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HPLC Analysis of Moxifloxacin in Rabbit Aqueous Humor after Topical Instillation of Mucoadhesive Hyaluronate Formulations

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Abstract
Here we provide an extremely simple, rapid, direct, sensitive and specific HPLC method to quantify moxifloxacin in rabbit aqueous humor. Separation of moxifloxacin was performed on a Kromasil C8 column using a ternary pre-mixed mobile phase of acetonitrile, methanol and 0.02 M KH_2PO_4 buffer solution containing 1% triethylamine (pH 3.0 with phosphoric acid) (15:20:65, v/v/v). UV detection at 292 nm was used with a flow rate of 1 ml/min. The developed method fulfills the validation requirements of FDA. Consequently, it is suitable to investigate the pharmacokinetic profile of ophthalmic formulation based on moxifloxacin. As an example, we investigated the pharmacokinetic profile of two new ophthalmic moxifloxacin mucoadhesive formulations based on sodium hyaluronate. The pharmacokinetic profiles outlined showed a strong bioavailability increase for moxifloxacin-hyaluronate based formulation with respect to the marketed conventional eye drop.

Keywords
Moxifloxacin, rabbit, pharmacokinetic, HPLC, sodium hyaluronate, mucoadhesion, bioavailability.

INTRODUCTION

Topical ocular instillation of drugs is the preferred route of administration for the treatment of a variety of eye disorders. On the other hand, the ocular bioavailability of ophthalmic drugs is considerably reduced due to very powerful protective mechanisms of the eye. In fact, blinking together with the rapid turnover of lachrymal fluid and efficient drainage apparatus causes a short precorneal residence time of the instilled drugs. Moreover, the special anatomic structure of the cornea prevent the absorption of drugs [1]. Consequently, repetitive instillations of eye drops are needed to ensure effective therapeutic drug level in tear film and ocular tissues. Unfortunately, frequently use of highly concentrated solutions may increase the possibility of causing both ocular and systemic side-effects [2].

In order to increase the quantity of drug capable to reach the target ocular tissues the precorneal residence time should be augmented. Sodium hyaluronate is a naturally occurring mucopolysaccharide consisting of residues of D-glucuronic acid and *N*-acetyl-D-glucosamine and is present in skin, synovial fluid as well as in the vitreous and aqueous humor. It is reported that sodium hyaluronate is capable of increasing the precorneal residence time and the bioavailability of various ophthalmic drugs. Mucoadhesive properties of sodium hyaluronate was demonstrated by *in vitro* and *in vivo* studies [3-4].

Moxifloxacin (1-cyclopropyl-7[*S*,*S*]-2,8-diazabicyclo[4.3.0] non-8-yl-6-fluoro-8-methoxy-1,4-dihydro-4-oxo-3-quinolone carboxylic acid hydrochloride, Fig. 1) is a fourth-generation antibiotic drug of the fluoroquinolone family which inhibits the bacterial enzymes DNA gyrase and topoisomerase IV. Moxifloxacin has a broad spectrum, with enhanced activity against Streptococci and Staphylococci and moderate to excellent activity against clinically relevant infection from gram-negative ocular pathogens [5-6].

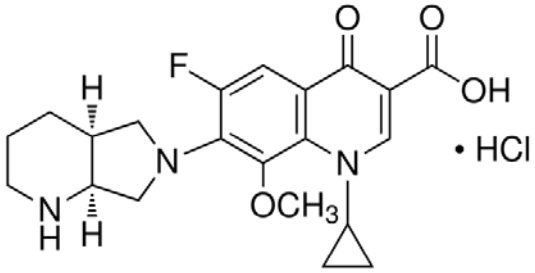

Fig. 1 Moxifloxacin Hydrochloride.

The increased use of moxifloxacin requires the development of simple, rapid, reliable and low-cost analytical method for the pharmacokinetic study and clinical monitoring. Recently, some HPLC methods using the expensive UPLC [7] system, mass spectrometry [8-9] and fluorescence detectors [10-14] were developed for determination of moxifloxacin in biological fluids. In this study, we proposed a specific, sensitive, simple, reliable low-cost HPLC-UV method for moxifloxacin determination in rabbit aqueous humor.

