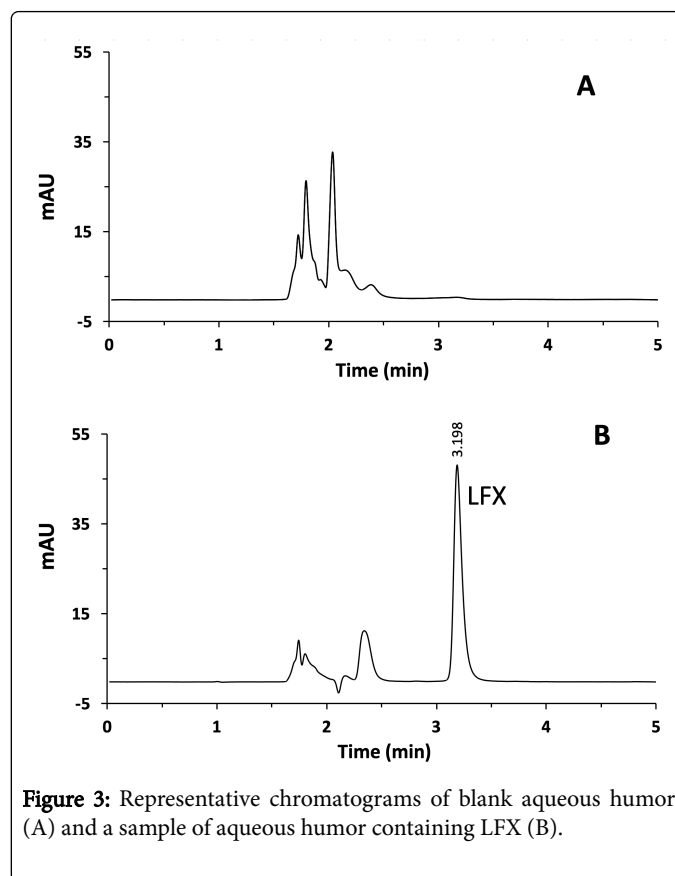
Selectivity

Identification of LFX was performed by HPLC-DAD analysis by comparing the retention time and the UV spectra of the samples with those of authentic reference samples. Potential interferences from AH constituents were excluded by analyzing the chromatograms of blank samples. In addition, peak-purity tests using photodiode-array detector spectra were used to show that the analyte peaks cannot be attributed to more than one component.

Peak purity is a technique able to detect the occurrence of impurities that can coelute together the analyte peak. This approach is based on the comparison of different UV spectra registered during the peak elution. If the peak is pure then the spectra taken at several peak points should all be identical, except for concentration differences. The peak purity factors were calculated by OpenLab® Agilent software. They were found to be >999 (considering 1000 as 100% match) and were within the threshold limit set to 990 (Figure 2) [31,32].

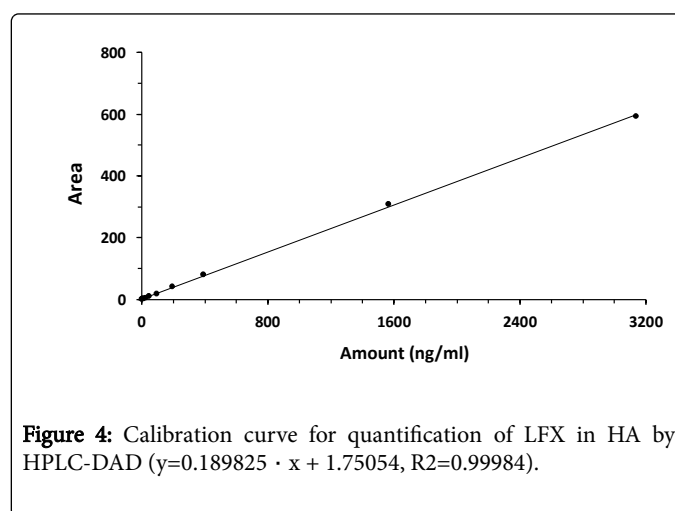


Under the developed chromatographic conditions and sample preparation procedures, the retention time of LFX is about 3.2 min and each run can be completed within 5 min. Representative chromatograms of blank AH and a sample of AH containing LFX are reported in Figure 3.



Calibration and linearity

The AH calibration curve was constructed using ten calibrators (3.1-3137.5 ng/mL). Calibration curve was evaluated at the beginning of six consecutive days (n=6). The equation of the calibration curve obtained was $y=0.189825x+1.75054$. The correlation coefficient was 0.99984 ($x=ng/ml$, $y=area$) (Figure 4 and Table 1). The present method showed a larger linear range with respect to the method of Gupta and co-worker, which determines LFX in AH by UPLC-UV [28].



Parameters	Results
Range (ng/ml)	3.1-3137.5
Slope	0.189825
Intercept	1.75054
R ²	0.99984
LOD (ng/ml)	0.77
LOQ (ng/ml)	3.11

Table 1: Results of validation of the HPLC-DAD determination of LFX in Rabbit Aqueous Humor.

