Preclinical Pharmacokinetic Evaluation of Gatifloxacin Mucoadhesive Formulations in Rabbit Eye using HPLC

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Abstract
In order to improve the frequent dosing regimen reported for the commercial aqueous gatifloxacin eye drops, two new ophthalmic mucoadhesive formulations based on high molecular weight sodium hyaluronate were prepared. The pharmacokinetic profiles outlined showed a strong bioavailability increase for gatifloxacin-hyaluronate formulations with respect to aqueous conventional eye drop. Gatifloxacin quantification in aqueous humor of albino rabbits was performed by an extremely simple, rapid, direct, sensitive and specific HPLC-UV method. Separations were obtained on an Adsorbosphere HS C18 column using CH3CN and an aqueous solution of 10 mM SDS, 10 mM TBAA and 25 mM citric acid (pH 2.5) (50:50 v/v). Detection was performed at 293 nm using a flow rate of 1.0 ml/min. The developed method fulfills the validation requirements of FDA and EMEA and it is suitable for the pharmacokinetic study in rabbit eye.

Keywords: Gatifloxacin, rabbit, pharmacokinetic, HPLC, sodium hyaluronate, mucoadhesion, bioavailability.

INTRODUCTION
Ocular bioavailability of topical conventional eye drops is considerably reduced due to very strong protective mechanisms of the eye. In fact, many instilled formulations have short preconneal residence time due to rapid turnover of lacrimal fluid, blinking, drainage by gravity and by nasolacrimal duct, and conjunctival absorption. Moreover, the particular anatomic structure of the cornea further reduces the absorption of drugs at 1-10% of the administered dose. Consequently, repetitive instillations of eye drops are needed to ensure effective therapeutic drug level in tear film and ocular tissues [1-4]. Unfortunately, frequently use of concentrated solutions can increase the possibility of triggering both ocular and systemic side-effects [5-7]. In order to increase the quantity of drug capable to reach the target ocular tissues the preconneal residence time of instilled drugs should be augmented. The use of bioadhesive polymers can ensure an increase of ocular residence time, via their enhanced viscosity and mucoadhesive properties [8-9]. Sodium hyaluronate is a naturally occurring mucopolysaccharide consisting of residues of D-glucuronic acid and N-acetyl-D-glucosamine and is present in many body tissues including skin, synovial fluid as well as in the vitreous and aqueous humor. It is reported that sodium hyaluronate due to its mucoadhesive properties is capable of increasing the preconneal residence time and the bioavailability of various ophthalmic drugs [10-11]. Fluoroquinolones are an important class of synthetic antibiotics widely used in anti-infective chemotherapy due to their remarkably broad spectrum of activity [12-13]. Gatifloxacin (GTFX) is a fourth-generation antibiotic drug of the fluoroquinolone family which inhibits the bacterial enzymes DNA gyrase and topoisomerase IV [14]. Gatifloxacin is chemically named as 1-cyclopropyl-6-fluoro-8-methoxy-7-(3-methylpiperazin-1-yl)-4-oxo-quinoline-3-carboxylic acid (Fig. 1). Gatifloxacin is an 8-methoxy fluoroquinolone derivative with a 3-methylpiperazinyl substituent at C7, utilized as antibacterial agent with a broad spectrum action against Gram-positive and Gram-negative bacteria. In fact, gatifloxacin has a good activity against clinically important pathogens, including Streptococcus pneumoniae, Haemophilus influenzae, Chlamydia pneumoniae, Moraxella catarrhalis, Mycoplasma pneumoniae and Legionella [15].