The developed method was used to determine the pharmacokinetic profile of moxifloxacin in rabbit following the topical administration of a commercial eye drop and two sustained release mucoadhesive hyaluronate formulations with improved bioavailability.

MATERIALS AND METHODS

Chemicals

Water, methanol and acetonitrile (HPLC grade) were from Merck (Milan, Italy). Moxifloxacine hydrochloride was purchased from sigma Aldrich (Milan, Italy). Sodium hyaluronate was obtained from HTL (Javené France). All other chemicals were reagent grade.

Animal

New Zealand albino rabbits (Charles River, Calco, Italy) weighing 1.8–2.2 kg, free of any signs of ocular inflammation or gross abnormality, were used. The animals were kept in restraining boxes during the experiments and were not anesthetized except when paracentesis was performed.

Preparation of mucoadhesive hyaluronate formulations

Commercial eye drop formulation containing moxifloxacin hydrochloride (Vigamox, Alcon Italia S.p.A.) was purchased at a local pharmacy. Commercial eye drop (MX-ED) was added of weighted amount of sodium hyaluronate powder in order to obtain two formulations with a biopolymer concentration of 0.5% (MX-SH-0.5) and 1% (MX-SH-1.0) (weight/volume). Osmolarities of the final hyaluronate formulations were slightly increased (5-15 mOsm/L) with respect to the initial value. Similarly, the pH remains unchanged when compared to the starting MX-ED solution.

HPLC Analysis

HPLC separations were performed on a HP 1100 chromatographic system (Agilent Technologies, Milan, Italy) equipped with a HP ChemStation software, a binary pump G1312A, a diode array detector G1315A and a thermostated column compartment G1316A. Separations were performed on Kromasil C8 column (250mm×4.6mm, 5µ particle size, Sigma-Aldrich, Milano, Italy). The isocratic mobile phase (1 ml/min) consisted of acetonitrile, methanol and 0.02 M KH_2PO4 buffer solution (containing 1% triethylamine, pH 3.0 with phosphoric acid) (15:20:65, v/v/v). The wavelength was set at 296 nm and the column was maintained at 30° C.

Sample preparation

A simple liquid-liquid extraction was used for the extraction of moxifloxacin in rabbit aqueous humor. To 0.5 ml of aqueous was added 40 µl of NaOH, 0.5 M and the resulting mixture was mixed for 10 s on a vortex. Successively, was performed an extraction with 5 ml of CH_2Cl_2. After vortexed for 5min, the mixture was centrifuged at 10,000 rpm for 5min. The organic layer was transferred into another glass tube and evaporated to dryness at 40° C under a nitrogen flow. The final residue was reconstituted in 100 µl aliquots of KH_2PO_4 buffer solution and 20 µl was injected into the HPLC.

Calibration

A stock solution of moxifloxacin (1.0 µg/ml) was prepared by dissolving an appropriate amount of moxifloxacin in acetonitrile. Working standard solutions of moxifloxacin were daily prepared by adequate dilution with acetonitrile of calculated amount of the stock solution. To prepare the aqueous humor calibration standards, aliquots of 50 µl of aqueous were spiked with increasing concentrations of working standard solutions to give moxifloxacin concentrations in the range 50-1600 ng/ml. Calibration standards were processed according to sample preparation procedure above mentioned and were analyzed by HPLC.

Validation

The HPLC method was validated in terms of linearity, specificity, sensitivity, precision and accuracy. In addition, the stability of moxifloxacin in aqueous humor at 4°, 20° C and after freeze-thaw cycles was evaluated [15].

Ocular pharmacokinetic study

Albino rabbits were randomly divided in three groups of seven animals and treated with the formulations under investigation. Each rabbit received a single instillation of 50 µL into the conjunctival sac of both eyes. The first group was treated with moxifloxacin commercial formulation (MX-ED), whereas the other two groups were treated with the mucoadhesive hyaluronate formulations (MX-SH-0.5 and MX-SH-1.0). Aqueous moxifloxacin levels were monitored at 0.5, 1, 2, 4, 6, 8 and 12 and h after instillation. Before paracentesis the rabbits were anesthetized by an intravenous injection of 25 mg/kg of ketamine. Aqueous humor (150 mL) was withdrawn through the limbus, with a syringe with a 26 G needle and stored at -20° C until HPLC analysis. Pharmacokinetic key parameters to evaluate the formulations bioavailability such as, C_{max}, T_{max}, K_{el} and AUC were determined [16].