Determination of the limit of detection and quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) of the method were quantified in AH samples spiked with progressively lower concentrations of LFX. LOD was found to be 0.77 ng/ml, with a signal/noise ratio of about 3:1 (n=6), while the LOQ was found to be 3.11 ng/ml, with a signal/noise of about 10:1 (n=6) (Table 2). The outlined method was more sensible when compared to literature method with a 16-20 fold improvement in LOQ and LOD, respectively [28].

True concentration	Amount found ^a (ng/ml)	Recovery %	RSD %
Intra-day			
50	49.71 ± 2.74	99.42	5.51
500	491.21 ± 5.63	98.24	1.15
3000	3001.25 ± 10.21	100.04	0.34
Inter-day			
50	48.27 ± 2.33	96.54	4.83
500	491.61 ± 6.99	98.32	1.42
3000	2993.51 ± 16.92	99.78	0.56
^a n=6			

Table 2: Intra- and inter-day accuracy and precision in the analysis of LFX in rabbit aqueous humor.

Accuracy and Precision

The precision and accuracy were determined at three concentration levels in rabbit AH spiked with three known amount of LFX (50, 500 and 3000 ng/mL) followed by their comparison with the calibration curves prepared on the same day. Precision was expressed as the %RSD of measured concentrations, while accuracy was expressed as recovery % (amount found/nominal amount x 100) of drug added to the blank AH samples. Table 2 showed that the developed method was highly reproducible with low intra-and inter-day variation.

In fact, the intra-day and the inter-day recoveries are comprised in the range 98.24-100.04% and 96.54%-99.78% respectively. The accuracies were less than 6.00% in both cases.

Sample stability

LFX was found to be stable in AH at 20°C in the dark for 1 h and at 5°C for 1 day with average recovery of 97.61 and 99.54%, respectively. The freeze-thaw data experiments showed that three cycles can be accepted without losses greater than 10%. Analogously, the stock solutions stability in mobile phase revealed no significant losses of LFX for at least 4 days at 5°C in the dark.

Ocular pharmacokinetic study

AH concentration-time profile of LFX after topical administration of Oftaquix[®] is reported in Figure 5. The pharmacokinetic key parameters to evaluate the formulations bioavailability C_{max}, T_{max}, T_{1/2}, K_{el} and AUC are reported in Table 3 [31].

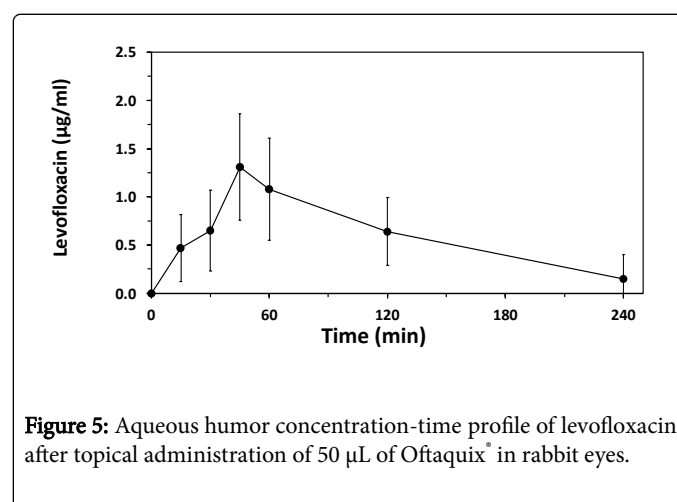


Figure 5: Aqueous humor concentration-time profile of levofloxacin after topical administration of 50 µL of Oftaquix[®] in rabbit eyes.

Market Formulation ^a	
C _{max} (µg/ml)	1.31 ± 0.55
T _{max} (min)	45 ± 0
T _{1/2} (min)	99.80 ± 14.3
K _{el} (min ⁻¹)	0.0069 ± 0.0011
AUC (µg·ml ⁻¹ ·min)	143.55 ± 78.5
^a n=12 rabbits (24 eyes)	

Table 3: Aqueous humor pharmacokinetic key parameters after single instillation of commercial Oftaquix[®].

The AH peak concentration (C_{max}) was 1.31 µg/ml and was reached at 45 min (T_{max}). The apparent rates of the rabbit aqueous clearance was described by the apparent half-life (99.80 min) and by the K_{el} (0.0069 min⁻¹). The AUC was 143.55 µg·ml⁻¹·min and is in accordance with literature data [15].

Conclusions

The goal of the developed chromatographic method was to obtain quantification and separation of LFX from other endogenous compounds present in the rabbit AH. We preferred to develop an isocratic elution in order to avoid the tedious re-equilibration time of gradient analysis. Various experimental parameters such as pH and

mobile phase composition together with column oven temperature were optimized. The method was validated according to ICH guidelines and uses a low-cost and very rapid sample preparation that guarantees good recovery of LFX from the biological sample. The total run time does not exceed 5 min per analysis with no peak interference from endogenous compounds. The proposed method was found to be suitable for LFX determination in aqueous humour and could be efficiently applied for the analysis of LFX in the eye tissues in preclinical research. In addition, it was demonstrated that proteins distribution in AH of various mammalian species such bovine, monkey, dog, human and rabbit is almost identical when the blood/aqueous barrier is intact [33]. Hence, the proposed HPLC method for quantification of LFX in aqueous humour of rabbits could be utilised for investigation in humans and other species.

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