Gatifloxacin was approved in 1999 in the USA as Tequin® (Bristol-Myers Squibb) for once-daily therapy in acute bacterial exacerbation of chronic bronchitis, acute sinusitis, community-acquired pneumonia, pyleonephritis, gonorrhea, and cystitis [16]. Successively, systemic administration of gatifloxacin was associated with disturbance in glucose homeostasis [17]. In fact, it was demonstrated that gatifloxacin-induced hyperglycemia can usually occurs following consecutive administrations of gatifloxacin, while hypoglycemia can occurs after single dose [18]. Consequently, in 2006 Tequin was removed from the North American market [19]. In 2011 the Indian health authorities adopted the same measures and the systemic gatifloxacin was withdrawn from the local market [20]. Topical gatifloxacin formulations, such as Zymar® (0.3% GTFX) and Zymaxid™ (0.5% GTFX) by Allergan Labs, (Irvine, CA, USA), were introduced respectively in 2003 and 2010. Both are indicated for the treatment of bacterial acute...
conjunctivitis caused by susceptible microorganisms, and are also employed in an off-label way for prophylaxis of endophthalmitis following ocular surgery [21]. There have been no reports of dysglycemia associated with topical gatifloxacin use, and actually there are present worldwide several generic gatifloxacin ophthalmic preparations. According to prescribing information of Zymar® and Zymaxid™ the dosing regimen for both medications is quite frequent (on day 1, one drop every two hours (up to 8 times); on days 2-7, one drop two to four times daily) [22-23]. This frequent administration regimen could reduce the compliance of patient and consequently the efficacy of the treatment. For this reason, we designed and prepared two new sustained release mucoadhesive hyaluronate formulations with improved bioavailability. In addition, we proposed and validated a specific, sensitive, simple, reliable low-cost HPLC-UV method for gatifloxacin determination in rabbit aqueous humor.

**MATERIALS AND METHODS**

**Chemicals**

Water and acetonitrile (HPLC grade) were from Merck (Milan, Italy). Gatifloxacin sesquihydrate and ciprofloxacin were obtained 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 gatifloxacin formulations**

Conventional aqueous eye drop formulation (GTFX-ED), similar to the commercial available gatifloxacin formulations, was prepared dissolving 300 mg of gatifloxacin sesquihydrate, 10 mg of edetate disodium and 880 mg of NaCl in 100 ml of water, the pH was adjusted to 5.7 with NaOH/HCl with a resulting osmolarity of 303 mOsm/L (measured by an Osmomat 030, Gonotec, Berlin, Germany). The obtained solution was sterilized by filtration through 0.22-mm membrane filter. An appropriate amount of sterile GTXF-ED formulation was added of weighted quantity of sodium hyaluronate powder in order to obtain two formulations with a biopolymer concentration of 0.6% (GTFX-SH-0.6) and 1.2% (GTFX-SH-1.2) (weight/volume). Osmolarities of the two final hyaluronate based formulations were slightly increased (3-5 mOsm/L) with respect to the initial value. Analogously, the pH of the new formulations remains unchanged when compared to the starting GTXF-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 Adsorbosphere HS (C18) column (250mm×4.6mm, 5µ particle size, Alltech, Milano, Italy). The isocratic mobile phase consisted of CH3CN and an aqueous solution of 10 mM sodium dodecyl sulfate (SDS), 10 mM tetrabutylammonium acetate (TBAA) and 25 mM citric acid (pH 2.5) (50-50 v/v). The flow rate was 1.0 ml/min, the column temperature was 25 °C and the detection wavelength was 293 nm.

**Sample preparation**

A simple liquid-liquid extraction was used for the extraction of gatifloxacin in rabbit aqueous humor. To 100-150 µl of aqueous humor, ciprofloxacin internal standard (50 µl of working stock solution) equivalent to 800 ng was added and vortex-mixed for 60 s. Successively, was performed an extraction with 3 ml of acetonitrile. After vortexed for 5 min, the mixture was centrifuged 10 min at 10,000 rpm. The organic layer was transferred into another glass tube and evaporated to dryness at 40° C under a gentle nitrogen flow. The final residue was reconstituted in 100 µl of mobile phase solution, filtered through 0.45 m Spartan-T syringe filters and 20 µl was injected into the HPLC.