Statistical Analysis.

Statistical differences of in vivo data are determined using repeated measure analysis of variance (ANOVA) followed by the Bonferroni-Dunn post hoc pairwise comparison procedure. A probability, P, of less than 0.05 is considered significant in this study.

RESULTS AND DISCUSSION

Method development

The HPLC method described in this paper was adapted from Xu and coworker [17] that developed a very simple and low-cost HPLC-UV analysis of moxifloxacin in human plasma. In the present paper we modified and validated the above mentioned method for the analysis of moxifloxacin in rabbit aqueous humor. The specificity of the method was evaluated by analyzing blank drug-free rabbit aqueous humor samples after single topical administration of moxifloxacin formulations. The eventual interferences from endogenous matrix constituents of rabbit aqueous humor were unequivocally excluded by analyzing the chromatograms of blank and spiked plasma samples. Under the outlined chromatographic conditions and sample processing procedure, the retention times of moxifloxacin was approximately 7.5 min and each run can be completed within 10 min.

Calibration and linearity

Moxifloxacin aqueous levels were calculated from linear regression of external standards of the drug, relating peak area and concentration. The standard curve was linear over the range 50-1600 ng/ml. The equation for the standard curve relating the moxifloxacin concentration (x, ng/ml) to peak area (A) was $A = 7117.41 + (914.47 \cdot x)$. The correlation

coefficient of standard curve was 0.9986 (n=6). These results show that within the concentration range used there is an excellent correlation between peak area ratio and concentration of moxifloxacin.

Determination of the limit of detection and quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) of the present method were determined in aqueous humor samples spiked with progressively lower concentrations of moxifloxacin. LOD was found to be 15 nM with a signal/noise ratio of about 3:1 (n=6). LOQ was found to be 50 nM with a signal/noise of about 10:1 (n=6).

Accuracy and Precision

The precision and accuracy of the extraction procedure were investigated at three concentrations levels in rabbit aqueous humor spiked with three levels of known amount moxifloxacin (10, 100 and 1000 ng/mL).

The accuracies of the assays were determined by comparing the measured concentration to its true value. The precision of the method was evaluated by replicate analysis and was expressed as RSD. The intra-day precision and accuracy were checked by replicate analysis on the same day. The inter-day precision and accuracy were evaluated on five consecutive days on fresh rabbit aqueous humor.

Table 1

Intra- and inter-day accuracy and precision in the analysis of moxifloxacin in rabbit aqueous humor

True conc. (nM/ml)	*Accuracy[a] Mean±SD*	*RSD[b] (%)*
Intra-day		
10	98.33±4.15	4.22
100	99.78±3.22	3.23
1000	99.89±2.11	2.11
Inter-day		
1	98.11±4.99	5.09
10	99.12±3.51	3.54
100	99.97±2.99	2.99

[a,b] n=6

Table 1 showed that the assay was highly reproducible with low intra- and inter-day variation. In fact, the intra-day and the inter-day recoveries range from 98.33 to 99.89% and from 98.11 to 99.97% respectively, whereas the accuracies were less than 2.44% in both cases.

Stability

Moxifloxacin was found to be stable in aqueous humor at 20° C for 24 h and at 5° C for 1 day with average recovery of 96.6 and 98.1%, respectively. The freeze-thaw data analysis showed that three cycles can be accepted without losses greater than 10%. Similarly, the stock solutions stability in mobile phase revealed no significant losses for at least 4 days at 20° C.

Ocular pharmacokinetic study

The aqueous humor concentration-time profile and the pharmacokinetic key parameters data are presented in Fig. 2 and table 2, respectively. The hyaluronate mucoadhesive formulations appear to offer significant sustained drug levels in aqueous humor compared with the reference commercial formulation.