**Calibration**

A stock solution of gatifloxacin (100.0 µg/ml) was prepared by dissolving an appropriate amount of gatifloxacin sesquihydrate in acetonitrile. Working standard solutions of gatifloxacin were daily prepared by adequate dilution with acetonitrile of calculated amount of the stock solution. To prepare the aqueous humor calibration standards, aliquots of 100-150 µl of aqueous humor were placed in each Eppendorf tube and were spiked with increasing concentrations of working standard solutions to give gatifloxacin concentrations of 50, 100, 200, 400, 800, 1500 and 3000 ng/ml. Calibration standards were processed according to sample preparation procedure above mentioned and were analyzed by HPLC.

**Validation**

Method validation was performed according to the principles of FDA and EMEA for bioanalytical method validation [24-25]. The developed method was validated for selectivity, linearity, precision and accuracy, recovery, limits of quantification and detection, and stability.

**Ocular pharmacokinetic study**

Albino rabbits were randomly divided in three groups of six animals and treated with the formulations under investigation. Each rabbit received a single instillation of 50 µL into the conjunctival sac of both eyes as a single bolus dose via a micropipette. Then, the two eyelids are closed softly for 30 seconds. The first group was treated with gatifloxacin aqueous formulation (GTFX-ED), whereas the other two groups were treated with the mucoadhesive hyaluronate formulations (GTFX-SH-0.6 and GTFX-SH-1.2). Aqueous gatifloxacin levels were monitored at 15, 30, 60, 90, 120, 180, 240, 300 and 360 min after instillation. Before paracentesis the rabbits were anesthetized by an intravenous injection of 25 mg/kg of ketamine. Aqueous humor (100-150 µL) 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, Cmax, Tmax, T1/2, Kel and AUC were determined [26].
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 and selectivity
The increased use of gatifloxacin in ophthalmic field requires the development of simple, fast, reliable and low-cost analytical method for the pharmacokinetic study and clinical monitoring. Recently, some HPLC methods using mass spectrometry [27-28], fluorescence [29-30] and ultraviolet detection [31-34] were developed for determination of gatifloxacin in biological fluids. In this study, we proposed a specific, sensitive, simple, reliable low-cost HPLC-UV method for gatifloxacin determination in rabbit aqueous humor. The developed method was used to determine the pharmacokinetic profile of gatifloxacin in rabbit following the topical administration of a conventional eye drop and two sustained release mucoadhesive hyaluronate formulations with improved bioavailability.

The HPLC method described in this paper was adapted from Liang and co-worker [35] that developed a very simple and low-cost HPLC-UV analysis of gatifloxacin in human plasma. In the present paper we modified and validated the above mentioned method for the analysis of gatifloxacin in rabbit aqueous humor samples.

The selectivity of the method was evaluated by analyzing aqueous humor samples from six different and randomly selected rabbits. The eventual interferences from endogenous matrix constituents of rabbit aqueous humor were unequivocally excluded by analyzing the chromatograms of blank and spiked aqueous humor samples. Under the outlined chromatographic conditions and sample processing procedures, the retention times of ciprofloxacin (internal standard) and gatifloxacin are approximately 5.6 and 6.5 min, respectively, and each run can be completed within 10 min.

Calibration and linearity
The aqueous humor calibration curve was constructed using seven calibrators (50-300 ng/ml). Peak area ratios of drug-to-internal standard were plotted vs. real concentrations.

Calibration curve was determined at the beginning of six consecutive days (n = 6). Weighted linear regression was performed on calibration data using 1/S² as weighting factor. This weighting factor was selected as it yielded the best fit of peak area ratios versus concentration of gatifloxacin [36]. The calibration curves were linear over the specified range. The mean ± S.D. of the slope and intercept were 0.00083±0.00009 and -0.0099±0.065, respectively. The coefficient of determination (r²) was > 0.9992 for all calibration curves. The present method showed a better lower limit of linear range than that reported by Liang and co-worker in plasma (from 100 ng/ml to 50 ng/ml) [35].

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 diluted concentrations of gatifloxacin. LOD was found to be 18 ng/ml with a signal/noise ratio of about 3:1 (n=6). LOQ was found to be 50 ng/ml 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 gatifloxacin (50, 500 and 3000 ng/mL) followed by their comparison with the calibration curves prepared on the same day and on three different days. Precision was expressed as the % RSD of measured concentrations, whereas accuracy was expressed as recovery % (amount found/nominal amount x 100) of drug added to the blank aqueous humor samples. 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 98.67 to 99.64% and from 98.42 to 99.71% respectively, whereas the accuracies were less than 5.00 % in both cases.

Stability
Gatifloxacin 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 97.31 and 98.54%, 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 22°C.

<table>
<thead>
<tr>
<th>True conc. (ng/ml)</th>
<th>Amount found* (ng/ml)</th>
<th>Recovery (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-day</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>49.75 ± 2.22</td>
<td>99.50</td>
<td>4.46</td>
</tr>
<tr>
<td>500</td>
<td>493.34 ± 6.98</td>
<td>98.67</td>
<td>1.41</td>
</tr>
<tr>
<td>3000</td>
<td>2989.25 ± 12.11</td>
<td>99.64</td>
<td>0.40</td>
</tr>
<tr>
<td>Inter-day</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>49.21 ± 2.43</td>
<td>98.42</td>
<td>4.94</td>
</tr>
<tr>
<td>500</td>
<td>494.21 ± 7.45</td>
<td>98.84</td>
<td>1.51</td>
</tr>
<tr>
<td>3000</td>
<td>2991.21 ± 13.31</td>
<td>99.71</td>
<td>0.44</td>
</tr>
</tbody>
</table>

* n=6
In the group treated with GTFX-0.6, probably due to the rapid increase in the AUC (from 60 to 90 min) for GTFX-ED, GTFX-SH-0.6 and GTFX-SH-1.2, respectively and the decrease in Kₐ (from 0.0127 to 0.0101 and 0.0075 for GTFX-ED, GTFX-SH-0.6 and GTFX-SH-1.2, respectively). Interestingly, in the group treated with the reference formulation, the aqueous level of gatifloxacin were undetectable after 300 min, probably due to the rapid preocular clearance of the drug. The AUCs were significantly increased in the group treated with the hyaluronate formulations with respect to the reference formulation, with an increase in the AUC values of 2.8- and 4.4-fold for GTFX-SH-0.6 and GTFX-SH1.2, respectively. The increased bioavailability of the hyaluronate based formulations could be due to the prolonged preocular residence time of sodium hyaluronate as demonstrated in previous study using gamma scintigraphic and fluorescence methods [10-11, 37-38]. In addition, it was demonstrated that sodium hyaluronate is able to determine concomitant changes in tear film thickness [38].

### Table 2

Aqueous humor pharmacokinetic key parameters after single instillation of commercial (GTFX-ED) and hyaluronate mucoadhesive formulations of gatifloxacin (GTFX-SH-0.6 and GTFX-SH-1.2).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>GTFX-ED</th>
<th>GTFX-SH-0.6</th>
<th>GTFX-SH-1.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cₘₐₓ (ng/ml)</td>
<td>0.48</td>
<td>1.31</td>
<td>1.78</td>
</tr>
<tr>
<td>Tₘₐₓ (min)</td>
<td>60</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>T₁/₂ (min)</td>
<td>54.75</td>
<td>68.56</td>
<td>91.89</td>
</tr>
<tr>
<td>Kₐ (min⁻¹)</td>
<td>0.0127</td>
<td>0.0101</td>
<td>0.0075</td>
</tr>
<tr>
<td>AUC (µg·ml⁻¹·min⁻¹)</td>
<td>56.77±10.51</td>
<td>160.12±28.11</td>
<td>248.32±33.21</td>
</tr>
</tbody>
</table>