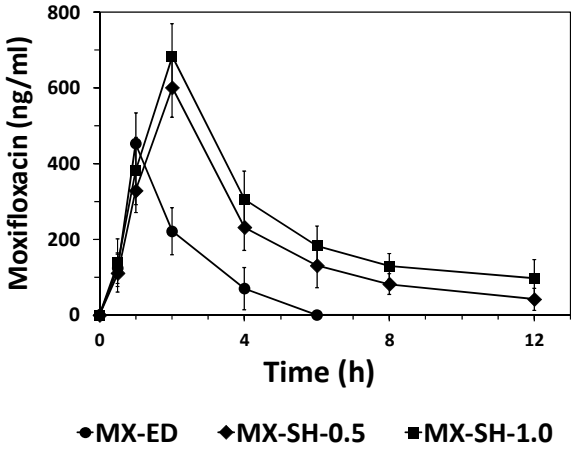

Fig. 2 Aqueous humor concentration-time profiles of moxifloxacin after ocular instillation in rabbit of MX-ED, MX-SH-0.5 and MX-SH1.0 formulations.

Table 2

Aqueous humor pharmacokinetic key parameters after single instillation of commercial (MX-ED) and hyaluronate mucoadhesive formulations of moxifloxacin (MX-SH-0.5 and MX-SH-1.0).

	MX-ED[a]	*MX-SH-0.5[a]*	*MX-SH-0.5[a]*
C_{max} *(ng/ml)*	453.5	601.0	684.5
T_{max} *(h)*	1	2	2
K_{el} *(h^{-1})*	0.29	0.21	0.21
AUC ($ng \cdot ml^{-1} \cdot h$)	875.18±115	2512.38±280	3528.38±332

[a] n=6 rabbits (12 eyes)

The aqueous humor peak concentration (C_{max}), following administration of commercial formulation, was significantly lower ($p<0.05$) compared to the hyaluronate formulations, with a 1.50- and 1.21-fold decrease for MX-SH-1.0 and MX-SH-0.5, respectively. For all the other point the value obtained with hyaluronate formulations were significantly higher ($p<0.05$) at all times except at 0.5 h. Consequently, the apparent rate of the rabbit aqueous clearance were slower than that observed with the commercial formulation, as demonstrated by the increase in the apparent half-life (from 2.40 h to 3.29 h and 3.36 h for MX-ED, MX-SH-0.5 and MX-SH1.0, respectively) and the decrease in K_{el} (from 0.29 to 0.21 for MX-ED, MX-SH-0.5/MX-SH1.0, respectively). In addition, the T_{max} values were increased from 1 to 2 h for groups treated with the hyaluronate formulations with respect to the commercial formulation group. Interestingly, in the group treated with commercial formulation the aqueous level of moxifloxacin were undetectable after 4 h, probably due to the rapid precorneal drainage of the drug. The AUC were significantly increased in the group treated with the hyaluronate formulations with respect to the commercial formulation, with an increase in the AUC values of 2.9- and

4.0-fold for MX-SH-0.5 and MX-SH1.0, respectively. This increased bioavailability performances of the hyaluronate based formulations could be attributed to the longer precorneal residence time of sodium hyaluronate as demonstrated in previous study [18-19]. Interestingly, all the pharmacokinetic parameters related to the hyaluronate formulations (MX-SH-0.5 and MX-SH1.0) were not statistically significant when compared each other. We speculate that this evidence may be indicative that corneal surface, when treated with hyaluronate formulation with biopolymer concentration superior to 0.5%, begins to saturate in terms of hyaluronate mucoadhesive binding interactions with the mucous layer of the tear film. In addition, it is reported that there is a certain viscosity range which maximally increases the precorneal residence time, and upon this range there is no further improve of bioavailability [20-21].

CONCLUSIONS

Here we provide an extremely simple, rapid, direct, sensitive and specific method to quantify moxifloxacin in rabbit aqueous humor. Linearity, accuracy and precision were found to be acceptable. The experiments show that the HPLC-UV method developed in this study could represent an useful alternative to the existing procedures since its simplicity and rapidity. Moreover, it fulfills the validation requirements of FDA for the analysis of drugs with respect to selectivity, precision and accuracy. Consequently, it is suitable to investigate the pharmacokinetic profile of ophthalmic formulation based on moxifloxacin. As an example, we evaluated the pharmacokinetic profile of two new moxifloxacin mucoadhesive formulations based on sodium hyaluronate. The pharmacokinetic profiles outlined clearly indicate an increase of bioavailability of the hyaluronate based formulation when compared to the marketed conventional ophthalmic solution.

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