* n=6 rabbits (12 eyes)

**Ocular pharmacokinetic study**

The HPLC method developed was successfully used to quantify gatifloxacin in aqueous humor samples following topical instillation of gatifloxacin ophthalmic formulations. 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 increase in the aqueous humor gatifloxacin levels with respect to the reference formulation. The aqueous humor peak concentration (Cₘₐₓ), following administration of reference formulation, was significantly lower (p<0.05) compared to the hyaluronate formulations, with a 2.7- and 3.7-fold decrease for GTFX-SH-0.6 and GTFX-SH-1.2, respectively. The gatifloxacin levels obtained with both hyaluronate mucoadhesive formulations were significantly higher (p<0.05) in the range 60-180 min, compared to the reference formulation. In the same time range, the gatifloxacin levels obtained with GTFX-SH-1.2 were significantly higher (p<0.05) with respect to GTFX-SH-0.6 at all times except at 60 min. The concentration values registered at 15 and 30 min for all three formulations were not statistically significant each other (p>0.05). At 240, 300 and 360 min solely the GTFX-SH-1.2 formulation showed higher and significant concentration levels compared to both GTFX-SH-0.6 and GTFX-ED formulations.

**Fig. 2** Aqueous humor concentration-time profiles of gatifloxacin after ocular instillation in rabbit of GTFX-ED, GTFX-SH-0.6 and GTFX-SH-1.2 formulations.

Tₘₐₓ values were increased from 60 to 90 min for groups treated with the hyaluronate formulations with respect to the conventional formulation group. Consequently, the apparent residence time of sodium hyaluronate as demonstrated in previous study using gamma scintigraphic and fluorescence methods [10-11, 37-38]. In addition, it was demonstrated that sodium hyaluronate is able to determine concomitant changes in tear film thickness [38].
CONCLUSIONS

The most frequently reported adverse events related to commercial gatifloxacin eye drops (i.e. Zymar® and Zymaxid™), which need frequent dosing regimen, were conjunctival irritation, keratitis, papillary conjunctivitis, increased lacrimation and altered taste [22–23]. In addition, it was reported a rare unique case of corneal crystalline deposits from the use of gatifloxacin 0.5% after combined cataract extraction and trabeculotomy surgery [39].

In this study we designed and prepared two new sustained release mucodhesive hyaluronate formulations of gatifloxacin in order to improve the dosing regimen reported for the conventional marketed eye drops. To overcome the insufficient bioavailability of conventional eye drops the best approach is to use sustained drug delivery systems instead of a simple rise in concentration of the active ingredient. In fact, in some cases, the instillation of highly concentrated eyedrops can cause adverse effects and cellular damage in ocular tissues [5–7]. The pharmacokinetic profiles outlined clearly indicate an increase of bioavailability of the hyaluronate based formulations when compared to the conventional ophthalmic preparation. However, additional preclinical and clinical studies will be needed to confirm whether the hyaluronate gatifloxacin formulations developed in this study could ensure a less-intense dosing regimen with respect to the commercial preparations.

The pharmacokinetic profile outlined was established using a sensitive, reliable and rapid HPLC method with UV detection and simple liquid–liquid extraction procedure. The HPLC method was developed, optimized and validated for gatifloxacin determination in rabbit aqueous humor. Various experimental parameters such as mobile phase composition, flow rate, pH of the mobile phase and column oven temperature were optimized. The method uses an economic and relatively fast sample preparation that ensures good recovery of gatifloxacin from aqueous humor samples. The validation of the assay was performed according to the FDA and EMEA guidelines. The total run time does not exceed 10 min per sample with no peak interference from endogenous compounds. This method could be efficiently applied for the analysis of gatifloxacin in biological fluids for evaluating eye pharmacokinetics in preclinical research.

REFERENCES